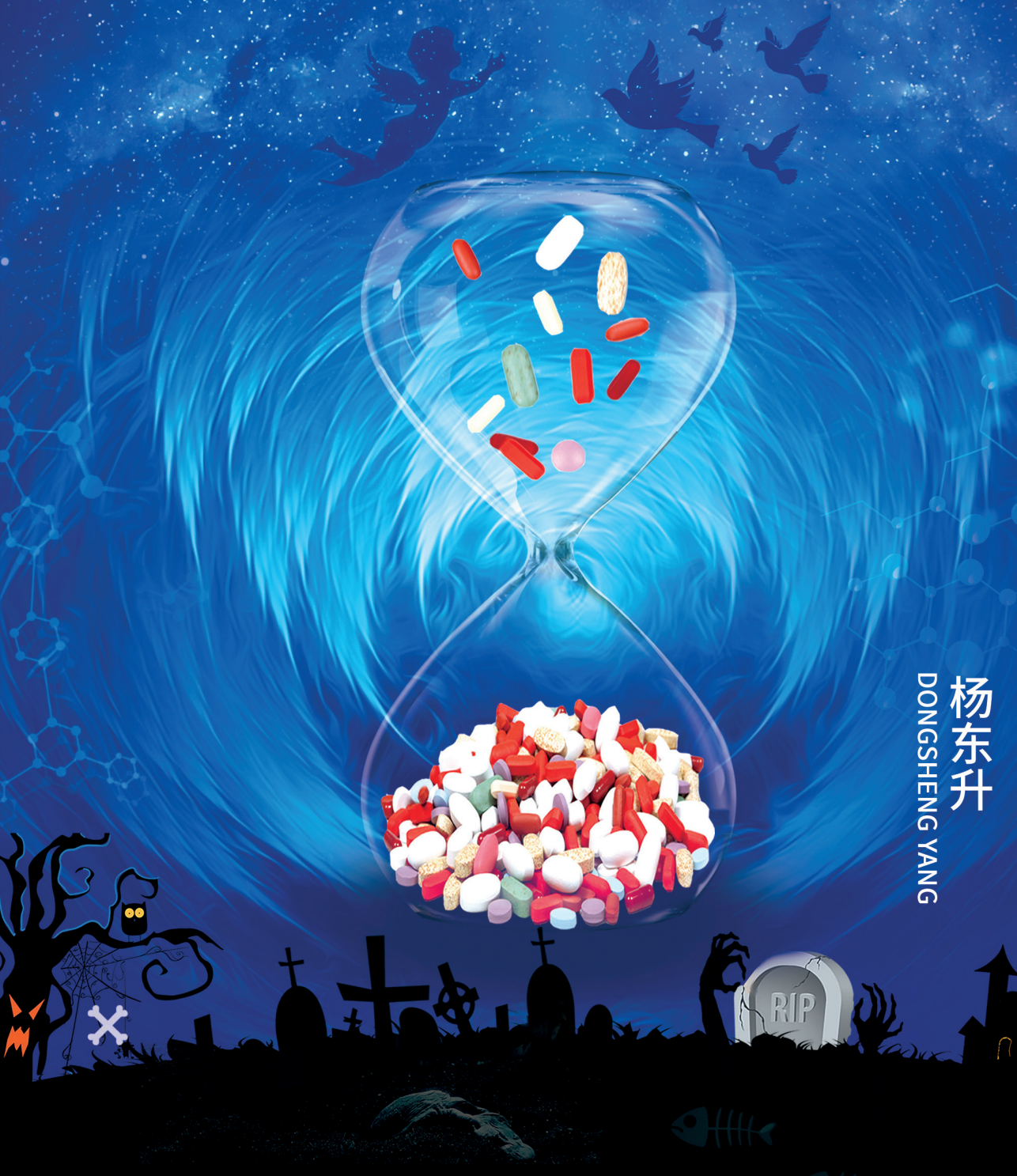
RISK FACTORS FOR ANTIMICROBIAL RESISTANCE IN EUROPEAN LIVESTOCK FARMING



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Risk Factors for Antimicrobial Resistance in European Livestock Farming

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Risk Factors for Antimicrobial Resistance in European Livestock Farming

Risicofactoren voor Antimicrobiële Resistentie in de Europese Veehouderij
(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General Introduction

Background

Nearly 100 years have passed since Alexander Fleming (1928) first discovered the inhibitory effect of the fungus *Penicillium rubens* on *Staphylococcus aureus*.[1, 2] As one of the greatest inventions in the 20th century, benzylpenicillin was purified and extensively marketed and used at the forefront of the clinical fight against infection.[1, 3, 4] Subsequently, other classes of antibiotics including (semi-)synthetic antibiotics have been introduced for clinical treatment of humans. This includes, for example, the discovery of sulfonamides by Bayer chemists Josef Klarer and Fritz Mietzsch in the early 1930s,[5] and later the discovery of quinolones (e.g. nalidixic acid) by George Y. Lesher in the early 1960s[6, 7] and oxazolidinones (e.g. linezolid) by Pharmacia & Upjohn Company in the 1990s.[8]

In order to kill or control the growth of pathogenic bacteria, antibacterial agents have multiple action mechanisms, such as interfering with bacterial cell wall synthesis (e.g. penicillins and carbapenems), inhibiting metabolic pathways (e.g. trimethoprim and sulfonamides), disruption of bacterial membranes (e.g. polymyxins) or interfering with protein synthesis (e.g tetracyclines, aminoglycosides).[9] According to the definition of the World Health Organization (WHO),[10] after losing sensitivity to one or more of these actions, bacteria will no longer be inhibited by these antimicrobials in their growth and activity as before, which is called antimicrobial resistance (AMR).

AMR leads to a decrease in the effectiveness of available antimicrobial agents, which may, in turn, compromise the treatment outcomes of infectious diseases, causing the death of thousands of people in clinical treatment each year.[11, 12] In humans, the abundance of different AMR species varies. According to one report from the WHO,[13] high AMR rates of *E. coli* were found towards ampicillin, cotrimoxazole, tetracycline and nalidixic acid in community sites in South Africa and India. Moreover, the geographical variation of human AMR is also evident, indexed by the highly varying prevalence of AMR between countries.[14-18] For example, the surveillance reports from European Centre for Disease Prevention and Control (ECDC) in 2019 showed that the percentage of resistance in *E. coli* reported in Southern and Eastern Europe was generally higher than that in Northern Europe during the period 2015-2018.[17]

Antimicrobial resistance in animals

Geographical variation of antimicrobial resistance in animals

With the extensive research and reports of AMR in humans, research interests have been triggered in animal AMR. Similar to the findings in humans, geographical variation in AMR abundance is also reported in animals.[19-23] For example, in a multi-country study that used metagenomic shotgun sequencing to quantify AMR, high between-country variation of AMR abundance was observed in pigs.[19] Similarly, in a study that included farm animal data from seven European countries during 2010-2011,[23] *E. coli* isolated from farm animals in Belgium generally showed the highest AMR level compared to other countries. Variation between countries might be attributed to differences in the veterinary prescription practices, farm production systems, or the occurrence of infectious diseases.[19, 20, 24] This can also be inferred from the high AMR levels in animals in low- and middle-income countries (LMICs), where farm hygiene and farm management are poor or livestock antimicrobial use (AMU) regulations are generally limited.[25-27]

Few studies[28, 29] have quantified or compared within-country or within-farm variation of AMR in animals. To enable further understanding of AMR epidemiology in farm animals, we explored the between- or within-country variation and within-farm variation of AMR, which will be further discussed in chapters 2 and 4 of this thesis.

Variation of antimicrobial resistance between animal species

AMR was also found to vary considerably between animal species. According to the European Union (EU) 2017/2018 Farm Animal AMR Report,[30] although high levels of resistance to ampicillin, trimethoprim & sulfonamides and tetracycline were commonly observed in *E. coli* isolates from pigs, poultry and veal calves, a high level of ciprofloxacin resistance was only found in *E. coli* isolated from broilers and turkeys. A similar difference between animal species was reported in France, where the prevalence of *E. coli* isolates resistant to aminoglycosides was much higher in cattle (77.6%) than in swine (42.6%) and poultry (17.2%).[29] The underlying reason is the different prescription patterns of antimicrobial drugs in the treatment of different infectious diseases between animals (e.g. respiratory diseases in poultry, mastitis in cattle).[29-32] This can also be illustrated by the results of studies in companion animals. For example, elevated levels of resistance to amoxicillin-clavulanate have been found in isolates that cause urinary tract infections in dogs and cats in European countries, which can be attributed to the frequent use of amoxicillin-clavulanate in companion animals to treat upper respiratory tract infections.[33-35] In chapters 2, 4, and 5 of this thesis, the AMR levels in samples from various animal species are quantified and compared to assess the variation of AMR or specific antimicrobial resistance genes (ARGs) between multiple animal species.

Antimicrobial use in animals

Generally, AMR is considered to be the evolutionary response of microorganisms to selective pressure induced by exposure to antimicrobial agents.[36] In clinical treatment of humans and animals, the use, misuse, and overuse of antimicrobials have been recognized as key drivers of acquired AMR.[1, 37, 38] Therefore, it is particularly important to understand the purpose of antimicrobial administration and the class and dosage of antimicrobials used for clinical treatment in different animals. There are four main purposes for using antimicrobial agents in animals: prophylaxis, therapy, metaphylaxis and growth promotion (Table 1).

Table 1. Types of antimicrobial use in animals.

AMU purposes	Administration population	Diseased population
Prophylaxis	Individual/group*	None
Therapy	Individual/group*	Individual/group*
Metaphylaxis	Group*	Few
Growth promotion	Group*	None

* Group treatment for farm animals.

Prophylaxis refers to the prior administration of antimicrobials to animals to prevent certain infectious diseases during periods of high risk. For example, prophylactic antimicrobial use in dairy cows generally occurs at the end of the lactation period to prevent new mastitis infections during the dry cow period and at early lactation.[39-41] In pigs, the use of prophylactic antimicrobials usually occurs when piglets are weaned or pigs from different litters are mixed in pens or among different herds which makes them susceptible to respiratory or intestinal infections.[39, 42, 43]

The application of antimicrobials to treat and control an existing infectious disease is called therapeutic use. For the purpose of treating infectious diseases in different farm animals, the use of antimicrobials varies by animal species (Table 2). For example in cattle, penicillin preparations have been gradually used by veterinarians to treat mastitis.[44, 45] While in pigs, tetracyclines and penicillins are commonly used mainly for respiratory diseases.[46, 47] In large farms, AMU for farm animals is usually empirical due to high testing costs or lack of laboratory testing. This means that even when only a few animals are identified ill, the farm usually administers antimicrobials directly to the entire herd to control the spread of disease, which is called metaphylaxis.[39, 48]

Table 2. Commonly used therapeutic antimicrobials in different animal species in Europe.

	Cattle/Calf[46]	Pigs[46]	Broilers/Turkeys[49-52]	Cats/dogs[46]
Infectious disease (AMU)	Mastitis (Penicillins/cephalosporins)	Respiratory disease (Tetracyclines/penicillins)	Omphalitis (Quinolones)	Skin diseases (Penicillins)
	Respiratory disease (Macrolides/phenicols)	Diarrhoea (Polymyxins/macrolides)	Gastrointestinal problems (Extended spectrum Penicillins)	Respiratory disease (Tetracyclines/penicillins)
	Diarrhoea (Polymyxins/quinolones)	*Streptococcus suis* infection (Penicillins)	*Enterobacteriaceae* infections (Polymyxins)	Urinary tract infection (Quinolones)

This table is summarised from the therapeutic AMU data in the period from 2013 to 2015. Cephalosporins: 1st ~ 4th generation cephalosporins. Quinolones: Fluoroquinolones + quinolones.

Antimicrobials are also used for growth promotion in farm animals. In the mid-1950s, researchers discovered that antimicrobial agents could contribute to the growth of animals and thus to meat production.[53, 54] Since then non-therapeutic antimicrobial growth promoters (AGPs) have been extensively used in farm animals as a growth promotor, next to the use for infectious disease treatment in animals.[53, 55] The use of AGPs is banned by the EU (since 2006) and the United States (since 2017), as described in more detail later.

Antimicrobial use monitoring approaches

National monitoring programs of antimicrobial use

For the purpose of controlling AMU in animals, it is important to systematically register the level of AMU. Correspondingly, national and international monitoring of antimicrobial consumption in animals is gradually being implemented.[22, 43, 46, 56-60] The first national AMU monitoring program in animals originated in Denmark, where data has been collected since 1995 and annually reported by the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP).[61, 62] DANMAP not only implements AMU monitoring but also aims to figure out the reasons for AMU changes in Denmark every year.[56, 61, 63] For example, from the 2013 DANMAP report,[63] the total consumption of antimicrobials (active compounds) in poultry increased by 57% compared to that in 2012. The main reasons concerned the widespread respiratory disease in turkey flocks at the beginning of 2013 and the increased incidence of diarrhoea in broiler flocks. This comprehensive monitoring method has played an immeasurable role in the control of veterinary antimicrobial

consumption in Denmark.[56, 63, 64] Subsequently, AMU monitoring systems for animals have also been developed in many other countries, such as Finland,[65] the Netherlands,[57, 66] and Belgium[67, 68].

International monitoring programs of antimicrobial use

As early as 1998, a resolution of the World Health Assembly urged member states to take measures to promote the rational use of antimicrobials and reduce AMU in farm animals.[13] Subsequently, in 2001, the consultation on monitoring AMU in food animals for the protection of human health encouraged national or international strategies and systems to establish an inventory for AMU monitoring in food animals.[13, 69] Then in September 2009, the European Medicines Agency launched the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project.[58] Since then, data on the sales of veterinary antimicrobials have been collected and reported in a harmonised manner from the Member States that have established monitoring programs.[24, 58-60] In LMICs, AMU monitoring and control are more challenging. This is mainly due to a series of reasons including imperfect policy frameworks, limited attention to infection prevention, and lack of technology and resources.[69, 70] In these countries, given the expected growing demand for protein from animal foods, it is expected that AMU and AMR problems will continue to increase in the future.[70-72] To address this issue, international technical agencies have formulated measures to assist AMU monitoring in animal production in developing countries.[70, 73]

Regulations of controlling antimicrobial use in animals

Controlling the use of AGPs

The results of veterinary AMU monitoring have provided a clearer direction for controlling AMU in animals. AGP is the first application of antibiotics that was restricted for use in animals by the government. This is because after AGPs were widely used in farm animals, infectious disease specialists and microbiologists discovered resistant bacteria of public health concern in the intestinal microbiota of farm animals and farmworkers on farms using AGPs.[48, 74-78] For example, studies have shown that the use of avoparcin in animals contributed to the increase of vancomycin-resistant enterococci (VRE) in contaminated retail meats and in humans.[48, 77, 78] VRE can cause severe infections in hospitalized patients and then lead to increased morbidity and mortality in patients. As a result, regulations have been introduced to ban the use of AGPs on farms.[79-81]

Examples include the ban by the EU in 2006[79] and the ban by the Food and Drug Administration (FDA) in the United States in 2017[80].

Controlling the use of CIAs

As early as 2003, Denmark implemented legislation by requiring susceptibility testing before using fluoroquinolones in animals, and the use of this antimicrobial class needs to be reported to the authorities.[82, 83] Then the classification of critically important antimicrobials (CIAs) was released by the Food and Agriculture Organization (FAO), the WHO and the World Organisation for Animal Health (OIE) in 2007 and has been updated to the sixth revised edition in 2018, which aims to reduce the use of CIAs in food animal production.[84-86] Subsequently, in 2010 and 2014, Denmark banned the use of cephalosporins in pigs and cattle, respectively, on a voluntary basis.[56, 83, 87] Afterwards, more and more countries have started to control the use of CIAs in animals.[56, 82, 83, 87-89] for example in the Netherlands, in 2011, the Dutch Health Council recommended in their report on AMU and AMR in food animal production that the use of 3rd- and 4th-generation cephalosporins should be banned and the long-term use of fluoroquinolones should be stopped.[88] Furthermore, since 2013, the Netherlands has required veterinarians to perform susceptibility tests before prescribing fluoroquinolones and 3rd- and 4th-generation cephalosporins.[89]

Other potential determinants of antimicrobial resistance in animals

When AMU in animals is more and more strictly controlled, it may have a negative impact on the treatment and production of animals, which is of concern. Studies have shown that after AGPs were banned, therapeutic antimicrobials have been increasingly used in farm animals.[57, 90-92] A report from Sweden showed that after one year of banning AGPs, piglet mortality increased by 1.5%.[93] Fortunately, most studies have shown that animal production did not change much after AMU was reduced or restricted.[90, 93-95] For instance, reports from Denmark and the United States both show that a negative impact of banning the use of AGPs in farm animals is negligible or even non-existent. This may be related to the optimization of production practices, such as improved diet and later weaning.[90, 96, 97] Considering multiple factors that affect the health and production of farm animals, it can be hypothesized that these factors may also be directly or indirectly related to AMR in farm animals. This has been proved by increasing evidence that in addition to AMU, many other farm characteristics may also affect AMR levels in farm animals.[98-101]

Herd characteristics related to antimicrobial resistance in farm animals

First of all, some conventional herd characteristics have been found to be associated with AMR levels in farm animals.[101-104] For example, compared with small-size calf farms, large-size calf farms showed a higher prevalence of Methicillin-resistant *Staphylococcus aureus* (MRSA)[105] and a higher proportion of resistant *E. coli*[106]. Moreover, according to reports on the association between animal age and AMR, pigs (non-antibiotic-exposed) in the finishing unit showed significantly larger AMR levels in faecal coliforms than pigs in farrowing houses or pasture.[107]

Biosecurity measures related to antimicrobial resistance in farm animals

In addition to herd characteristics, farm biosecurity has also been of interest to researchers in recent years. Biosecurity refers to all on-farm measures related to livestock health and disease prevention, including external biosecurity (all measures designed to prevent pathogens from entering or leaving the herd) and internal biosecurity (all measures designed to reduce the spread of pathogens within the herd).[108, 109] A high level of biosecurity, including good hygiene,[109-111] farm management and husbandry practices,[112, 113] could improve the quality of life and health status of farm animals, thereby reducing AMR levels in farm animals. Dorado-García et al. in 2015 reported that the presence of free-ranging farm cats and sheep on calf farms was significantly positively associated with a higher prevalence of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* (LA-MRSA) in calves.[105] In addition, one study conducted in an MRSA-free environment in Norway suggested that farmworkers may have played a role in the introduction of LA-MRSA isolates from humans into pigs.[114] Meanwhile, some studies have shown that biosecurity only has a limited influence on AMR abundance in farm animals.[98-100, 115] For example, Van Gompel et al. have shown that farm internal biosecurity is positively related to macrolide resistance abundance in pig faeces, especially related to its sub-category 'cleaning and disinfection' level. Considering the limited published evidence and the conflicting results, it is difficult to draw firm conclusions about the relationship between farm factors and farm animal AMR. To elucidate such complex influences of farm factors on farm animal AMR, more in-depth risk analyses incorporating as many farm characteristics as possible across multiple animal species are needed. In this thesis, these analyses will be discussed in chapters 3 to 6.

One health approach for monitoring and controlling antimicrobial resistance

Antimicrobial resistance in the environment

As mentioned above, the farm factors affecting AMR in farm animals are complex. However, in the more complex ecosystem, many more environmental factors need to be taken into account. Studies have indicated that in addition to humans and animals, the environment is also a reservoir of AMR, and environmental microorganisms have been considered the source of most antimicrobial resistance determinants.[116, 117] In the environment, the primary 'natural' AMR is considered to be the result of a Darwinian competitive selection process by fungi and bacteria producing antimicrobial agents (e.g. chloromycetin resistance produced by *Streptomyces venezuelae* or streptomycin resistance produced by *Streptomyces griseus*).[118, 119] In addition, studies have shown that the rapid increase in AMR levels in the environment is mainly a man-made situation superimposed in nature, such as the direct use of antimicrobials in food production environments, the excretion of human and animal containing antimicrobial residues, and the waste discharge from pharmaceutical factories.[37, 120-122]

Potential transmission of antimicrobial resistance between animals, humans and the environment

Numerous studies have shown that there is direct or indirect (e.g. food, airborne, environment) AMR transmission between animals and humans.[123-128] This is partly due to the transfer of food-borne resistant bacteria from farm animals to humans through the traditional farm-to-fork route (consumption of meat and eggs).[124, 129-131] Alternatively, direct contact with companion animals that carry AMR bacteria in the home[123, 132, 133] and occupational exposure to livestock carrying AMR bacteria may also play a role in AMR transmission.[126-128] In the indirect pathways that include the environment as a route of transmission, AMR can be introduced to humans from animals through the environment by inhalation (e.g. exposure to airborne dust containing animal manure particles) or contact with contaminated surfaces.[134, 135] The results reported by Luiken et al. have provided evidence of a potential link between animal manure, farm dust and farmworker resistome.[125] One explanation for this is the clonal spread of existing resistant bacteria, with or without zoonotic potential (e.g. *Salmonella enterica*, LA-MRSA).[125, 136, 137] In addition, horizontal gene transfer contributes to bacteria acquiring ARG determinants (e.g. extended-spectrum beta-lactamases (ESBL)).[136, 138, 139] In summary, AMR transmission between animals, humans, and the environment is interconnected, which means that simply controlling AMR in humans or animals may not be effective in cutting off this transmission. To address this problem in the entire

ecosystem, a multi-faceted, collaborative and cross-sectional approach has emerged, which is called the One Health approach.[135, 140]

One Health approach in controlling antimicrobial resistance

The One Health approach has gained momentum as a possible key to controlling AMR. For the successful implementation of this approach, harmonized surveillance standards and open systems are urgently needed to measure and compare AMR evolution in various countries. Around ten years ago, the WHO gathered and formed an international and multidisciplinary expert group called the Advisory Group for Integrated Surveillance of Antimicrobial Resistance (AGISAR).[141] Subsequently, the AGISAR developed a five-year strategic framework to provide member states with key information on the design of integrated AMR surveillance programs to monitor food-borne and zoonotic bacterial AMR.[142] In 2016 in Europe, the Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) published the first comprehensive report of antimicrobial consumption and bacterial resistance occurrence in humans and food animals using the relevant EU monitoring networks.[143]

In terms of the environment, the necessity of a comprehensive monitoring system has been recognized,[144, 145] and Global Sewage Surveillance Project (GSSP) has initiated monitoring AMR in sewage in 102 countries in the world.[146] Sewage samples from 60 countries in GSSP have been analysed using metagenomic methods for AMR abundance and distribution by the National Food Research Institute of the Technical University of Denmark (DTU Food).[147] The result showed high diversity and geographical variation of AMR across the world, and improving sanitation and health may be able to limit the global burden of AMR. Moreover, considering the limited level of surveillance systems in LMICs, the WHO has developed a global AMR surveillance program that includes only one indicator (i.e. ESBL), which is also called the 'Tricycle protocol'.[148] Nevertheless, data on AMR in dust (related to the farm environment) has not been included in these sampling plans, either in GSSP or in the Tricycle protocol. Therefore, in chapter 2 of this thesis, we performed AMR quantification on a large number of samples from humans, various animal species and sample sources in food production chains and explored possible relationships, which may provide clues for One Health approaches of AMR controlling.

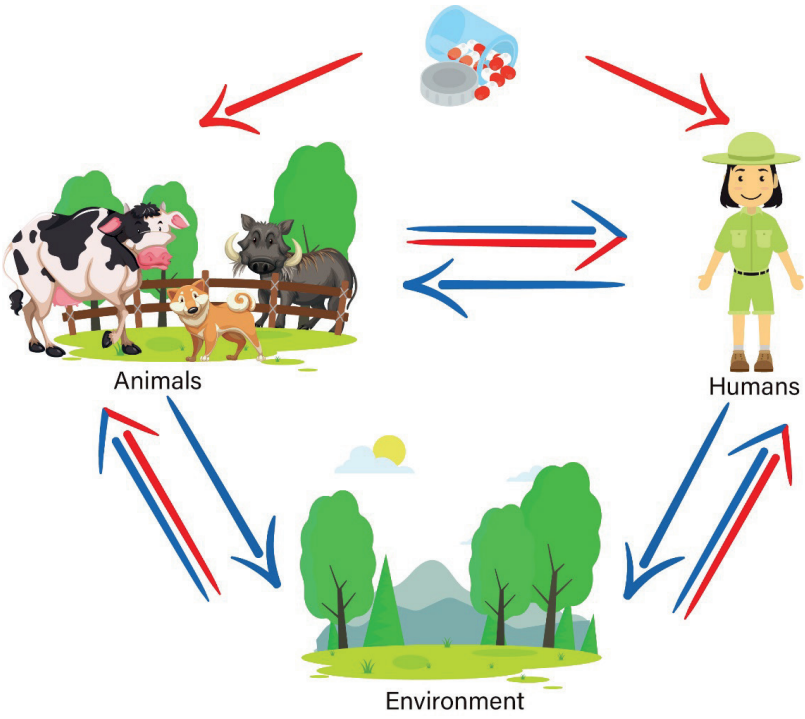

Figure 1. The potential transmission of AMR between animals, humans and the environment.

Red arrows: AMR introduction through the oral route. Blue arrows: AMR emission and exposure.

Quantitative assessment of antimicrobial resistance and related issues

Phenotypic quantification of antimicrobial resistance

Monitoring AMR in different animal sources in various countries greatly profits from a standardized and appropriate quantification method. There are two general types of AMR quantification - phenotypic quantification and genetic quantification with the first being the most classic method of antimicrobial susceptibility testing (AST) of bacterial isolates.[149] This method can be used for qualitative (e.g. agar disk diffusion) determination of the susceptibility of bacteria by evaluating the size of the inhibition zone diameter.[150] In addition, agar or broth dilution method have been developed to quantify the susceptibility by determining the minimum inhibitory concentration (MIC) that can inhibit the visible growth of bacteria.[151] These methods have

been extensively used for AST and quantification of selected bacteria isolated from human or animal sources.[152, 153]

Genetic quantification of antimicrobial resistance-qPCR

However, as many bacteria remain unculturable or difficult to culture, genotypic testing methods have been emerging to compensate for the shortcomings of the traditional phenotypic quantification methods. As a molecular method, PCR can be used to identify specific ARGs in purified DNA of single bacterial isolates and also complex bacterial communities. Since its discovery, PCR methodology has undergone many improvements, such as the application of probes to improve the specificity of the assay and the development of real-time quantitative PCR (qPCR). qPCR is faster and more sensitive than classical gel-based PCR and can detect ARGs quantitatively (by capturing fluorescent signals).[154]

Although there have been studies using qPCR method for AMR quantification and follow-up studies, these studies are generally limited to certain species or one specific country.[155-157] The different sampling sources (e.g. faeces, swabs) and various selected ARG targets make it difficult to compare these results. In order to achieve comparability of AMR levels between countries or between different species, we used a harmonized qPCR method to quantify the abundance of ARGs (*aph(3')-III, ermB, sul2, tetW*) in a large number of samples from nine European countries, which will be further discussed in chapters 2 and 4 of this thesis.

Genetic quantification of antimicrobial resistance-NGS

In recent years, to detect a more complete picture of (or novel types of) AMR and ARGs or determinants in DNA of bacterial communities, metagenomic sequencing has been increasingly used. Metagenomic sequencing started with Sanger sequencing, also called the first-generation sequencing, which incorporates deoxynucleotide triphosphate analogues into a growing chain of oligonucleotides, known as "synthetic sequencing".[158] On the basis of the first generation of testing, next-generation sequencing (NGS) was developed which achieves massive parallelization of sequencing reactions, thereby increasing the number of DNA fragments sequenced in one run.[159, 160]

The NGS method has completely changed the field of AMR research, and the plummeted costs have rapidly increased its popularity.[161] In addition, NGS technology enables random and large-scale targeting of the entire microbial community, which is currently called "metagenomic shotgun sequencing" (MG-SS).[162, 163] In the field of AMR, there are roughly two types of approach: functional metagenomics and sequence-based

metagenomics.[164] Functional metagenomics, which makes it possible to explore previously unknown ARGs, begins with the construction of a metagenomic library by cloning and heterologous DNA expression in *E. coli*. Subsequently, AMR activity of the metagenomic library is screened by antimicrobial selection media and sequencing.[117, 165, 166] Compared with functional metagenomics, sequence-based metagenomics does not require cloning into expression vectors or screening through selective media, but directly performs shotgun sequencing on total DNA extracted from samples. Finally, the sequences are subjected to computational pre-processing (e.g. base calling, quality trimming), actual sequence analysis (e.g. read assembly, alignment) and post-processing (epidemiological analysis) through a metagenomic workflow.[163, 167]

As a tool for AMR characterization, NGS can bring insights into the emergence and spread of AMR. The value of NGS in AMR epidemiology and surveillance purposes is often emphasized.[168-170] However, the cumulative evidence comparing NGS and phenotypic methods for susceptibility testing of individual bacterial isolates is considered limited or poor, which means replacing antibiotic susceptibility testing with NGS for clinical use has not been considered appropriate.[171] A possible direction to advance the current understanding in this regard is to conduct different quantitative methods in parallel in one study. Next to NGS for single isolates, NGS is also used for community DNA. In this thesis, we explore whether qPCR can be an alternative to NGS for the precise quantification of AMR targets in community DNA, which may be a cost-effective approach in certain instances. Therefore, in order to explore the possible consistency and comparability between AMR quantified by metagenomics and qPCR, we evaluated the correlation of specific ARGs quantified by both methods in different animal species (pigs, broilers, veal calves and turkeys) in chapters 2, 4 and 6.

Objective/outline

Objectives

Focusing on risk factors of AMR in the livestock production chain, this thesis includes samples from farm animals, humans and the environment in Europe. Studies in the thesis aim to identify sources of AMR variation by comparing AMR abundance between countries, between/within farms and across species. Variation of AMR abundance in the pig and broiler production chain was assessed, and potential links between AMR in animals, the livestock environment, and humans were explored. To determine potential risk factors of AMR, comprehensive data on farm-related characteristics were included for veal calves, pigs, broilers, and turkeys. To

assess differences in risk factor analysis results for AMR quantified by different methods, we simultaneously compared results from both qPCR and metagenomics for pigs and broilers, and all three quantification methods (qPCR, NGS, MIC) for turkeys. Correlation analysis between qPCR data and metagenomic data of pooled faecal samples was conducted to evaluate whether qPCR can be an alternative to NGS for AMR quantification in the community samples. Correlation analysis between individual faecal data and pooled faecal data was conducted to assess whether the sample size per farm is sufficient to represent the overall AMR level at that farm.

Outline

Chapter 2

In order to make a comprehensive assessment of AMR levels in animals, humans and the environment in Europe, we collected and analysed 9,572 samples from animals and various related sources of nine European countries in chapter 2. AMR variation across the production chain was evaluated in pigs and broilers. To explore the variation sources (between-country variation, between- or within-farm variation) of AMR abundance, we amplified four ARGs (*aph(3')-III, ermB, sul2, tetW*) for all individual faecal samples using qPCR and analysed the variance components per target in veal calves and turkeys. The association between AMU for pigs and broilers and relative ARG abundance in their pooled faeces was assessed. The correlation between pooled qPCR data and previously published metagenomic data in pigs and broilers was investigated. The potential association between AMR in animals, the production environment, and humans was explored.

Chapter 3-4

Quantification technologies have highlighted AMR occurrence in livestock and humans (chapter 2), however, there is less information on AMR determinants. We explored the relationship between AMR and AMU, as well as other farm characteristics (e.g. age and weight of animals, number of farmworkers, and farm biosecurity), for veal calves (chapter 3), and pigs and broilers (chapter 4) (Figure 2). In chapter 4, we also conducted correlation analyses between median-individual qPCR data and previously published pooled metagenomic data to compare the abundances of ARG targets in two datasets. In addition, variance components (between-country variation, between- or within-farm variation, farm characteristics) of AMR were analysed. The sample size of individual faecal samples per farm was evaluated.

Chapter 5

In chapter 5, we used an updated AMR reference database and alternative bioinformatics/computational approaches to quantify the resistome in farm animals (pigs, broilers, and veal calves) in nine European countries. Associations between updated resistome data and potential risk factors (e.g. AMU, biosecurity) were analysed in pigs and broilers to verify previously published associations. We also analysed determinants of the resistome in veal calves.

Chapter 6

To compare the risk factor analysis results of AMR quantified by different methods, three AMR quantification approaches (NGS, qPCR, and MIC) were used in turkeys (chapter 6) (Figure 2). Potential farm characteristics (e.g. AMU, biosecurity) associated with AMR were determined.

Finally, in chapter 7, all results presented in chapter 2 to chapter 6 were discussed and compared with previous studies in farm animals associated with AMR.

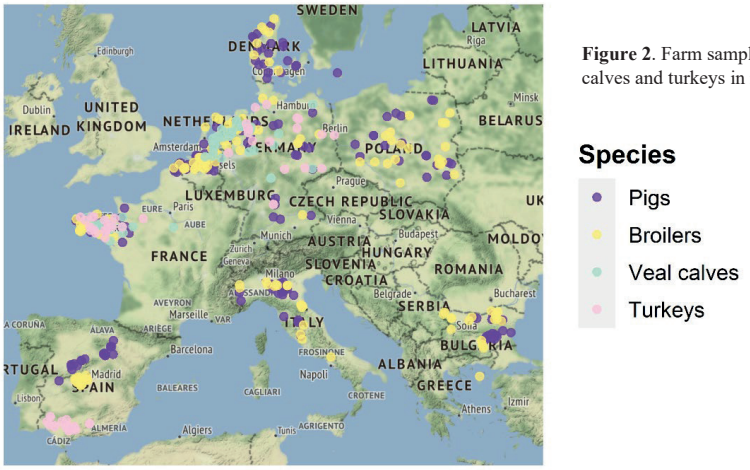

Figure 2. Farm sampling of pigs, broilers, veal calves and turkeys in nine European countries.

The EFFORT project

The studies in this thesis are embedded in the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project (www.effort-against-amr.eu), which aims to investigate the epidemiology of AMU and AMR in the meat and poultry production chain in Europe. Data collection, questionnaires, lab experiments and data analyses were implemented between 2014 and 2017.

Previous studies in EFFORT have investigated AMR abundance in metagenomic data (pigs and broilers)[19, 98, 100] or MIC data (pigs, broilers, veal calves, turkeys)[20] and identified potential risk factors for AMR. However, AMR data quantified by qPCR has not been explored until this thesis. The research in this thesis summarises all the AMR data quantified by qPCR in EFFORT, where more than 9500 samples were collected from animals, the environment and humans in nine European countries. Potential links between AMR in animals, humans and livestock environments are discussed and all related on-farm risk factors are identified. The results of previously published risk factor analyses of metagenomic or MIC data are confirmed in this thesis. In addition to this, we assessed within-farm AMR variation, which is not possible with EFFORT metagenomic data, as it used pooled faeces samples (faecal samples aggregated within a farm). In addition, we compared the contribution of all risk factors to AMR variation, which indicated that AMU and farm biosecurity were the main components of AMR variation among all risk factors.

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Chapter 2

Antimicrobial resistance genes *aph(3')-III, ermB, sul2* and *tetW* abundance in animal faeces, meat, production environments and human faeces in Europe

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Under revision

Abstract

Background: qPCR is an affordable method to quantify antimicrobial resistance gene (ARG) targets, allowing comparisons of ARG abundances along animal production chains.

Objectives: We present a comparison of ARG abundances across various animal species, related production environments and humans in nine European countries. ARG variation sources (country, farm, determinants) were quantified. The relationship between farm-level antimicrobial use (AMU) and ARG levels was determined.

Methods: A cross-sectional study was conducted in nine European countries comprising 9,572 samples. qPCR was used to quantify ARG (*aph(3')-III, ermB, sul2, tetW*) and 16S rRNA abundances. ANOVA was used for ARG abundance comparisons. Variance component analysis was conducted to explore variance sources. Random-effects meta-analysis was performed to determine the AMU-ARG relationship in pigs and broilers. Correlation was used to assess the agreement between qPCR data and previously published metagenomic data.

Results: ARG abundance varied strongly between animal species, environments and humans. This variation was dominated by between-farm variation (pigs) or within-farm variation (broilers, veal calves and turkeys). A decrease in ARG abundances along pig and broiler production chains (from 'farm to fork') was observed. A high relative ARG abundance was observed in humans occupationally exposed to livestock. In pigs and broilers, corresponding AMU-ARG positive association was observed. ARG abundance showed a high correlation (Spearman's $\rho>0.7$) between qPCR data and metagenomic data of pooled samples.

Conclusion: This study shows qPCR analysis is a valuable tool to assess ARG abundances in a large collection of livestock-associated samples. The geographical and between-farm variation of ARG abundances could partially be explained by AMU and farm biosecurity levels. ARG abundances in human faeces were related to livestock antimicrobial resistance exposure.

Introduction

Antimicrobial resistance (AMR) poses a threat not only to humans but also to animals worldwide.[1] High antimicrobial usage (AMU) leads to the selection of resistant bacteria, which limits therapeutic options in animals and humans.

A variety of methods have been carried out to quantify AMR levels in faeces, such as conventional testing of phenotypes of antimicrobial susceptibility of selected organisms.[2, 3] Besides, next-generation sequencing (NGS) is emerging as a new method to detect genetic determinants conferring AMR in selected isolates or metagenomically in DNA of bacterial communities.[4-6] However, due to the high costs and technological constraints of NGS methods, the number of samples per study is often limited, or animal samples are pooled (e.g. at the farm or herd level).[4] Consequently, in pooled samples, within-farm variation of antimicrobial resistance gene (ARG) abundance cannot be determined. Compared to NGS, real-time polymerase chain reaction (qPCR) is an affordable and widely applied method that can also provide precise quantification of certain ARG targets,[7] allowing comparisons of ARG abundance between and within sampling sites. Furthermore, using qPCR, associations between farm-level risk factors and ARG abundances can be efficiently determined in relatively large datasets.

Direct or indirect exposure of humans to livestock, companion animals, or animal products are known risk factors for AMR acquisition.[8-10] Intensity and frequency of contact with live animals have been shown to represent a risk factor for carriage of AMR bacteria such as Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* (LA-MRSA) or Extended-spectrum beta-lactamases (ESBL)-producing bacteria.[11, 12] Although the correlation of ARG abundances between animals and humans has been studied using qPCR,[13-15] the insights are limited with respect to geographical distribution or variation across different sample types.

As part of the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project, we used qPCR to quantify four frequently occurring ARGs (*aph(3')-III, ermB, sul2* and *tetW*) in a total of 9,572 samples collected in nine European countries. We analysed samples from animal faeces, meat, production environments and human faeces. The objectives of the current study are 1) to describe ARG abundances among the different sample types and countries; 2) to quantify ARG variation sources (between countries, between and within farms, and determinants) based on variance component analysis (VCA); 3) to determine associations between AMU and ARGs in pig and broiler faeces; 4) to determine the correlation between ARG abundances quantified by qPCR and metagenomics.

Methods and materials

Study population and sampling procedure

Between 2014 and 2017, we collected a large set of samples (9572 samples) from various sources. The sampling procedures have been partially described before.[5, 6, 14, 16-24] Faecal samples were taken from farm animals (pigs,[5, 14, 24] broilers,[6, 24] veal calves,[16] turkeys,[17] fish (intestines were collected)), companion animals (cats, dogs),[18] and wild boars. Carcass samples were taken from pigs[14] and broilers at slaughterhouses, raw meat (pork,[14] chicken, turkey, veal, and trout) samples were purchased at food stores. We also collected environmental samples (electrostatic dustfall collector (EDC)[20, 21] and gloves of slaughterhouse workers[14]) and human faeces (humans occupationally exposed to pigs or broilers at Dutch and German farms or slaughterhouses, and control subjects from the Dutch 'Lifelines' cohort[14, 19, 22, 23]). Animal samples were collected from nine European countries (Belgium/BE, Bulgaria/BG, Germany/DE, Denmark/DK, Spain/ES, France/FR, Italy/IT, the Netherlands/NL, and Poland/PL). More details about faecal sampling are described in the Supplement (*'Sampling procedure of faeces'*).

Before DNA extraction, animal faecal samples were stored at 4°C, transported to the laboratory within 24 h and stored at -80°C.[14, 16] Exposed EDC cloths were put into a small re-sealable bag with sterile tweezers and frozen at -80°C.[20] Carcass, meat, and gloves samples were collected in a stomacher bag (Interscience, 400 ml, UK), transported and stored at 4°C, followed by further preparations.[14] Human faecal samples were refrigerated directly after collection, transported and stored on dry ice.[14, 22, 23]

ARG targets selection

Within the EFFORT project, the ARG target choice was based on several criteria, including 1) the prevalence and relative abundance of ARG targets in previous metagenomic shotgun sequencing analyses of pig and broiler faeces.[4] Although gene targets of particular public health concerns (eg. *mecA, vanA*, ESBL-encoding genes) were considered for inclusion, the proportion of samples with quantifiable levels of these genes was generally too low, as judged from previous metagenomics findings (e.g. the initially chosen vancomycin resistance gene *vanA* was excluded from the targets as it could not be measured in a pilot subset of samples); 2) the inclusion of targets of different antibiotic classes; 3) limited correlation of the chosen gene concentrations to avoid redundancy; and 4) a PCR protocol should either exist or be achievable. More details about the selection process are described in the Supplement (*'ARG targets selection process'*).

DNA extraction, qPCR and sequencing

DNA of animal and human faeces was extracted using the modified QIAmp Fast DNA Stool Mini Kit (Cat. No. 51604; Qiagen, The Netherlands) as described before.[14, 16] DNA of EDC and gloves was respectively extracted using the modified NucleoSpin® 8 Plant II Kit and NucleoSpin®96 Food Kit (Macherey-Nagel, Germany), while DNA of meat was extracted using the modified Nucleospin® Food Kit (Macherey-Nagel, Germany).[14] Following DNA extraction, qPCR was conducted to quantify the abundance of four antimicrobial ARGs (*aph(3')-III, ermB, sul2, tetW*) along with the 16S rRNA gene as a measure of total bacterial DNA. More details on the qPCR process have been described in previous papers[14, 16] and the Supplement (*'qPCR process'*). qPCR quality control comprised of a number of elements is described in the Supplement (*'Quality control and quantification of qPCR results'*).

Absolute ARG abundance was calculated as the $\log_{10}$ transformed ARG copy number per unit of the sample. Relative ARG abundance was calculated using 16S rRNA as a general bacterial molecular marker to normalize for the size of the bacterial community in animal and human samples.

DNA of pooled faecal samples from pigs, broilers and veal calves was extracted at the Technical University of Denmark (DTU) with the same extraction method and shipped on dry ice for shotgun metagenomic sequencing at the Oklahoma Medical Research Foundation (OMRF; Oklahoma City, OK, USA). In total, pooled faecal DNA collected from 181 pig farms, 178 broiler farms and 61 calf farms were shotgun sequenced on the HiSeq3000 platform (Illumina), resulting in > 36 billion sequences (18 billion paired-end reads). More details on the subsequent processing of the metagenomic data were described in our previous study.[4-6]

Comparison of ARG abundances between sample types, farms and countries

For all samples, the variation in relative abundances of four ARG targets (*aph(3')-III, ermB, sul2, tetW*) in the selected samples was visualized in box plots. We compared ARG loads between animals from nine different countries, between different environmental samples collected along Dutch pig and German broiler production lines, and between different human populations.

Principle component analyses (PCA) were performed to evaluate the similarities and differences in the distributions of the four relative ARG abundances across faecal samples from different species. The PCAs were run using the 'R' package *vegan*[25] on R version 4.0.3. Log_{10} transformation was used before PCA due to the right-skewed distribution of the data.[26]

Except for PCA results, all comparisons of AMR abundance in this study were conducted using a classic or Welch's analysis of variance (ANOVA) depending on the homogeneity of variance.[27, 28] In case of a significant difference in ANOVA ($p<0.05$), post-hoc tests (Games-Howell Post-Hoc Test[29] or Tukey's Honest Significant Difference test (Tukey HSD)[30]) were carried out to test differences between groups. Unless otherwise specified, appropriate post-hoc test p-values are reported in the comparison.

Variance component analyses

Variance component analysis (VCA) was conducted per ARG target with a null model (AMR ~ country + farm) to evaluate the geographical (between countries), between-farm, and within-farm variation of relative ARG abundance in faecal samples of four animal species (pigs, broilers, veal calves and turkeys) using the 'R' package *VCA*[31] on R version 4.0.3.[26] In addition, to be consistent with our study in pigs and broilers,[24] we also determined the variation contribution of farm characteristics (e.g. AMU, biosecurity measures) in veal calves and turkeys by adjusting these factors into the null model. More details were described in the Supplement (*'Variance component analyses in veal calves and turkeys'*).

Random-effects meta-analyses in pigs and broilers

We examined the relationship between AMU and relative ARG concentrations as assessed by qPCR for pooled faeces of pigs and broilers. Farm AMU datasets (group treatments) that were collected using a standardized questionnaire completed by farms were available for analysis.[5, 6, 32, 33] A meta-analysis with random effects by country was performed using the 'R' package *metafor*[34] as described before.[5, 6] The effect size of the association was adjusted using Benjamini–Hochberg false discovery rate (FDR) with 0.1 as a threshold. More details about the meta-analyses are described in the Supplement (*'Random-effects meta-analyses in pigs and broiler farms'*).

Correlation between pooled qPCR and pooled metagenomic data

As data were not normally distributed, Spearman's rank correlation was used to evaluate the correlation between relative abundances of four ARGs between pooled qPCR data and earlier published pooled metagenomic data.[4] To match the ARG targets of qPCR, all downstream gene abundances of *aph(3')-III, ermB, sul2* and *tetW* were collected from the metagenomic data [fragments per kilobase reference per million bacterial fragments (FPKM)] and summed per gene target. FPKM was $\log_{10}$ transformed after adding a pseudo-count of 1.

To be consistent with the correlation analysis in our study in pigs and broilers,[24] and turkeys[17], we assessed the correlation between individual qPCR data and pooled metagenomic data in veal calves. The median value of 7 samples per farm was used for the veal calf qPCR data.

Results

Table 1 describes the 9,572 samples analyzed by qPCR. Faeces, farm dust and retail meat samples from pigs and broilers were collected among all nine countries, while the other samples were collected in three or even fewer countries. Human samples were collected in the Netherlands and Germany, including pig and broiler farmworkers and their families (n=127), pig and broiler slaughterhouse workers (n=669), and a healthy control population (n=46) [14, 19, 23] (Table 1, Supplementary Table S2). After a quality check involving the evaluation of compliance with technical requirements and the limit of detection (LOD) and Limit of quantification (LOQ), 7,084 (74%) samples had detectable gene levels for *ermB* and 6,700 (70%) for *tetW*, while in 4,892 (80%) samples, *aph(3')-III* and in 4,543 (74%) samples, *sul2* could be detected (Table 1). Of all the samples, only a small amount of ARG targets (generally less than 10% of the total sample size) were detected in fish faeces.

Comparison of relative ARG abundances in animal faeces and dust between countries

Among all sample types (Figure 1, Supplementary Figure S1, Supplementary Table S3), the highest mean relative abundance of *tetW* was generally seen in farm animals, especially in pigs ($p>0.05$). For *aph(3')-III,* the mean relative abundance was highest ($p>0.05$) in broiler slaughterhouse faeces, and lowest ($p>0.05$) in wild boar faeces. For *sul2*, the mean relative abundance was highest in veal calf faeces ($p<0.01$), and lowest in wild boar ($p>0.05$). *For ermB and tetW,* the mean relative abundance was highest in dust collected on broiler farms and lowest in retail pork, but this was only significant for *tetW* in broiler farm dust ($p<0.05$). Cats showed higher ($p>0.05$) mean relative abundance than dogs for four ARGs.

For faeces samples, the country distribution of the relative abundances of four ARG targets varied highly between animal species (Figure 2). For broiler faeces samples, the lowest mean relative abundance was found in Denmark for *ermB*, *sul2* and *tetW*, and in Spain for *aph(3')-III* (only significant for *ermB* ($p<0.01$). For pig faeces samples, the lowest mean relative abundance was found in the Netherlands for all ARGs ($p>0.05$). The relative abundance of *aph(3')-III* in the Netherlands was significantly lower than all the other countries except

Table 1. Overview of all sample types and numbers of samples with detectable qPCR targets.

Description	Final sample country					Final sample number No. (%)					Total sample number
	16S	*aph(3')-III*	*ermB*	*sul2*	*tetW*	*16S*	*aph(3')-III*	*ermB*	*sul2*	*tetW*	
Pig faeces (farms)	All nine countries	All nine countries	All nine countries	All nine countries	All nine countries	1230 (97)	1084 (86)	1259 (100)	1042 (82)	1262 (100)	1265
Broiler faeces (farms)	All nine countries	All nine countries	All nine countries	All nine countries	All nine countries	884 (97)	847 (93)	902 (99)	840 (93)	898 (99)	908
EDC (pig farms)	All nine countries	All nine countries	All nine countries	All nine countries	All nine countries	462 (92)	431 (86)	477 (95)	436 (87)	475 (94)	503
EDC (broiler farms)	All nine countries	All nine countries	All nine countries	All nine countries	All nine countries	501 (90)	430 (77)	530 (95)	341 (61)	526 (94)	559
Pooled pig faeces (farms)	All nine countries	All nine countries	All nine countries	All nine countries	All nine countries	165 (92)	168 (94)	176 (98)	168 (94)	175 (98)	179
Pooled broiler faeces (farms)	All nine countries	All nine countries	All nine countries	All nine countries	All nine countries	169 (95)	170 (96)	177 (99)	170 (96)	176 (99)	178
Retail pork	All nine countries	.	All nine countries	.	BG, DK, ES, PL	848 (98)	.	140 (16)	.	8 (1)	862
Retail chicken	All nine countries	.	All nine countries	.	All nine countries	875 (99)	.	592 (67)	.	383 (43)	882
Turkey faeces (farms)	DE, ES, FR	DE, ES, FR	DE, ES, FR	DE, ES, FR	DE, ES, FR	290 (95)	263 (87)	299 (98)	264 (87)	301 (99)	304
Veal calf faeces (farms)	DE, FR, NL	DE, FR, NL	DE, FR, NL	DE, FR, NL	DE, FR, NL	405 (96)	339 (81)	415 (99)	387 (92)	420 (100)	420
Fish faeces (farms)	ES, FR, PL	FR, PL	ES, FR, PL	ES, FR, PL	ES, PL	270 (77)	5 (1)	27 (8)	140 (40)	6 (2)	352
Wild boar faeces	IT, PL	IT, PL	IT, PL	IT, PL	IT, PL	156 (78)	43 (22)	83 (42)	30 (15)	197 (98)	200
Cat faeces	BE, IT, NL	BE, IT, NL	BE, IT, NL	BE, IT, NL	BE, IT, NL	141 (95)	117 (79)	121 (81)	43 (29)	148 (99)	149
Dog faeces	BE, IT, NL	BE, IT, NL	BE, IT, NL	BE, IT, NL	BE, IT, NL	148 (99)	104 (69)	92 (61)	82 (55)	146 (97)	150
Retail turkey	DE, ES, FR	.	DE, ES, FR	.	DE, ES, FR	143 (99)	.	112 (77)	.	59 (41)	145
Retail veal	DE, ES, FR	.	DE, ES, FR	.	ES	130 (100)	.	38 (29)	.	2 (2)	130
Retail fish	FR, NL, PL	.	FP, PL	.	FR	122 (99)	.	6 (5)	.	1 (1)	123
Human faeces (pig farms)	NL, DE	NL, DE	NL, DE	NL, DE	NL	93 (99)	90 (96)	93 (99)	57 (61)	93 (99)	94
Human faeces (poutry farms)	NL, DE	NL, DE	NL, DE	NL, DE	DE	33 (100)	33 (100)	32 (97)	26 (79)	33 (100)	33
Pig faeces (slaughterhouses)	NL	NL	NL	NL	NL	59 (98)	58 (97)	60 (100)	20 (33)	60 (100)	60
Pig carcass (slaughterhouses)	NL	.	NL	.	NL	166 (84)	.	28 (14)	.	21 (11)	198
Gloves (pig slaughterhouses)	NL	.	NL	.	NL	310 (96)	.	178 (55)	.	148 (46)	324
Pig meat (slaughterhouses)	NL	.	.	.	.	190 (96)	.	.	.	.	198
Human faeces (pig slaughterhouses)	NL	NL	NL	NL	NL	479 (99)	441 (91)	480 (99)	296 (61)	480 (99)	483
Human faeces control	NL	NL	NL	NL	NL	46 (100)	46 (100)	46 (100)	17 (37)	46 (100)	46
Broiler faeces (slaughterhouses)	DE	DE	DE	DE	DE	60 (97)	47 (76)	62 (100)	55 (89)	62 (100)	62
Broiler carcass (slaughterhouses)	DE	.	DE	.	DE	198 (98)	.	159 (79)	.	146 (72)	202
Gloves (broiler slaughterhouses)	DE	.	DE	.	DE	231 (94)	.	208 (84)	.	190 (77)	247
Broiler meat (slaughterhouses)	DE	.	DE	.	DE	127 (98)	.	109 (84)	.	54 (42)	130
Human faeces (broiler slaughterhouses)	DE	DE	DE	DE	DE	180 (97)	176 (95)	183 (98)	129 (69)	184 (99)	186
Overall	All nine countries	All nine countries	All nine countries	All nine countries	All nine countries	9111 (95)	4892 (80)	7084 (74)	4543 (74)	6700 (70)	9572

Final sample number: All samples left after a quality check (technical standard, limit of detection (LOD) and limit of quantification (LOQ)). .: Missing values due to lack of information. BE: Belgium, BG: Bulgaria, DE: Germany, DK: Denmark, ES: Spain, FR: France, IT: Italy, NL: the Netherlands, PL: Poland. Some results have been reported or partly reported in previous papers: Veal calf faeces (farms).[16] Pig faeces (slaughterhouses), pig carcass (slaughterhouses), gloves (pig slaughterhouses), pig meat (slaughterhouses) and human faeces (pig slaughterhouses).[14] Turkey faeces (farms).[17] EDC (pig farms), EDC (broiler farms).[21] Pig faeces (farms), broiler faeces (farms).[24]

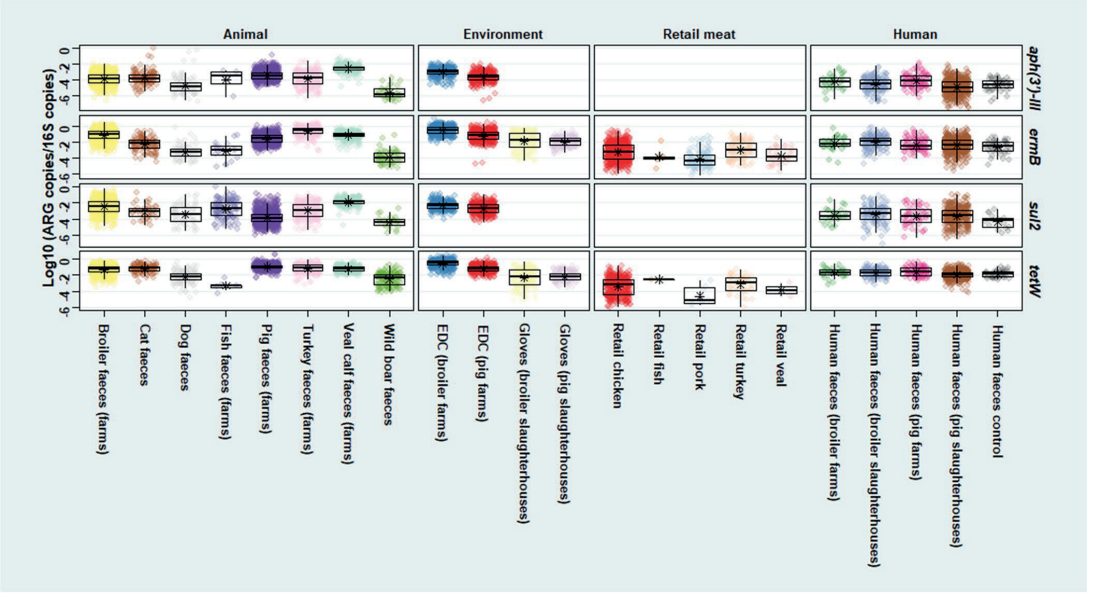

Figure 1. Relative abundances of four targets (*aph(3')-III, ermB, sul2, tetW*) in all samples.

Relative abundance of gene target was calculated by $\log_{10}$(gene copies/*16S* copies). Asterisk shows the mean by sample type. Pooled faeces, slaughterhouse pig and broiler faeces, and slaughterhouse carcass samples were not included in this figure.

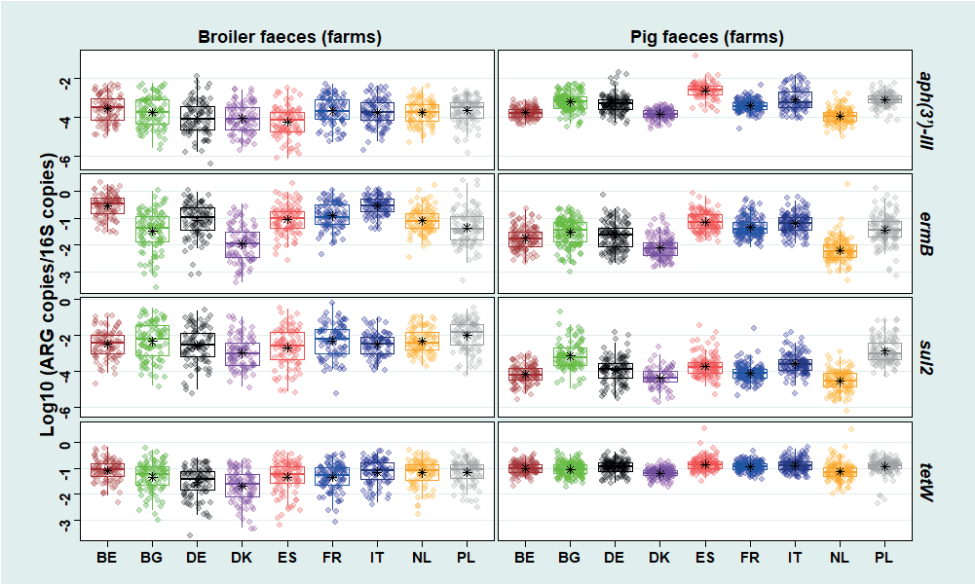

Figure 2. Relative abundances of four targets (*aph(3')-III, ermB, sul2, tetW*) in pig and broiler faeces sampled in nine EU countries.

Relative abundance of gene target was calculated by log10 (gene copies/*16S* copies).

Asterisk shows the mean by country. BE: Belgium, BG: Bulgaria, DE: Germany, DK: Denmark, ES: Spain, FR: France, IT: Italy, NL: the Netherlands, PL: Poland.

Denmark ($p<0.01$) (Figure 2). For faeces of other animal species (cat, dog, veal calves), the Netherlands generally showed the lowest relative abundance of all ARGs (Supplementary Figure S2). This was only significant for *aph(3')-III* ($p<0.05$) and *tetW* in veal calves ($p<0.01$).

In dust samples, we observed similar between-country variation. For broiler dust samples, the lowest mean relative abundance was found for *aph(3')-III* ($p<0.01$) and *tetW* ($p>0.05$) in Italy, and for *ermB* ($p>0.05$) in Denmark, and *sul2* ($p>0.05$) in France. For pig dust samples, the lowest relative abundance was found for *aph(3')-III*, *ermB* and *sul2* in Denmark, and for *tetW* in Italy. This was only significant for *sul2* in Denmark ($p<0.01$) (Supplementary Figure S3).

Relative ARG abundances across the pig and broiler production chain

We found an overall decreasing trend of the mean relative abundance of four ARGs along the production chain of pigs in the Netherlands and broilers in Germany, i.e. from farm to slaughterhouse to meat in the food store (Figure 3-4). Farm dust showed a higher ($p<0.01$) mean relative ARG abundance than faeces recovered from the same farms, except for *tetW* in pigs. In addition, we found a significantly higher ($p<0.01$) mean relative abundance of *aph(3')-III* in human faeces sampled in pig farms than in human faeces sampled in pig slaughterhouses. A significantly higher ($p<0.05$) mean relative *sul2* abundance was observed in human faeces sampled in pig slaughterhouses than in faeces of control subjects (Figure 3). Meanwhile, faeces from broiler farmers showed a higher mean relative abundance of all ARG targets than faeces from broiler slaughterhouse employees, which was significant for *aph(3')-III* ($p<0.01$) (Figure 4). Furthermore, we found higher mean relative *ermB* and *tetW* abundance in pig/broiler slaughterhouse carcasses than in slaughterhouse meat and retail meat, which was significant for *ermB* ($p<0.01$) (Figure 3-4).

With respect to environmental samples, absolute concentrations are difficult to compare due to the different metrics used (e.g. copies/m^2 surface/day for EDC, versus copies/g for faeces, and copies/glove for gloves).

Determination of ARG patterns' variation by animal species

The faecal resistome, here measured as the frequency distributions of four ARG targets (*aph(3')-III, ermB, sul2* and *tetW*) in faeces, differed between animal species (Figure 5). High distribution overlap of ARG abundances was found between farm animals and differed from wild boars and dogs. The results of fish were not shown in Figure 5 as there were too many missing values per gene target. In the production chain of pigs and broilers, farm dust samples generally showed higher abundance for all ARGs (Supplementary figure S4). Faeces and production environment samples showed higher relative *tetW* and *ermB* abundance than human faeces in both farms and slaughterhouses. ARG distribution overlapped obviously between carcass and gloves and differed from human samples (Supplementary figure S5-S8).

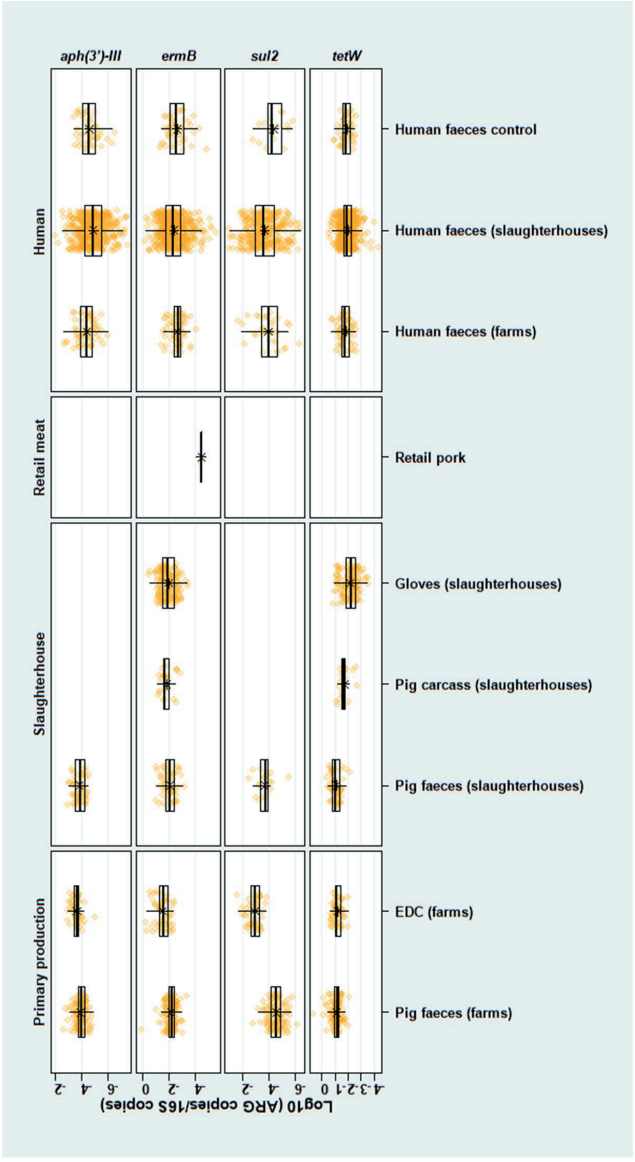

Figure 3. Relative abundance of four targets (*aph(3')-III, ermB, sul2, tetW*) in the exposure chain of pigs in the Netherlands.

Relative abundance of gene target was calculated by $\log_{10}$(gene copies/*16S* copies). Only pig farms and slaughterhouses in the Netherlands were involved. Human faeces of control subjects were collected from the 'Lifelines' cohort in the Netherlands. Asterisk shows the mean by sample type in the Netherlands.

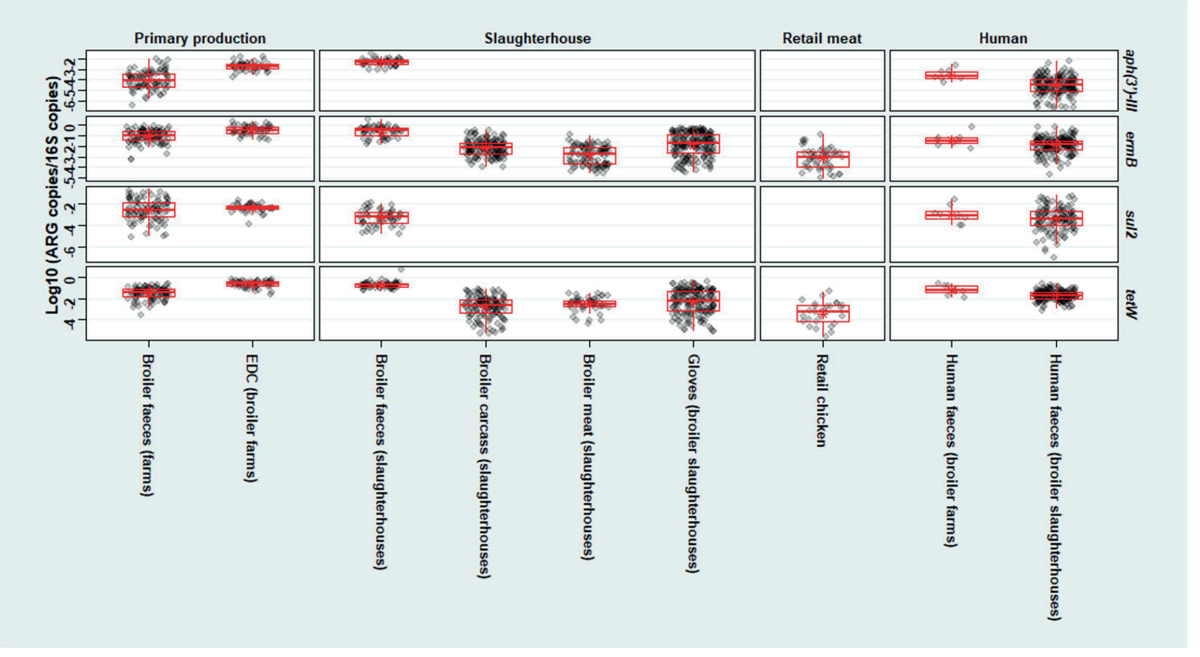

Figure 4. Relative abundance of four targets (*aph(3')-III, ermB, sul2, tetW*) in the exposure chain of broilers in Germany.

Relative abundance of gene target was calculated by log10 (gene copies/*16S* copies). Only broiler farms and slaughterhouses in Germany were involved. Asterisk shows the mean by sample type in Germany.

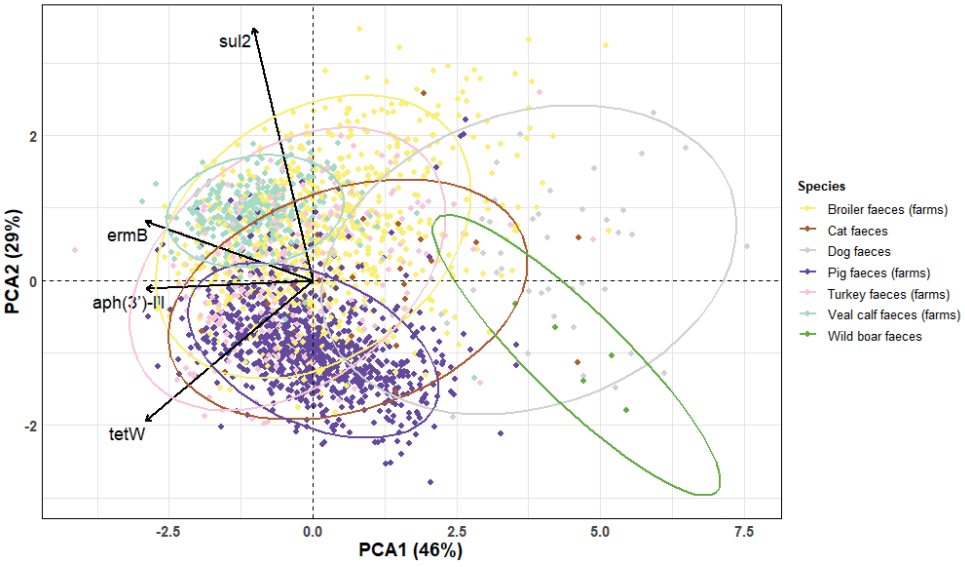

Figure 5. Principal component analysis biplot of relative ARG abundances in animal faeces samples.

ARG targets: *aph(3')-III, ermB, sul2, tetW.* Relative abundances of the gene targets were calculated by $\log_{10}$(gene copies/*16S* copies). Symmetric scaling was used. Circles indicate 95% confidence ellipses that were computed with the assumption of multivariate normal distribution of the data.

Variance component analyses of relative ARG abundances

In the null model for pigs, broilers, veal calves and turkeys, we generally found a low (absolutely and relatively) within-farm AMR variation and a high geographical and between-farm variation in pig faeces. In the other three animals species, we generally found a high (absolutely and relatively) within-farm variation in faeces AMR (Figure 6, Supplementary Table S4).

In the adjusted VCA model for veal calves and turkeys (Supplementary Table S5-S6), we found that between-country and between-farm variation could partly be explained by AMU and biosecurity measures. For example, in the adjusted model for *sul2* in veal calves, the use of trimethoprim and sulfonamide accounted for 6.78% of the total variation, while the contribution of between-country variation decreased from 11.49% to 6.07%. In the adjusted model for *ermB* in turkeys, the biosecurity measure 'Visitor access more than once a month' accounted

for 13.70% of the total variation, while the contribution of between-farm variation decreased from 46.56% to 30.66%. For pigs and broilers, the adjusted VCA models are described elsewhere.[24]

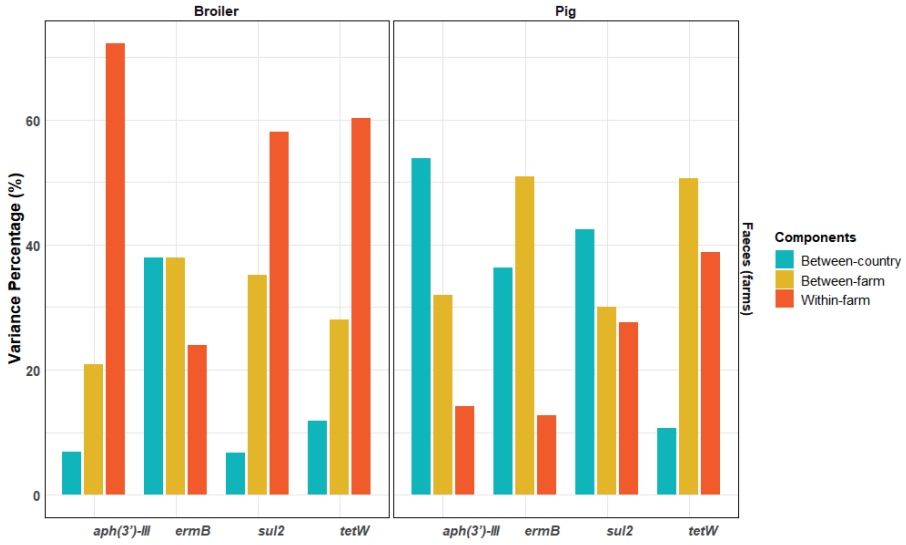

Figure 6. Variance component percentages of relative ARG abundances in faecal samples from pigs and broilers.

Relative abundance of gene target was calculated by $\log_{10}$(gene copies/*16S* copies). Variance percentages of three components (between-country variance, between-farm variance and within-farm variance) were calculated using the variance component analysis.

Random-effects meta-analyses in pig and broiler farms

For 179 pig farms and 178 broiler farms, AMU and relative ARG abundance data were available for analysis. In the meta-analyses that involved all possible AMU and ARG combinations, we found a statistically significant (FDR $p < 0.1$) positive association between relative *ermB* abundance and macrolides use in pigs and broilers (Figure 7, Supplementary Table S7-S8). Furthermore, in pigs, we found a statistically significant (β=0.56, FDR $p < 0.1$) positive association between total AMU during the fattening period and relative *aph(3')-III* abundance (Supplementary Table S7). In broilers, we found a statistically significant (FDR $p < 0.1$) positive association between aminoglycosides use and relative *aph(3')-III* abundance, and between tetracyclines use and relative *tetW* abundance (Supplementary Table S8).

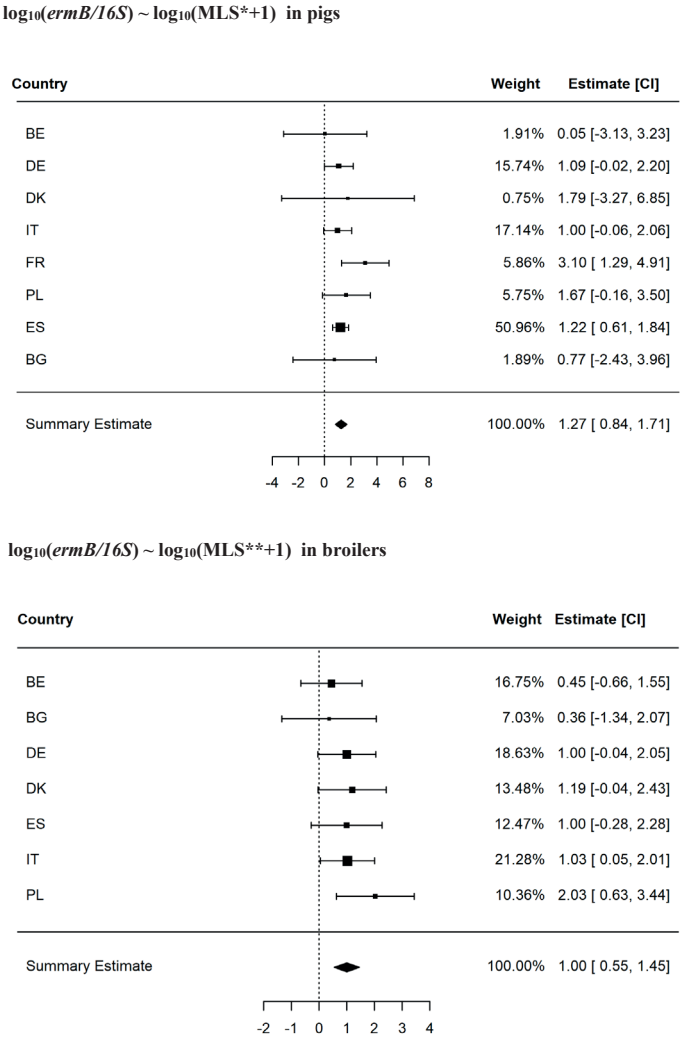

Figure 7. Meta-analysis of relative *ermB* abundance and MLS use in pigs and broilers.

*: Macrolide + lincosamide + spectinomycin use in fatteners in pig farms. **: Macrolide + lincosamide + spectinomycin use in broiler farms. The relative *ermB* abundance was measured from pooled faecal samples in pig and broiler farms.

Comparison of ARG abundances between qPCR results and metagenomic data

In total, respectively 4, 15, 19 and 5 gene variants in the metagenomic data were allocated to *aph(3')-III*, *ermB*, *sul2* and *tetW*. For pigs and broilers, we found a high correlation ($\rho>0.7$, $p<0.01$) between qPCR data and

metagenomic data of pooled faecal samples for four ARG targets, except for *tetW* ($\rho<0.7$, $p<0.01$) in pigs (Figure 8). Meanwhile, for veal calves, a low correlation ($\rho<0.7$, $p<0.01$) was observed for all ARGs between median individual qPCR data and pooled metagenomic data (Supplementary Figure S9).

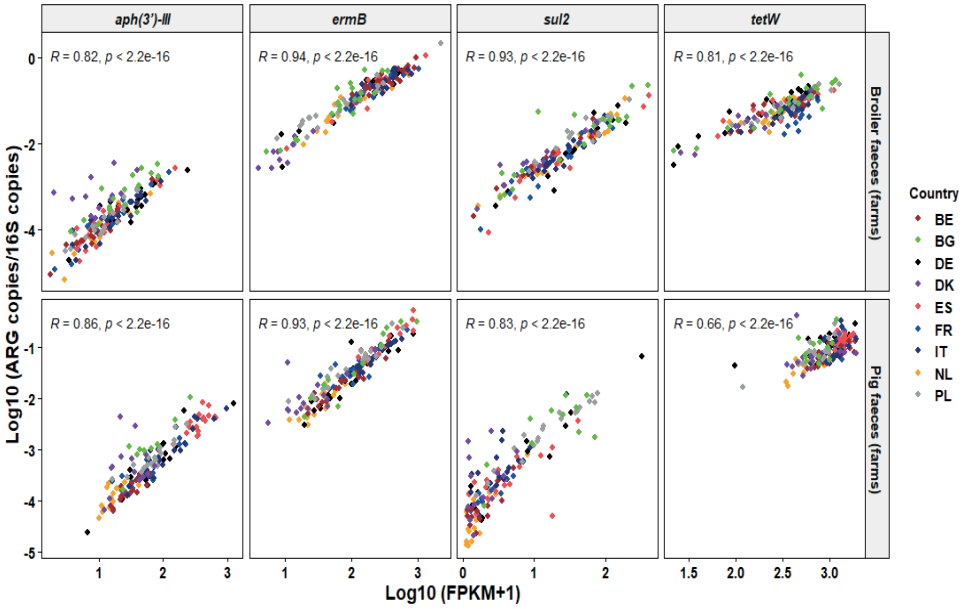

Figure 8. Spearman's rank correlation of relative ARG abundances and ARG FPKM abundances in pooled faeces samples from pig and broiler farms.

ARG targets: *aph(3')-III, ermB, sul2, tetW*. Relative abundance of gene target was calculated by $\log_{10}$(gene copies/*16S* copies). FPKM: Fragments per kilobase reference per million bacterial fragments.[35] FPKM was log10 transformed after adding a pseudo-count 1. BE: Belgium, BG: Bulgaria, DE: Germany, DK: Denmark, ES: Spain, FR: France, IT: Italy, NL: the Netherlands, PL: Poland

Discussion

To the best of our knowledge, this is the first study that applied qPCR on a large scale to explore the association between ARG abundances from different sources, animal species and humans in nine European countries in more than 9,500 samples. ARG abundances varied for four ARG targets (*aph(3')-III, ermB, sul2* and *tetW*) across different sample types. A significant decrease in relative ARG abundances was observed along both pig

and broiler production chains (from farm to slaughterhouse to food store). In addition, the geographical variation (between countries) and between-farm variation in pigs, broilers, veal calves and turkeys could partially be explained by AMU and biosecurity levels. Furthermore, we found a statistically significant positive association between AMU and ARG abundances in both pigs and broilers. A high correlation between qPCR and metagenomically assessed ARGs in pooled faeces samples was found in pigs and broilers.

Comparison of ARG abundances between animal species

We explored the abundances of different ARGs across animals and speculate that the observed variation is partly a result of differences in AMU exposure. For example, the highest mean relative abundance of *tetW* was observed in farm animals, especially in pigs. This could be explained by previous findings in EFFORT,[32, 33] in which a higher proportion of tetracyclines use was found in pig farms (15.3%)[32] than in broiler farms (11%)[33] among nine countries. Still, as there is a strong association between the faecal microbiome and the resistome,[4, 20, 22] the difference in ARG abundances between species can also be related to differences in microbiome compositions. In fish samples, we only detected a small amount of ARGs. This may be due to the sampling procedure in our study, as fish faecal samples were collected from frozen guts, which made it difficult to separate fish intestines and faecal contents, leading to a high proportion of host DNA. As a result, AMR levels of fish faeces may be underestimated. In the future, more appropriate methods for collecting fish faeces samples are worthy of studying.

Wild boars showed the lowest mean ARG abundance among all animal species, which is likely the result of negligible AMU exposure of wild animals.[36, 37] This is consistent with previous results of European wild animal AMR studies.[38-40] Last, in companion animals, we saw higher relative ARG abundance in cat faeces than in dog faeces. Similar results were described in previous studies.[18, 41] But as previous EFFORT study showed no AMU-AMR association in companion animals,[18] this higher ARG abundance in cat faeces may be due to the fact that cats roam more freely than dogs,[42] through which cats are probably exposed to more environmental sources than dogs.

ARG abundances decline across the pig and broiler production chain

In pig and broiler production chains, a decline in relative ARG abundances was seen from primary production to slaughterhouse and retail meat. The possible explanation is that individuals and livestock may be exposed to AMR through animal faeces and dust in farms. In this study, the relative ARG abundance in farm dust was higher than in farm animal faeces in pig and broiler farms, which is consistent with previous findings using

metagenomic data.[20] The explanation may be that microorganisms in dust sources such as animal faeces[20, 21] and constituents (skin, feather)[43-45] are richer in ARGs. The relative ARG abundance in retail chicken meat was found to be significantly lower than that of carcasses and meat samples of pig and broiler slaughterhouses. One potential explanation is that the production steps along the slaughterhouse line, including the cooling process, reduce bacterial loads and related AMR.[14, 46] More research is needed in the future to link these changes to specific production steps.

Geographical and between-farm and within-farm variation of AMR in pigs, broilers, veal calves and turkeys

For pigs and broilers, substantial between-country variation in ARG levels was previously reported using metagenomics analysis of pooled faecal samples.[4-6] Our qPCR results on samples from the same farms, also allow quantification and comparison of within-farm variation per country. This was not possible in metagenomics analyses, in which samples were generally pooled on the farm level. To put between-country variations in perspective, the results in our study showed a considerable within- and between-farm variation for ARG levels in pig and broiler farms, which can be larger than the between-country variation. In addition, for the relative gene abundance in pig faeces samples, within-farm variation was in most cases smaller than between-farm variation which was comparable to between-country variation, pointing to single farms as relevant epidemiological units to test ARG prevalence (and determinants) in pig farms. In stark contrast, within-farm variation dominated the relative ARG abundance in broiler faeces, while the smallest variation was found between countries. This overlap in ARG abundance between countries could probably be explained by broilers' pyramid breeding structure.[47]

For veal calves and turkeys, we found a high within-farm variation. Considering the large variation in individual characteristics,[48, 49] it is not surprising that the AMR abundance varies highly among animals per farm. The other explanation is the limited sample size (sample numbers per farm (7/5) and country numbers (3)) in veal calves and turkeys. The different farm management and practices may also be associated with the distribution of AMR variation.

Determinant variation of AMR in veal calves and turkeys

In our previous adjusted VCA models of pigs and broilers,[24] we observed that geographical and between-farm variation were partly explained by AMU. However, in the present study on veal calves and turkeys, we found

that in addition to AMU, the geographical and between-farm variations were also partly explained by farm biosecurity measures. Although the small sample size of veal calves and turkeys may have reduced statistical power in our study, our results provide evidence that both sources of variation (country and farm) contributed by AMU and farm biosecurity.

Relationship between AMU and relative ARG abundances in pigs and broilers

To the best of our knowledge, there is currently no study that compares AMR abundance using qPCR and metagenomics in the same animal samples in parallel. In this context, we assume that the positive association with AMU can also be seen based on qPCR results of pigs and broilers like previous metagenomics studies.[5, 6] The meta-analysis results in the present study showed that this was indeed the case.

For broilers, we observed statistically significant positive associations between all corresponding AMR (except for *sul2*) and AMU, which is consistent with the previous studies[6]. More evidence was shown by the high correlation between pooled qPCR and pooled metagenomic data.[50] This also indicates that qPCR with pooled data can be an alternative cost-effective approach for the quantitative analysis of ARG targets when the project budget is limited.

However, compared with AMU-AMR associations in the previous EFFORT study in pigs,[5] we found that in this study, only the association between macrolides use and relative *ermB* concentration remained statistically significant (FDR $p < 0.1$). This points to one limitation of qPCR method that selected specific ARGs could not capture all the gene variation within a particular class of resistance genes.

ARG abundances in humans

Previous studies reported that farmworkers had higher nasal MRSA prevalence than slaughterhouse workers[51] and AMR exposure in pig farms was higher than in pig slaughterhouses[52]. Similar results were found in this study where pig farm workers showed significantly higher mean relative *aph(3')-III* abundance than pig slaughterhouse workers. Although the exposure difference, as well as other determinants (working hours, life history, etc.) within the production chain may affect workers' ARG carrying,[14] we argue that people working in pig/broiler farms are most likely to be exposed to ARGs than people working in pig/broiler slaughterhouses, especially considering the high farm dust levels.[20] In the future, more in-depth research is needed to reproduce and confirm these findings.

Conclusion

This study shows that qPCR analysis is a valuable tool to assess the abundances of selected ARGs in a large amount of livestock-associated samples collected across Europe. High variation of ARG abundances assessed using qPCR was found across animal species, environmental samples, and humans. A decreasing trend in ARG levels from 'farm to fork' was found for both pigs and broilers. The geographical and between-farm variation could be partially attributed to AMU and farm biosecurity levels. The corresponding AMU-AMR positive association has been found. Occupational livestock AMR exposure is related to the ARG abundances in human faeces.

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Transparency declarations

'Lifelines' research was conducted according to the protocols approved by the Medical Ethics Review Board of the Medical Center Groningen (NL) (Protocol METc2007/152). Written consent was received from all participants. The Medical Ethical Committee of the University Medical Centre Utrecht (NL) confirmed that the Dutch 'Medical Research Involving Human Subjects Act' did not apply for the study of the EFFORT research (Protocols 14–346/C, 14–403/C). Participants in EFFORT were compensated financially (100 euro per farm family, 25 euro per slaughterhouse employee).[4, 22]

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Supplementary Materials

Antimicrobial resistance genes *aph(3')-III, ermB, sul2* and *tetW* abundance in animal faeces, meat, production environments and human faeces in Europe

Methods

Sampling procedure of faeces

Faecal samples were collected at pig and broiler farms in all nine European countries, while only three countries were involved for the other farm animals (veal calves (DE, FR, NL), turkeys (DE, ES, FR) and fish (trout) (ES, FR, PL)). For each species, 25 fresh faecal samples were collected randomly from 20 conventional farms in each country that complied with the inclusion criteria as described previously etc.[1-6] Faecal samples were collected from animals as close to slaughter as possible, sterile spoons were used in the faecal samples collection, pooled faeces were prepared by mixing 25 individual faecal samples (contributing equal mass) from each herd, etc.[1-6] Raw meat samples (100 pork chops,[7] 100 chicken legs (drumsticks) with skin, 50 veal steaks, 50 turkey legs (drumsticks) with skin and 50 rainbow trouts) were collected at supermarkets from conventionally produced animals.

Faecal samples of 50 cats and 50 dogs that met the inclusion criteria (minimum age of 1 year, not living on the farm, etc) were collected in each of the three countries (BE, IT, NL). More details about the sampling procedure of cats and dogs have been described in the previous paper.[8]

Faecal samples of 100 individual wild boars from two countries (IT, PL) were included that met the inclusion criteria, including 1) the preferred body gross weight is ≥ 50 kg; 2) the animals to be included should be from at least 20 hunting events in one season. 3) preferably not more than 5 animals per hunting. 4) preferably all collected within a single hunting season. 5) preferably regionally distributed per country.

To explore the change of antimicrobial resistance gene (ARG) abundances across the exposure chain of certain animals (pigs and broilers),[6] farm dust samples[9, 10] (without being pooled) and slaughterhouse samples[7] (faeces, carcass, gloves and meat) were collected. From 2015 to 2017, we visited two pig slaughterhouses in the Netherlands and three broiler slaughterhouses in Germany in total.

In addition, human faecal samples (from farmers, slaughterhouse workers and control subjects) were collected to compare with animal and environmental samples. Pig farmers, broiler farmers, veterinarians and their family members were asked to collect their faecal samples.[11] Furthermore, human faecal samples were collected from the pig slaughterhouses in the Netherlands and the broiler slaughterhouses in Germany.[7, 12] Human faecal samples from the Dutch 'Lifeline' cohort were used as nonexposed control population in this study.[11, 13]

ARG targets selection process

The choice of genes for study in quantitative polymerase chain reaction (qPCR) was based on results from initial metagenomic and qPCR analyses. When selecting qPCR targets, metagenomic data was available for animal faeces of pig and broiler. In addition, *tetW* had been analysed by qPCR in a subset of pig faecal samples, resulting in estimates of the limit of detection of *tetW* by qPCR. First, combining the limit of detection of qPCR of *tetW* with the *tetW* qPCR signal and the relative abundance of *tetW* in metagenomics analyses of selected samples, a cut-off for the relative abundance of resistance genes in metagenomes was defined that samples had to meet in order to be quantifiable by qPCR in general. In other words, in this case, metagenomics was more sensitive than qPCR. Gene targets were considered for inclusion in qPCR if they were predicted to be quantifiable in ≥90% (for quantitative analyses) or 25% (for qualitative analyses) of all pig and broiler faecal samples, by comparison of the gene signal in metagenome and predicted qPCR detection limit. Quantitative analyses were predicted to be possible for resistance genes belonging to the classes of tetracyclines, aminoglycosides, and macrolides. With qualitative analyses, additionally, genes for resistance to the classes of glycopeptides and sulfonamides were also deemed possible. Several resistance genes which had been considered beforehand due to their high public health relevance (*mcr* for polymyxins resistance, bla_{CMY}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{Z}, and *mecA* for beta-lactam resistance, and *qnrB* for quinolones resistance) did not meet these cut-offs. Next, individual genes were chosen that had a high relative abundance within the respective antibiotic class and a moderate correlation with other resistance genes as judged from metagenomics analyses, namely *aph(3')-III* (aminoglycosides), *ermB* (macrolides), *sul2* (sulfonamides) and *tetW* (tetracyclines). The maximum Spearman correlations between the four genes were 0.47 (for broiler faeces, between *ermB* and *tetW*) and 0.72 (for pig faeces, between *aph(3')-III* and *ermB*). PCR primers were identified from the literature and evaluated versus the ResFinder database of sequence variants, resulting in slight primer changes for *ermB*. In addition, 25-73 bacterial strains with and without the presence of the studied resistance gene were PCR analyzed to determine the specificity and selectivity of these primers.

qPCR process

Not all samples have been subjected to qPCR for all five gene targets (*16S*, *aph(3')-III, ermB, sul2, tetW*). For meat, animal carcasses, and glove samples (3441 samples in total), qPCR was only performed for *16S*, *ermB* and *tetW*. This was decided based on tests of *aph(3')-III* and *sul2* in a subset of meat samples, showing that only a minor proportion of meat samples would result in quantifiable levels of these two genes.

The qPCR processes of 16S, *ermB* and *tetW* were described in (L. Van Gompel, et al.).[7] Analyses were based on centralized DNA extraction and qPCR (DNA extraction and PCR for a specific gene exerted only in one partner's lab), with the exception of meat DNA which was conducted in each partners' lab. This was decided after ring tests in which faecal and meat DNA was extracted at different laboratories and analysed by qPCR for *tetW* in one lab. For *aph(3')-III* and *sul2*, methods differed from *tetW/ermB* with respect to some details. Briefly, *aph(3')-III* and *sul2* qPCRs were performed in Poland (National Veterinary Research Institute, Puławy). For both DNA targets, the PCR reaction (10 µl) consisted of 5 µl SsoAdvanced™ Universal Probes Supermix (Bio-Rad, USA)), 3 µl DNA template, as well as primers (*aph(3')-III*: 400 nM; *sul2*: 100 nM) and a probe (*aph(3')-III*: 250nM, *sul2*: 100nM) (Table S1).

Large amounts of fat or humic acid in the DNA extract can inhibit PCR. To rule out qPCR inhibition, preliminary experiments (*tetW* and 16S qPCR) of a part of all samples (about 120 samples in total, distributed on different sample types) was carried out. Dilutions were established at which PCR inhibition did not occur, which were defined as the concentrations at which the observed concentration does not change from a given dilution to the next lower dilution. The precise dilutions were 1:500 for wild boar faeces, 1:100 for other faeces, 1:50 for dust, and 1:20 for meat and gloves. All samples were diluted centrally (with TE-buffer pH8 high EDTA (Thermo Fisher Scientific, Vilnius, Lithuania), and aliquots of these dilutions were sent to the participating labs. In addition, synthetic DNA coding for the blue fluorescence protein (bfp) was used as internal amplification control (IAC). In some cases (*tetW* in faecal samples), IAC was omitted as *tetW* concentrations were so high that IAC PCR was outcompeted (as judged from titrations of IAC versus *tetW*). The IAC identifies samples with substances that inhibit the PCR reaction by shifting the IAC signal to a higher threshold (Ct) value. Bfp primers (*aph(3')-III*: 400 nM; *sul2*: 100 nM) and a probe (*aph(3')-III*: 250nM, *sul2*: 100nM) were added.

Standard curves were constructed using synthetic DNA. A joint calibration curve is determined from all the individual plates in the iterative process. First, include all calibration curve samples, and perform a linear regression to generate the first calibration curve. Then, all individual measurement values that exceed 2 Ct in the joint calibration curve are declared as outliers and excluded from the calibration curve. The calibration curve was calculated again without these values, and this process was repeated until no further deviations are observed.

In each PCR plate, 8 dilutions of the standard curve were run. For different sample types, slightly different dilutions were used to adjust the expected Ct range. qPCR was centralized, i.e. preparation of each assay and

standard curves was centralized in one single laboratory. In total, 4 positive and 8 negative control samples (TE buffer pH8, Thermo Fisher Scientific, USA) were used per PCR plate. All samples and controls were run in two technical PCR duplicates.

Quality control and quantification of qPCR results

During quality control, all samples were firstly evaluated for amplification of the IAC (samples that showed a signal for the IAC that was greater than the mean+2 standard deviations of the IAC results of the calibration curves were flagged for possible inhibition). Secondly, the consistency of two PCR technical duplicates was checked based on inspection of their deviations, and acceptable deviations were derived for different Ct ranges, based on the distribution of deviations observed in all samples.

Furthermore, the limit of detection (LOD) and Limit of quantification (LOQ) values were determined for qPCR quantification. The LOD was defined with an optimisation of the number of false-positive or false-negative samples based on receiver-operator curves (ROC).[7, 14] Non-template controls were used to derive parameters for false-positive samples, and calibration curve samples were used to derive parameters for false-negative samples. The Youden index was used to determine the optimal Ct as a cut-off point between false-positive and false-negative sample annotations. The LOQ was defined as the highest Ct value based on the tolerable absolute deviation in log gene copies between true concentrations and the calibration curve. The percentage of the calibration curve sample within the 2 Ct range below the LOQ that deviates from its true concentration by more than 1 log copy is less than 5%.

LODs, LOQs and qPCR efficiencies were applied and computed as follows (LOD, LOQ, qPCR efficiency percentage): 16S – 3.11, 3.11, 103%; *aph(3')-III* – 0.22, 0.52, 101.6%; *ermB* – 1.52, 2.02, 94.3%; *sul2* – 0.92, 0.92, 93.6%; *tetW* – 0.64, 2.16, 97.1%.

Table S1: **Overview of target sequences in qPCR.**

Gene	Sequence/forward	Sequence/reverse	Sequence/probe	Reference	Modifications to standard protocols
16S	ACTCCTACGGGAG GCAGCAG (338F)	ATTACCGCGG CTGCTGG (518R)	n/a	Fierer *et al.* [15]	Primer concentrations: 200 nM Annealing temperature: 60°C /45 sec
ermB	GGTTGCTCTTGCAC ACTCAA	CAGTTGACGAT ATTCTCGATTG	AACTTACCCGCCAT ACCACAGATGTTC	Koike *et al.* [16]	Primer and probe concentrations: resp. 400 nM and 250 nM Annealing temperature: 61°C/1 min
tetW	CGGCAGCGCAAAG AGAAC	CGGGTCAGTAT CCGCAAGTT	6FAM-CTGGACGCT CTTACG-TAMRA -BHQ1	Walsh *et al.* [17]	Primer and probe concentrations: resp. 600 nM and 200 nM Annealing temperature: 59°C/45 sec
aph(3')-III	ACATATCGGATT GTCCCTATACGAA	TCGGCCAGA TCGTTATTCAGTA	AGACAGCCGCTT AGCCGAATTGGATT	Woegerbauer et al.[18]	Primer and probe concentrations: resp. 400 nM and 250 nM Annealing temperature: 60°C/20 sec
sul2	CGGCTGCGCTTC GATT	CGCGCGCAG AAAGGATT	CGGTGCTTCTGTC TGTTTCGCGC	Heuer et al. [19]	Primer and probe concentrations: resp. 100 nM and 100 nM Annealing temperature: 60°C/60 sec
Internal amplification control:					
Bfp	CAACGTCTATATCA TGGCCGAC	CCGTCCTCGAT GTTGTGG	HEX-TGAAGTTCGC CTTGATGCCGTTCT -BHQ1	de Rooij *et al.* [20]	Primer concentrations: *ermB* 400nM, *tetW* 600nM *aph(3')-III* 400nM, *sul2* 100nM

Variance component analyses in veal calves and turkeys

To determine the variance contribution of all the factors, variance component analysis (VCA) was conducted firstly in the null model (antimicrobial resistance (AMR) ~ country + farm) using the 'R' package *VCA*.[21] In addition, factors (antimicrobial use (AMU) and other farm characteristics) that were determined to be associated with AMR in our previous study in veal calves[4] and turkeys[5] were included in the null model. The variance components were inspected for each ARG target (*aph(3')-III, ermB, sul2, tetW*).

Random-effects meta-analyses in pig and broiler farms

Briefly, for each country, ARG targets were linearly regressed on AMU separately before estimates were combined in a meta-analysis by country. Before the meta-analysis, AMR data were standardized (mean=0, sd=1) to avoid countries having a large effect on the analysis weights. For both pigs and broilers, corresponding combinations of AMU and ARGs were tested firstly (aminoglycosides use vs *aph(3')-III*, macrolides use vs *ermB*, trimethoprim & sulfonamide use vs *sul2* and tetracyclines use vs *tetW*). Subsequently, all possible combinations of AMU and ARG targets were tested to determine the potential cross-selection of AMU against ARGs. In this process, a multiple testing correction was performed [Benjamini-Hochberg false discovery rate (FDR $p < 0.1$)]. For pigs, associations were only preserved when at least four countries were incorporated to be consistent with the previous analysis.[3] For broilers, all associations were involved due to low AMU levels.[2] Assumptions of all statistically significant associations (FDR $p < 0.1$) were checked using diagnostic tests.

Results

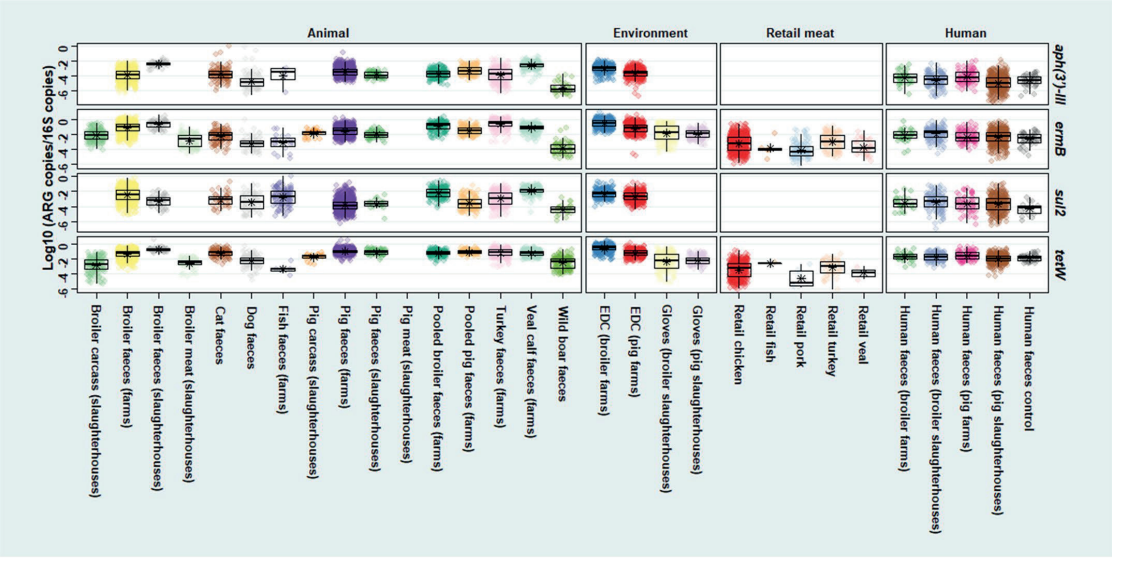

Figure S1. Relative abundances of four targets (*aph(3')-III*, *ermB*, *sul2*, *tetW*) in all samples.

All samples in our study are shown in this figure. Relative abundance of the gene targets was calculated by $\log_{10}$(gene copies/*16S* copies). Asterisk shows the mean by sample type. EDC: Electrostatic dustfall collector.

Table S2. Overview information table of all the sample types in the relative qPCR results.

Description	Final sample country				Final sample number No. (%)				Total sample number
	aph(3')-III	*ermB*	*sul2*	*tetW*	*aph(3')-III*	*ermB*	*sul2*	*tetW*	
Pig faeces (farms)	All nine countries	All nine countries	All nine countries	All nine countries	1058 (84)	1225 (97)	1014 (80)	1228 (97)	1265
Broiler faeces (farms)	All nine countries	All nine countries	All nine countries	All nine countries	826 (91)	878 (97)	819 (90)	874 (96)	908
EDC (pig farms)	All nine countries	All nine countries	All nine countries	All nine countries	426 (85)	456 (91)	427 (85)	454 (90)	503
EDC (broiler farms)	All nine countries	All nine countries	All nine countries	All nine countries	422 (75)	495 (89)	335 (60)	498 (89)	559
Pooled pig faeces (farms)	All nine countries	All nine countries	All nine countries	All nine countries	160 (89)	164 (92)	156 (87)	163 (91)	179
Pooled broiler faeces (farms)	All nine countries	All nine countries	All nine countries	All nine countries	166 (93)	169 (95)	163 (92)	168 (94)	178
Retail pork	.	All nine countries	.	BG, DK, ES, PL	.	138 (16)	.	8 (1)	862
Retail chicken	.	All nine countries	.	All nine countries	.	588 (67)	.	379 (43)	882
Turkey faeces (farms)	DE, ES, FR	DE, ES, FR	DE, ES, FR	DE, ES, FR	251 (83)	286 (94)	254 (84)	288 (95)	304
Veal calf faeces (farms)	DE, FR, NL	DE, FR, NL	DE, FR, NL	DE, FR, NL	330 (79)	400 (95)	372 (89)	405 (96)	420
Fish faeces (farms)	FR, PL	ES, FR, PL	ES, FR, PL	ES, PL	4 (1)	19 (5)	124 (35)	5 (1)	352
Wild boar faeces	IT, PL	IT, PL	IT, PL	IT, PL	36 (18)	62 (31)	24 (12)	154 (77)	200
Cat faeces	BE, IT, NL	BE, IT, NL	BE, IT, NL	BE, IT, NL	114 (77)	116 (78)	41 (28)	141 (95)	149
Dog faeces	BE, IT, NL	BE, IT, NL	BE, IT, NL	BE, IT, NL	102 (68)	91 (61)	80 (53)	144 (96)	150
Retail turkey	.	DE, ES, FR	.	DE, ES, FR	.	111 (77)	.	58 (40)	145
Retail veal	.	DE, ES, FR	.	ES	.	38 (29)	.	2 (2)	130
Retail fish	.	FP, PL	.	FR	.	6 (5)	.	1 (1)	123
Human faeces (pig farms)	NL, DE	NL, DE	NL, DE	NL, DE	89 (95)	92 (98)	56 (60)	92 (98)	94
Human faeces (broiler farms)	NL, DE	NL, DE	NL, DE	NL, DE	33 (100)	32 (97)	26 (79)	33 (100)	33
Pig faeces (slaughterhouses)	NL	NL	NL	NL	57 (95)	59 (98)	19 (32)	59 (98)	60
Pig carcass (slaughterhouses)	.	NL	.	NL	.	28 (14)	.	21 (11)	198
Gloves (pig slaughterhouses)	.	NL	.	NL	.	176 (54)	.	146 (45)	324
Pig meat (slaughterhouses)	.	.	.	.	.	.	.	.	198
Human faeces (pig slaughterhouses)	NL	NL	NL	NL	438 (91)	477 (99)	295 (61)	476 (99)	483
Human faeces control	NL	NL	NL	NL	46 (100)	46 (100)	17 (37)	46 (100)	46
Broiler faeces (slaughterhouses)	DE	DE	DE	DE	46 (74)	60 (97)	54 (87)	60 (97)	62
Broiler carcass (slaughterhouses)	.	DE	.	DE	.	157 (78)	.	144 (71)	202
Gloves (broiler slaughterhouses)	.	DE	.	DE	.	206 (83)	.	189 (77)	247
Broiler meat (slaughterhouses)	.	DE	.	DE	.	107 (82)	.	53 (41)	130
Human faeces (broiler slaughterhouses)	DE	DE	DE	DE	172 (92)	178 (96)	126 (68)	180 (97)	186
Overall	All nine countries	All nine countries	All nine countries	All nine countries	4776 (78)	6860 (72)	4402 (72)	6469 (68)	9572

Final sample number: All samples that left after a quality check (technical standard, LOD and LOQ). .: Missing values due to lack of information. BE: Belgium, BG: Bulgaria, DE: Germany, DK: Denmark, ES: Spain, FR: France, IT: Italy, NL: the Netherlands, PL: Poland. Some results have been reported or partly reported in previous papers: Veal calf faeces (farms).[4] Pig faeces (slaughterhouses), pig carcass (slaughterhouses), gloves (pig slaughterhouses), pig meat (slaughterhouses) and human faeces (pig slaughterhouses).[7] Turkey faeces (farms).[5] Electrostatic dustfall collector (EDC) (pig farms), EDC (broiler farms).[10] Pig faeces (farms), broiler faeces (farms).[6]

Table S3. Summary statistics of the qPCR results per sample type – relative abundances.

Description	Mean				10-90th percentile				Standard deviation			
	aph(3')-III	*ermB*	*sul2*	*tetW*	*aph(3')-III*	*ermB*	*sul2*	*tetW*	*aph(3')-III*	*ermB*	*sul2*	*tetW*
Pig faeces (farms)	-3.41	-1.59	-3.82	-0.99	-4.05--2.66	-2.34--0.91	-4.76--2.84	-1.31--0.67	0.54	0.54	0.78	0.27
Broiler faeces (farms)	-3.84	-1.11	-2.46	-1.31	-4.79--2.88	-2.00--0.30	-3.68--1.30	-2.09--0.72	0.75	0.69	0.90	0.54
EDC (pig farms)	-3.62	-1.22	-2.70	-1.19	-4.26--2.89	-2.01--0.42	-3.51--1.94	-1.75--0.69	0.57	0.64	0.61	0.41
EDC (broiler farms)	-2.97	-0.55	-2.35	-0.55	-3.54--2.49	-1.23-0.06	-2.93--1.68	-1.17--0.11	0.47	0.49	0.49	0.43
Pooled pig faeces (farms)	-3.27	-1.51	-3.59	-1.06	-3.91--2.40	-2.20--0.84	-4.47--2.36	-1.34--0.74	0.56	0.52	0.80	0.25
Pooled broiler faeces (farms)	-3.70	-0.95	-2.20	-1.20	-4.39--2.96	-1.85--0.33	-3.00--1.38	-1.70--0.78	0.56	0.59	0.66	0.36
Retail pork	.	-4.14	.	-4.62	.	-5.23--2.72	.	-5.66--3.17		0.99		1.18
Retail chicken	.	-3.26	.	-3.44	.	-4.70--1.84	.	-5.06--2.09		1.08		1.11
Turkey faeces (farms)	-3.84	-0.64	-2.95	-1.15	-5.19--2.81	-1.28--0.03	-4.12--1.78	-1.77--0.62	0.90	0.57	0.92	0.51
Veal calf faeces (farms)	-2.53	-1.15	-1.88	-1.23	-2.93--2.13	-1.57--0.74	-2.28--1.42	-1.66--0.86	0.38	0.39	0.39	0.32
Fish faeces (farms)	-4.01	-3.15	-2.76	-3.33	-5.50--2.92	-4.78--1.96	-4.21--1.41	-3.99--2.65	1.51	1.11	1.10	0.74
Wild boar faeces	-5.62	-3.89	-4.40	-2.51	-6.33--4.51	-4.84--3.10	-5.32--3.44	-3.65--1.64	0.74	0.79	0.75	0.78
Cat faeces	-3.75	-2.30	-3.11	-1.19	-4.77--2.83	-3.46--1.38	-4.27--1.90	-1.71--0.67	0.88	0.80	0.88	0.44
Dog faeces	-4.68	-3.25	-3.44	-2.24	-5.78--3.65	-4.26--2.34	-4.98--2.14	-3.04--1.44	1.04	0.77	1.11	0.69
Retail turkey	.	-2.99	.	-3.13	.	-4.45--1.66	.	-4.54--2.03		1.04		1.01
Retail veal	.	-3.72	.	-3.88	.	-4.89--2.49	.	-4.57--3.18		0.94		1.22
Retail fish	.	-3.84	.	-2.56	.	-4.70--2.80	.	-2.56--2.56		1.12		
Human faeces (pig farms)	-4.09	-2.35	-3.63	-1.57	-5.08—3.00	-3.24--1.28	-5.26--2.07	-2.30--0.88	0.84	0.76	1.14	0.55
Human faeces (broiler farms)	-4.23	-2.14	-3.57	-1.67	-5.07--3.08	-2.77--1.39	-4.85--2.47	-2.21--1.13	0.85	0.75	0.88	0.50
Pig faeces (slaughterhouses)	-3.86	-2.07	-3.67	-1.10	-4.36--3.36	-2.65--1.48	-4.13--2.99	-1.47--0.69	0.39	0.48	0.65	0.34
Pig carcass (slaughterhouses)	.	-1.87	.	-1.74	.	-2.63--1.35	.	-2.46--1.38		0.47		0.42
Gloves (pig slaughterhouses)	.	-1.98	.	-2.18	.	-2.70--1.33	.	-2.82--1.30		0.56		0.56
Pig meat (slaughterhouses)	.	.	.	.	.	.	.	.				
Human faeces (pig slaughterhouses)	-4.89	-2.41	-3.67	-1.97	-6.17--3.74	-3.62--1.38	-5.24--2.30	-2.57--1.39	0.96	0.89	1.09	0.50
Human faeces control	-4.59	-2.61	-4.35	-1.89	-5.34--3.74	-3.60--1.75	-5.42--3.21	-2.36--1.42	0.69	0.71	0.85	0.39
Broiler faeces (slaughterhouses)	-2.36	-0.66	-3.31	-0.74	-2.74--2.04	-1.40--0.17	-4.33--2.42	-1.02--0.51	0.33	0.50	0.73	0.28
Broiler carcass (slaughterhouses)	.	-2.19	.	-2.82	.	-3.18--1.24	.	-4.31--1.60		0.74		0.98
Gloves (broiler slaughterhouses)	.	-1.87	.	-2.30	.	-3.58--0.54	.	-3.91--1.05		1.10		1.13
Broiler meat (slaughterhouses)	.	-2.89	.	-2.55	.	-3.98--1.85	.	-3.40--1.74		0.86		0.63
Human faeces (broiler slaughterhouses)	-4.53	-1.96	-3.44	-1.72	-5.53--3.51	-2.96--1.15	-4.87--2.04	-2.32--1.15	0.85	0.75	1.13	0.47

.: Missing values due to lack of information. EDC: Electrostatic dustfall collector. All values are rounded at two digits behind the decimal point.

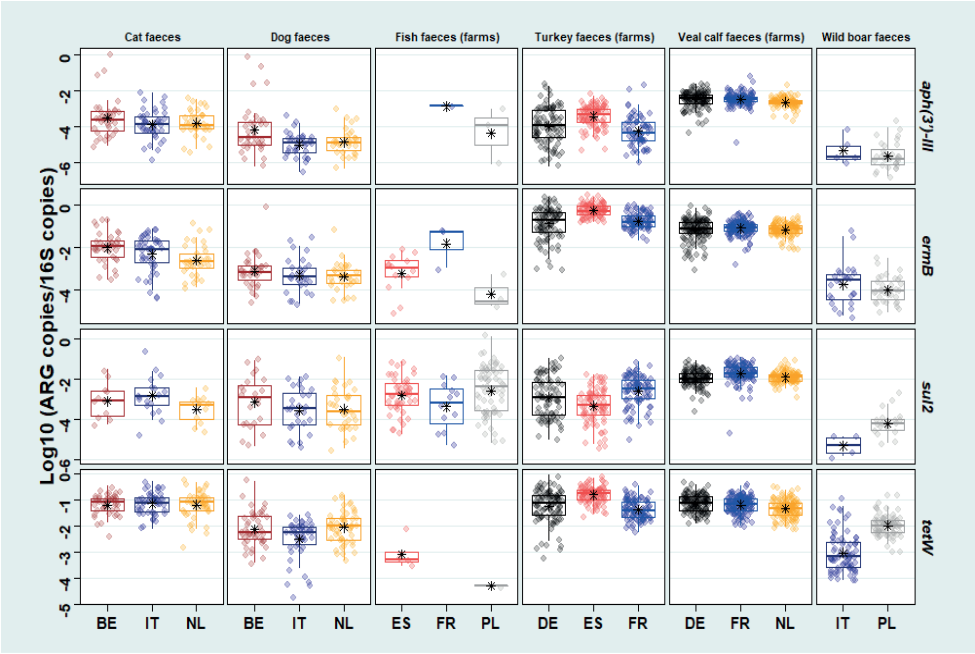

Figure S2. Relative abundance of four targets (*aph(3')-III, ermB, sul2, tetW*) in faeces samples involved three or fewer countries.

Relative abundance of gene target was calculated by log10 (gene copies/*16S* copies). Asterisk shows the mean by country.

BE: Belgium, BG: Bulgaria, DE: Germany, DK: Denmark, ES: Spain, FR: France, IT: Italy, NL: the Netherlands, PL: Poland

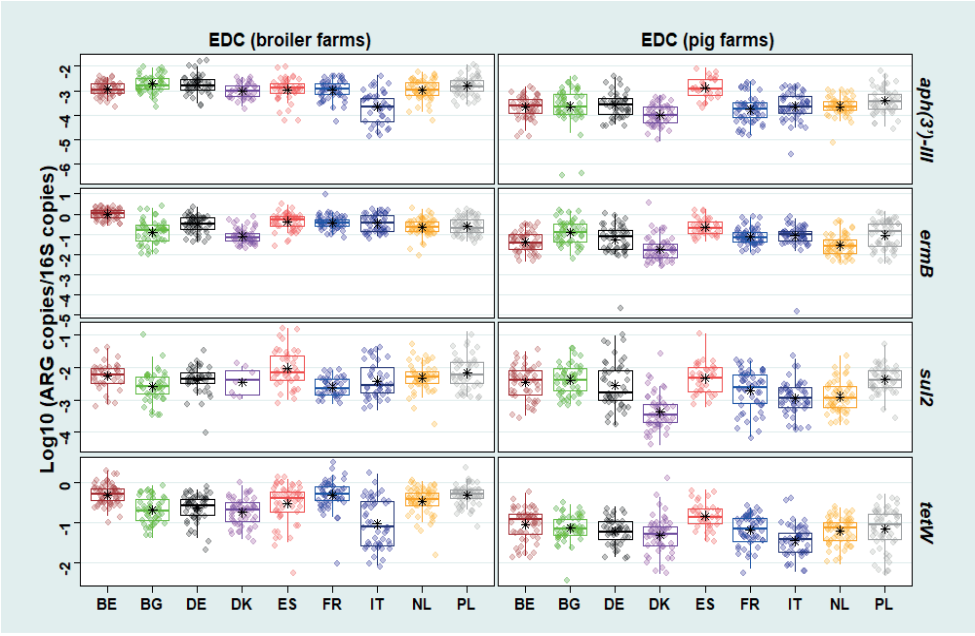

Figure S3. Relative abundances of four targets (*aph(3')-III, ermB, sul2, tetW*) in dust samples of pigs and broilers in nine countries.

Relative abundance of gene target was calculated by $\log_{10}$(gene copies/*16S* copies). Asterisk shows the mean by country. EDC: Electrostatic dustfall collector.

BE: Belgium, BG: Bulgaria, DE: Germany, DK: Denmark, ES: Spain, FR: France, IT: Italy, NL: the Netherlands, PL: Poland

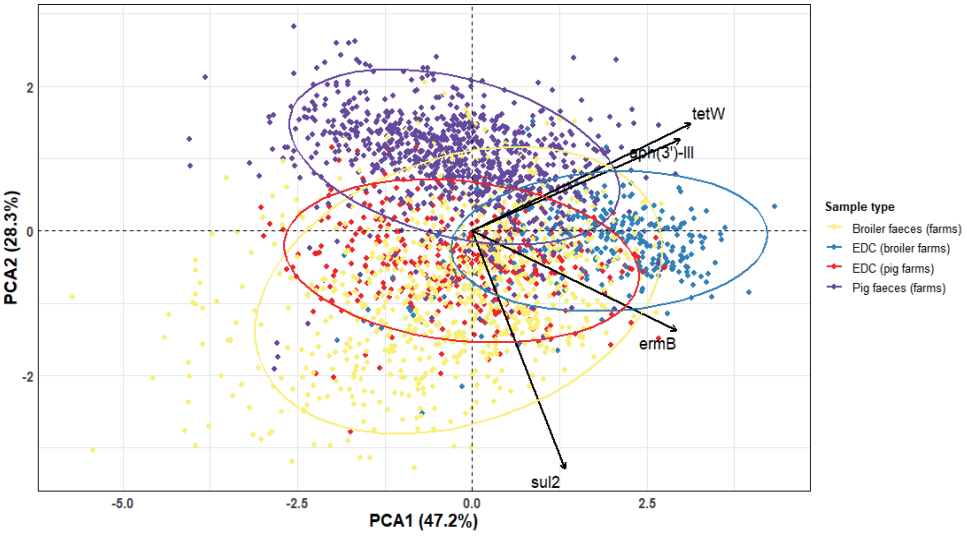

Figure S4. Principal component analysis biplot of relative ARG abundances in faeces/dust samples in pigs and broilers among nine countries.

Symmetric scaling was used. Arrows represent the direction of four ARG targets' maximum correlation with the PCA axes. Relative abundance of gene target was calculated by $\log_{10}$(gene copies/*16S* copies). Circles indicate 95% confidence ellipses that were computed with the assumption of multivariate normal distribution of the data.

ARG targets: *Aph(3')-III, ermB, sul2, tetW.* EDC: Electrostatic dustfall collector.

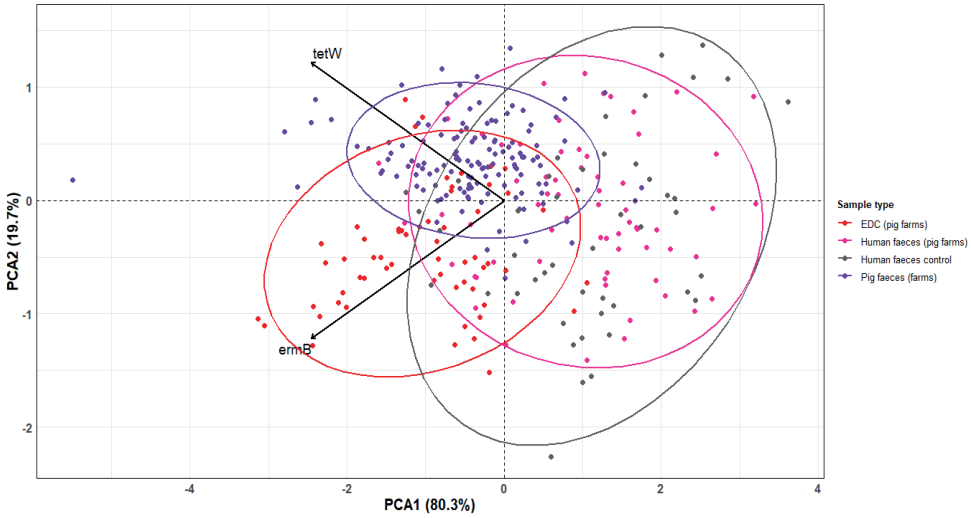

Figure S5. Principal component analysis biplot of relative ARG abundances in pig farms in the Netherlands.

Symmetric scaling was used. Arrows represent the direction of four ARG targets' maximum correlation with the PCA axes. Relative abundance of gene target was calculated by $\log_{10}$(gene copies/*16S* copies). Circles indicate 95% confidence ellipses that were computed with the assumption of multivariate normal distribution of the data.

ARG targets: *ErmB, tetW.* EDC: Electrostatic dustfall collector.

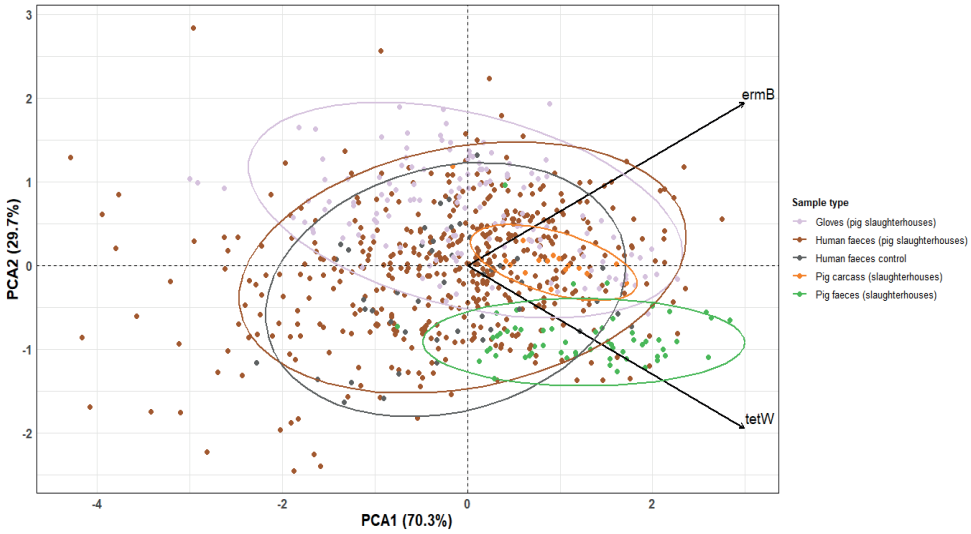

Figure S6. Principal component analysis biplot of relative ARG abundances in pig slaughterhouses in the Netherlands.

Symmetric scaling was used. Arrows represent the direction of four ARG targets' maximum correlation with the PCA axes. Relative abundance of gene target was calculated by $\log_{10}$(gene copies/*16S* copies). Circles indicate 95% confidence ellipses that were computed with the assumption of multivariate normal distribution of the data.

ARG targets: *ErmB, tetW.*

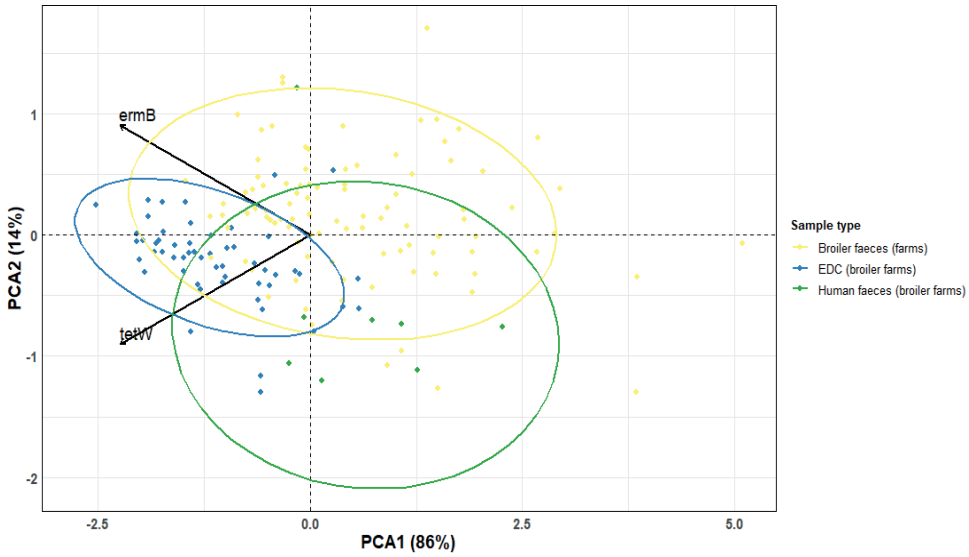

Figure S7. Principal component analysis biplot of relative ARG abundances in broiler farms in Germany.

Symmetric scaling was used. Arrows represent the direction of four ARG targets' maximum correlation with the PCA axes. Relative abundance of gene target was calculated by $\log_{10}$(gene copies/*16S* copies). Circles indicate 95% confidence ellipses that were computed with the assumption of multivariate normal distribution of the data.

ARG targets: *ErmB, tetW.* EDC: Electrostatic dustfall collector.

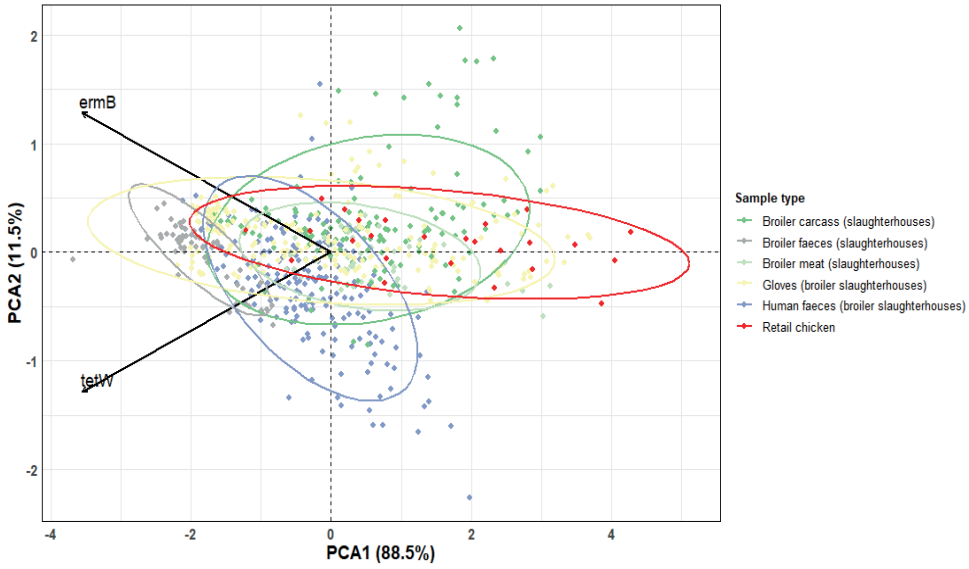

Figure S8. Principal component analysis biplot of relative ARG abundances in broiler slaughterhouses in Germany.

Symmetric scaling was used. Arrows represent the direction of four ARG targets' maximum correlation with the PCA axes. Relative abundance of gene target was calculated by $\log_{10}$(gene copies/*16S* copies). Circles indicate 95% confidence ellipses that were computed with the assumption of multivariate normal distribution of the data.

ARG targets: *ErmB, tetW.*

Table S4. Variance component analysis in faeces samples from pigs, broilers, veal calves and turkeys.

Animal species	Variables	DF				VC				%Total				SD			
		aph(3')-III	*ermB*	*sul2*	*tetW*	*aph(3')-III*	*ermB*	*sul2*	*tetW*	*aph(3')-III*	*ermB*	*sul2*	*tetW*	*aph(3')-III*	*ermB*	*sul2*	*tetW*
Pigs	**Between-country**	7.50	6.96	7.36	5.00	0.20	0.12	0.28	0.01	**53.83**	36.33	**42.40**	10.63	**0.44**	**0.34**	**0.53**	0.09
	Between-farm	144.43	159.63	121.98	138.12	0.12	0.16	0.20	0.04	32.00	**50.94**	30.00	**50.59**	0.34	0.40	0.44	**0.20**
	Within-farm	879.33	1044.03	835.9	1047.14	0.05	0.04	0.18	0.03	14.17	12.73	27.60	38.78	0.23	0.20	0.43	0.17
Broilers	**Between-country**	4.99	7.17	4.35	5.83	0.04	0.19	0.05	0.04	6.93	38.00	6.78	11.83	0.20	0.43	0.23	0.19
	Between-farm	52.47	134.50	89.46	80.07	0.12	0.19	0.28	0.08	20.82	**38.00**	35.14	27.96	0.34	**0.43**	0.53	0.29
	Within-farm	646	697.03	638.74	693.2	0.41	0.12	0.47	0.18	**72.25**	24.00	**58.07**	**60.22**	**0.64**	0.34	**0.68**	**0.42**
Veal calves	**Between-country**	0.55	NA	1.51	1.30	0.00	0.00	0.02	0.01	3.01	0.00	11.53	7.91	0.07	0.00	0.14	0.09
	Between-farm	36.48	49.34	27.32	3.16	0.06	0.09	0.04	0.03	43.15	**62.31**	24.40	28.43	0.25	0.31	0.20	0.17
	Within-farm	270.46	340.03	312.31	345.15	0.08	0.06	0.10	0.07	**53.84**	37.69	**64.07**	**63.66**	0.28	0.24	0.32	0.26
Turkeys	**Between-country**	1.59	1.65	1.56	1.74	0.15	0.10	0.14	0.08	16.94	27.42	15.43	27.50	0.39	0.32	0.38	0.28
	Between-farm	24.17	48.08	23.82	35.37	0.24	0.19	0.25	0.09	27.24	**50.63**	27.03	32.10	0.49	0.44	0.50	0.30
	Within-farm	191.06	225.07	194.01	227.23	0.49	0.08	0.54	0.12	**55.83**	21.95	**57.54**	**40.39**	0.70	0.29	0.73	0.34

DF: Degree of freedom. VC: Absolute variance per component. %Total: The proportion of the variance component. SD: Standard deviation.

All values are rounded at two digits behind the decimal point.

Table S5. Variance component analysis of all determinants in veal calves.

		aph(3')-III			*ermB*			*sul2*			*tetW*		
		VC	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**
Adjusted model	**AMU:**												
	Aminoglycosides used (ref: no)	0.01	*3.89*	0.08									
	Log10 TIDDDvet MLS				0.00	0.00	0.00						
	Log10 TIDDDvet trimethoprim & sulfonamide							0.01	6.78	0.10			
	Log10 TIDDDvet tetracyclines							0.00	0.00	0.00	0.00	0.00	0.00
	Herd characteristics:												
	Other animals present at the farm (ref: no)	0.01	6.52	0.10									
	Weight of calves at arrival (kg)	0.00	0.00	0.00	0.00	0.00	0.00						
	Number of calves at sampling							0.00	0.00	0.00			
	Biosecurity measures:												
	Agents used for cleaning (water/soaking/disinfectants)				0.01	6.45	0.10				0.01	6.58	0.08
	Country and farm variation:												
	Between-country	0.00	0.00	0.00	0.00	0.00	0.00	0.01	6.07	0.10	0.01	6.05	0.08
	Between-farm	0.06	**38.93**	0.25	0.09	**56.73**	0.30	0.03	20.20	0.18	0.03	23.89	0.16
	Within-farm	0.08	50.66	0.28	0.06	36.82	0.22	0.11	**66.94**	0.32	0.07	**63.47**	0.26
Null model	**Country and farm variation:**												
	Between-country	0.00	3.43	0.07	0.00	0.00	0.00	0.02	11.49	0.14	0.01	8.66	0.10
	Between-farm	0.07	**50.73**	0.27	0.10	**62.40**	0.31	0.04	24.62	0.20	0.03	28.02	0.17
	Within-farm	0.07	45.84	0.26	0.06	37.60	0.24	0.11	**63.89**	0.32	0.07	**63.32**	0.26

VC: Absolute variance per component. %Total: The proportion of the variance component. SD: Standard deviation. TIDDDvet: Mean treatment incidence (TI) based on defined daily doses administered (DDDvet), which indicates the average number of treatment days per 100 days. MLS: Macrolide + lincosamide + spectinomycin use. Trimethoprim & sulfonamide: Sulfonamide + trimethoprim/sulfonamide use. All values are rounded at two digits behind the decimal point. Values in bold indicate the highest Variance component percentage for each model.

Table S6. Variance component analysis of all determinants in turkeys.

		aph(3')-III			*ermB*			*sul2*			*tetW*		
		VC	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**
Adjusted model	**AMU:**												
	Aminoglycosides used (ref: no)	0.00	*0.00*	0.00									
	Log10 TIDDDvet MLS				0.01	1.57	0.08						
	Trimethoprim & sulfonamide use (ref: no)							0.09	8.68	0.30			
	Log10 TIDDDvet tetracyclines										0.00	0.59	0.05
	Herd characteristics:												
	Age of turkeys at sampling (standardized)				0.00	0.00	0.00						
	Other livestock present (ref: no)	0.00	0.24	0.05				0.01	1.32	0.12			
	Sampling season (ref: autumn. winter)							0.15	14.27	0.39			
	Biosecurity measures:												
	Visitor access more than once a month (ref: no)				0.06	13.70	0.24				0.08	21.08	0.27
	Outdoor access possible for turkeys (ref: no)				0.04	10.37	0.21				0.03	7.93	0.17
	Different age categories of turkeys present (ref: no)				0.03	6.23	0.16						
	Bird- and vermin proof grids placed on the air inlets (ref: no)	0.02	2.42	0.15									
	Staff keeps turkeys or birds at home (ref: no)	0.03	3.20	0.17									
	Country and farm variation:												
	Between-country	0.12	13.96	0.35	0.08	18.03	0.28	0.02	1.92	0.14	0.07	20.65	0.27
	Between-farm	0.23	25.96	0.48	0.13	**30.66**	0.36	0.22	21.29	0.47	0.06	16.80	0.25
	Within-farm	0.48	**54.22**	0.69	0.08	19.43	0.29	0.55	**52.52**	0.74	0.12	**32.94**	0.34
Null model	**Country and farm variation:**												
	Between-country	0.15	16.82	0.38	0.12	31.26	0.34	15.14	0.24	0.38	0.08	28.66	0.29
	Between-farm	0.24	27.62	0.49	0.18	**46.56**	0.42	0.25	26.81	0.50	0.09	30.51	0.30
	Within-farm	0.48	**55.55**	0.69	0.08	22.18	0.29	0.55	**58.05**	0.74	0.12	**40.83**	0.34

VC: Absolute variance per component. %Total: The proportion of the variance component. SD: Standard deviation. TIDDDvet: Mean treatment incidence (TI) based on defined daily doses administered (DDDvet), which indicates the average number of treatment days per 100 days. MLS: Macrolide + lincosamide + spectinomycin use. Trimethoprim & sulfonamide: Sulfonamide + trimethoprim/sulfonamide use. All values are rounded at two digits behind the decimal point. Values in bold indicate the highest VC percentage for each model.

Table S7. Meta-analysis of all AMU vs ARGs combinations in pig farms

AMU	ARG classes	Estimate (β)	95% CI	P-value	FDR	Countries and number of farms
Log10 TIDDDvet MLS use (fatteners)	*ermB*	1.27	[0.84,1.71]	p<0.01	p<0.01	BE-3, BG-2, DE-5, DK-6, ES-14, FR-1, IT-10, PL-4
Log10 TIDDDvet macrolide use (fatteners)	*ermB*	1.57	[0.83,2.32]	p<0.01	p<0.01	BE-2, DE-4, DK-2, ES-12, FR-1, IT-7, PL-3
Log10 TIDDDvet polymixin use (fatteners)	*ermB*	-1.06	[-1.57,-0.56]	p<0.01	p<0.01	BG-1, ES-11, FR-1, IT-2, PL-1
Log10 TIDDDvet polymixin use (sucklers)	*ermB*	-0.94	[-1.41,-0.48]	p<0.01	p<0.01	DE-1, DK-1, ES-12, IT-4, NL-1, PL-1
Total AMU (fatteners)	*aph(3')-III*	0.56	[0.21,0.90]	p<0.01	0.08	BE-10, BG-8, DE-11, DK-11, ES-20, FR-4, IT-15, NL-1, PL-15

All values are rounded at two digits behind the decimal point. ARG classes clusters in this table represent relative ARG abundance (log10(gene copies/16S copies)). Countries and number of farms: countries were excluded if no AMU was recorded on all farms from the respective country. Only associations that have an FDR p-value < 0.1 were shown. TIDDDvet: Mean treatment incidence (TI) based on defined daily doses administered (DDDvet), which indicates the average number of treatment days per 100 days. MLS: Macrolide + lincosamide + spectinomycin use.

Table S8. Meta-analysis of all AMU vs ARGs combinations in broiler farms

AMU	ARG classes	Estimate (β)	95% CI	P-value	FDR	Countries and number of farms
Log10 TIDDDvet MLS use	*ermB*	1.00	[0.55-1.45]	p<0.01	p<0.01	BE-8, BG-1, DE-5, DK-1, ES-3, IT-4, PL-3
Log10 TIDDDvet aminoglycosides use	*aph(3')-III*	1.30	[0.39-2.21]	p<0.01	0.06	ES-7, PL-2
Log10 TIDDDvet aminoglycosides use	*sul2*	1.32	[0.41-2.22]	p<0.01	0.06	ES-7, PL-2
Log10 TIDDDvet macrolides use	*ermB*	1.49	[0.38-2.61]	p<0.01	0.06	BE-1, ES-3, IT-4, PL-2
Log10 TIDDDvet tetracyclines use	*tetW*	0.60	[0.16-1.03]	p<0.01	0.06	BE-6, DE-2, DK-2, ES-1, NL-1, PL-8

All values are rounded at two digits behind the decimal point. ARG classes clusters in this table represent relative ARG abundance (log10(gene copies/16S copies)). Countries and number of farms: countries were excluded if no AMU was recorded on all farms from the respective country. Only associations that have an FDR p-value < 0.1 were shown. TIDDDvet: Mean treatment incidence (TI) based on defined daily doses administered (DDDvet), which indicates the average number of treatment days per 100 days. MLS: Macrolide + lincosamide + spectinomycin use.

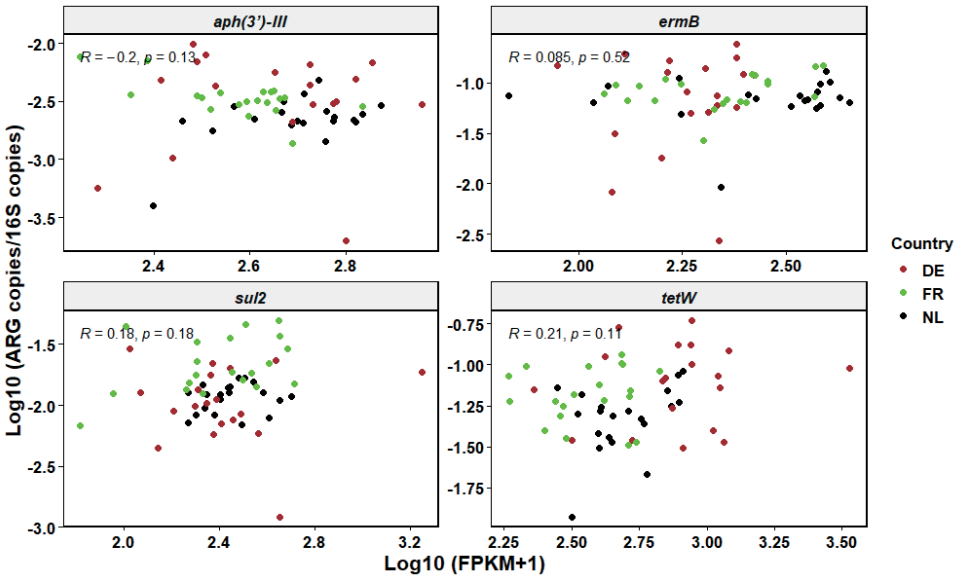

Figure S9. Spearman's rank correlation of individual qPCR data and pooled metagenomic data of veal calves.

ARG targets: *aph(3')-III, ermB, sul2, tetW.* FPKM: Fragments per kilobase reference per million bacterial fragments.[1] DE: Germany, FR: France, NL: the Netherlands.

The median of 7 individual qPCR results was calculated per farm before correlation analysis.

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Chapter 3

Association of antimicrobial usage with faecal abundance of *aph(3')-III, ermB, sul2 and tetW* resistance genes in veal calves in three European countries

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Abstract

Background

High antimicrobial use (AMU) and antimicrobial resistance (AMR) in veal calves remain a source of concern. As part of the EFFORT project, we determined the association between AMU and faecal antimicrobial resistance genes (ARGs) in veal calves in three European countries.

Methods

In 2015, we collected faecal samples of calves close to slaughter from farms located in France, Germany and the Netherlands (20 farms in France, and the Netherlands, 21 farms in Germany, 25 calves per farm). Standardized questionnaires were used to record AMU and farm characteristics. In total, 405 faecal samples were selected for DNA extraction and qPCR to quantify the abundance (16S normalized concentration) of four ARGs [*aph(3')-III*, *ermB*, *sul2* and *tetW*] encoding for resistance to frequently used antimicrobials in calves. Multiple linear mixed models with random effects for country and farm were used to relate ARGs to AMU and farm characteristics.

Results

We found a significant positive association between trimethoprim/sulfonamide use and the concentration of *sul2* in veal calves' faeces. A higher weight of calves at arrival was negatively associated with *aph(3')-III* and *ermB*. At farms with non-commercial animals present, we found lower *aph(3')-III* concentrations. Furthermore, farms using only water for the cleaning of stables had a significantly lower faecal *ermB* and *tetW* abundance compared to other farms.

Conclusion

A positive association was found between trimethoprim/sulfonamide use and *sul2* abundance in veal calves. Additionally, other relevant risk factors associated with ARGs in veal calves were identified, e.g. weight at arrival at the farm and cleaning practices.

Introduction

Global emergence of antimicrobial resistance (AMR) is considered a large threat to human health [1], which partly results from antimicrobial use (AMU) in animals [2]. In veal calf farming, high AMU and AMR remains a source of concern. The calf trading network is complex, calves from different farms are mixed and transported across regional and international borders [3]. Consequently, and because the immune system of a calf is not fully developed until approximately six months after birth [4], calves have an increased risk of developing infectious diseases, resulting in high AMU in veal calves [5].

Thus, to control the level of AMR in veal calves, there is an urgent need to quantify the abundance of antimicrobial resistance genes (ARGs) and to identify its potential risk factors. ARGs can horizontally be transferred between bacterial species. Compared with focusing on pathogenic resistant bacteria, studying ARGs can provide a more comprehensive overview of AMR in livestock [6]. To determine the potential determinants of AMR in veal calves, a previous study found a positive association between AMR and AMU in veal calves [7]. Interestingly, a longitudinal study has shown that the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in calves and stable air did not increase simultaneously with or directly after treatment with antimicrobials [8], suggesting that AMU may not be the only determinant for AMR in veal calves.

As part of the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project, our study aims to better understand the relationship between AMU and the abundance of selected ARGs in veal calves. In this study, we investigated the abundance of genes encoding resistance to four antimicrobial classes: aminoglycosides, macrolides, tetracyclines, and sulfonamides [2,6,8,9]. Antimicrobials that belong to these classes and the combined use of trimethoprim/sulfonamides (trim/sulfa) belong to the most widely used antimicrobials in European veal calves [3,6,9]. We determined the association between faecal ARGs to the above mentioned antimicrobial classes (*aph(3')-III*, *ermB*, *sul2* and *tetW),* and potential farm-related risk factors such as AMU, the weight of calves, and stable cleaning agents in France, Germany and the Netherlands.

Materials and methods

Study design and study population

Between January and December 2015, a cross-sectional study was conducted among 61 veal calf farms in France, Germany and the Netherlands. Country names were anonymized to “B”, “E” and “F” in line with previous EFFORT publications [6,10]. In each of the participating countries, conventional non-mixed white or rosé veal calf farms were visited. Sampling was spread over the entire year. Only individual farms rearing at least 200 animals per production round with no contacts through trade and using an all-in-all-out production system were included in the study. Farm selection was partially based on convenience (e.g. distance of the research institute to the farms). Therefore, the sampling of farms in each country cannot be considered representative of the entire veal calf sector in that country.

Faecal sampling and data collection

Faecal sample collection

At each farm, 25 individual calves were sampled in their last weeks before slaughter. Faecal samples were collected using sterile spoons during or directly after defaecation from faecal parts without floor contact. No animal ethics approval from the respective national authorities was needed (non-invasive faecal sampling process). Per calf, a minimum of 10g faeces was collected in a sterile faeces container. After collection, the samples were stored at 4°C and transported to the lab within 24 hours, homogenized and stored at -80°C until DNA extraction.

Data on AMU and farm characteristics

A standardized questionnaire was completed by the farmer, which included questions on AMU, farm and herd characteristics and farm management. AMU group treatment incidence (TI) based on calculated Defined Daily Doses Animal (DDDvet) were computed at farm-level. More technical details have been described before [11].

DNA extraction and qPCR

Of the 25 faecal samples collected at the farms, seven individual faecal samples were randomly selected per farm for qPCR detection and analysis. DNA was extracted by using the modified QIAmp

Fast DNA stool mini kit (Qiagen, cat. no. 51604). Following DNA extraction, qPCR was performed to quantify the relative abundance of ARGs. More details regarding the qPCR assays, quality control steps [internal amplification control (IAC), replicate consistency check] and 16S-normalization have been described before (16S, *ermB*, *tetW*) [12]. For *aph(3')-III* and *sul2*, the qPCR process slightly differed in PCR reaction composition (primers, probe): [*aph(3')-III* - 400nM, 250nM; *sul2*: 100nM, 100nM] [13,14]. Limit of detection/ quantification (LOD/LOQ) and qPCR efficiency percentage: *aph(3')-III* – 0.22, 0.52, 101.6%.; *sul2* – 0.92, 0.91, 93.6%. All genes were expressed as $\log_{10}$ gene copies.

Statistical analysis

SAS software version 9.4 and R software version 3.6.3 were used for statistical analysis. Potential risk factors were chosen based on the published literature on AMR risk factors in veal calves (age, weight, farm size, season) [7,15], or other livestock sectors (non-commercial animals [16], type of cleaning agents [17]).

After qPCR quality control (IAC and replicate consistency), values lower than the LOD were removed (16S gene) or replaced [*aph(3')-III*, *sul2*]. ARGs were replaced with half of the lowest untransformed value >LOD per gene and country before $\log_{10}$ transforming these again.

AMU data were strongly right-skewed, therefore $\log_{10}$ transformation was applied [log10(AMU +1)]. To take the between and within-country variation into account, we applied a linear mixed model with a random effect for both country and farm.

AMU and other farm-related factors (fixed effect variables) were first selected in a univariate analysis ($p<0.20$) and subsequently introduced into a multiple linear mixed model per ARG. Correlations between the included independent variables were checked, and only variables with low correlations were included in the full multivariable model [both continuous variables: Pearson correlation (ρ) < 0.7, both categorical variables: Chi-squared test ($p>0.05$) or in case of one continuous and one categorical variable: a One-way ANOVA ($p>0.05$)]. Subsequently, the full models (containing all possible risk factors and confounders) were manually reduced employing a backward selection based on the Akaike information criterion (AIC) to obtain the final model. To make the model coefficients better interpretable, all coefficients were exponentiated to obtain geometric mean ratios (GMR and 95%

confidence intervals). Before exponentiation, numeric variables were multiplied by the IQR as such that an increase in the independent variables correspond to an IQR increase of the determinant variables.

Results

Antimicrobial use

The type of antimicrobials and the amount of AMU varied across the three countries (Figure 1), macrolides (84%, 60%, 100% of farms in country B, E, F, respectively) and tetracyclines (84%, 90%, and 100% of farms in country B, E, F, respectively) were both widely used. In country B, aminopenicillins (95%), amphenicols (79%), and fluoroquinolones (89%) were also widely used. Two farms were excluded from the analysis due to missing qPCR data or when AMU was out of the range of expected values, resulting in 59 farms for data analysis. Since only eight of the 59 farms used aminoglycosides, TI_{DDDvet} for aminoglycosides was replaced by a binary variable indicating aminoglycoside use (yes/no).

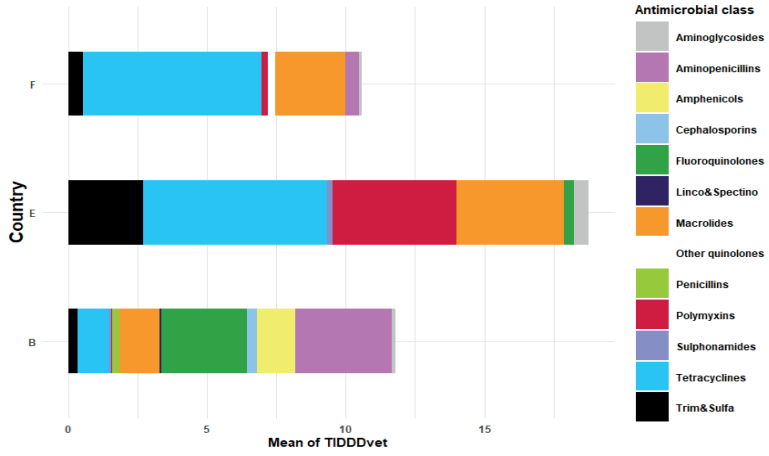

Figure 1: Overview of antimicrobial usage (AMU) in sampled veal farms in 3 countries.

In total, 61 veal farms were sampled in three countries, zero usage levels were included when calculating mean AMU. TI_{DDDvet} = mean treatment incidence (TI) based on defined daily doses administered (DDDvet), which indicates the average number of treatment days per 100 days.

Abundance of ARGs

After qPCR quality control and 16S-normalization, per ARG model, 124-137, 118-137 and 89-131 (min-max) samples respectively remained for analysis within country B, E and F. Significant differences between the three countries were found for the mean abundance of all targets (*aph(3')-III* ($p<0.01$), *sul2* ($p<0.01$), and *tetW* ($p=0.04$), one-way ANOVA), except for *ermB* ($p=0.08$).

Associations between farm characteristics and ARG abundances

Figure 2 illustrates the relationship between the ARGs and AMU for the different antimicrobial classes among the three countries. For each ARG, a final linear mixed model was fitted (Table 1).

A significant positive association was found between trim/sulfa use and the abundance of *sul2* in faecal samples from calves (GMR=1.37, $p<0.01$). Furthermore, a higher weight of calves at arrival at the farm was negatively associated with faecal *aph(3')-III* (GMR=0.82, $p=0.01$) and *ermB* (GMR=0.72, $p<0.01$) loads. We also found lower faecal *aph(3')-III* levels in calves at farms where non-commercial animals (e.g. cats, dogs, sheep) were present (GMR=0.71, $p=0.05$). Veal calves sampled at farms using only water for cleaning of stables carried lower *ermB* (GMR=0.5, $p=0.01$) and *tetW* (GMR=0.66, $p=0.04$) concentrations in their faeces compared to veal calves of farms which used both soaking agents and disinfectants. Agents used for cleaning stables' was not included in the final model of *aph(3')-III* because of correlation with 'Aminoglycosides used' ($p<0.01$, Chi-squared test).

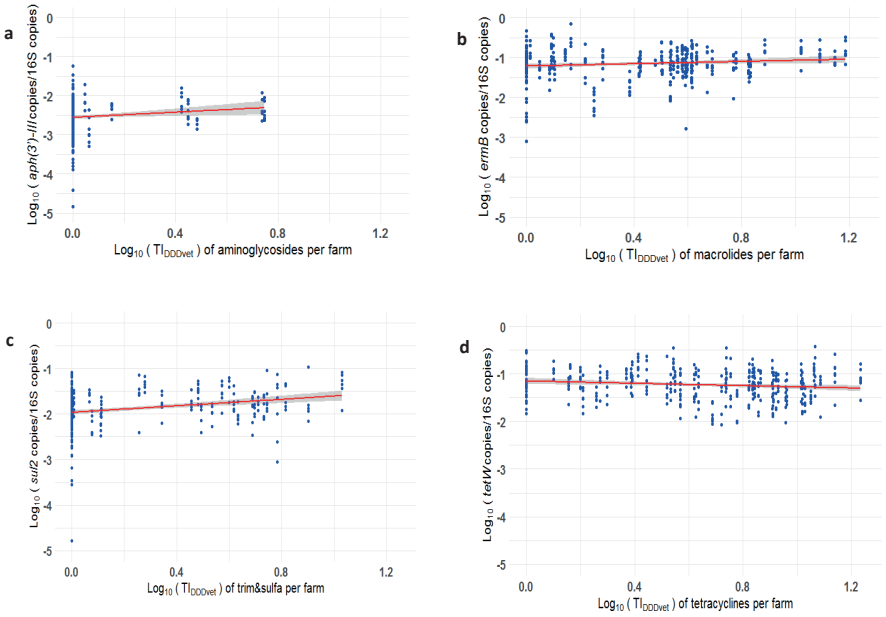

Figure 2. Association between AMU (farm-level, TI_{DDDvet}) and AMR (log_{10}(ARGs copies-16s copies)) in veal calf faeces (n=405), sampled at 59 farms in three countries.

a: Association between log_{10} transformed aminoglycoside usage and 16S normalized *aph(3')-III* abundance. **b:** Association between log_{10} transformed macrolide usage and 16S normalized *ermB* abundance. **c:** Association between log_{10} transformed trim&sulfa usage and 16S normalized *sul2* abundance. d**:** Association between log_{10} transformed tetracycline usage and 16S normalized *tetW* abundance.

Table 1: Association of AMU and veal calf farm characteristics with the relative abundance of *aph(3')-III*, *ermB*, *sul2* and *tetW* resistance genes

			GMR [95% CI]			
AMU	**IQR or %**	**median (25-75pct)**	***aph(3')-III***	***ermB***	***sul2***	***tetW***
Aminoglycosides used (ref: no)	14%	-	1.42 [0.91,2.20]			
Log TIDDDvet macrolides	0.52	0.5 (0.09-0.61)		1.07 [0.82,1.39]		
Log TIDDDvet trim&sulfa	0.49	0 (0-0.49)			**1.37 [1.09,1.74]**	
Log TIDDDvet tetracyclines	0.53	0.74 (0.39-0.92)			1.23 [0.99,1.52]	0.94 [0.75,1.17]
Farm characteristics						
Other animals present at the farm (ref: no)	57.6%	-	**0.71 [0.51,0.99]**			
Weight of calves at arrival (kg)	4.9	48 (45.3-50.2)	**0.82 [0.71,0.95]**	**0,72 [0.62,0.85]**		
Number of calves at sampling	785	729 (258-1043)			0.83 [0.64,1.06]	
Agents used for cleaning						
No cleaning	8.5%	-		0.67 [0.34,1.32]		0.75 [0.44,1.25]
Water only	22%	-		**0.5 [0.29,0.84]**		**0.66 [0.44,0.97]**
Soaking agents	6.8%	-		1.21 [0.58,2.53]		1.1 [0.65,1.88]
Disinfectants	45.8%	-		0.94 [0.59,1.50]		0.97 [0.69,1.37]
Soaking agents and disinfectants	16.9%	-		ref .		ref .

Final linear mixed model with a random effect for both country and farm after mutual adjustment for confounding from the univariate analysis ($p<0.05$), which was defined with the lowest Akaike's Information Criterion (AIC). Significant associations are marked in bold ($p<0.05$).

Discussion

In this study, we determined potential risk factors for faecal carriage of ARGs in veal calves close to slaughter. We found a significant positive association between the TI of trim/sulfa and the abundance of *sul2* in the overall model. Similarly, one Danish study suggested a positive link between the faecal presence of sulfonamide resistant *E. coli* in pigs and an increased trim/sulfa administration [18]. However, for aminoglycoside, macrolide and tetracycline resistance, we did not demonstrate

significant associations between AMU and the respective ARG. An explanation for the absence of additional associations might be that antimicrobials administered in the early rearing phase do not affect faecal ARG abundances in the period just before slaughter. Within our study, a considerable number of farms reported no use of specific antimicrobial classes, while the corresponding ARGs could still be demonstrated in faecal samples of the respective farms. Therefore, we hypothesize that actual AMU in veal calves only partially explains AMR, which is in line with results within EU broiler farming that showed a similar resistome composition in untreated flocks compared with treated flocks [10].

We found a negative association between the weight of calves at arrival at the farm and faecal *aph(3')-III* carriage, and *ermB* carriage, respectively. A Swiss study also found significantly higher AMR abundance among lower weight pigs [19]. Possibly, the weight of a calf at arrival could be an indicator for lower health status of this calf, resulting in the administration of a higher number of AMU treatments during its lifespan or a higher dosage of AMU upon arrival at the farm [7]to reduce the risk of infectious disease transmission.

Surprisingly, we found that veal calves from farms using only water for cleaning had significantly lower *ermB* and *tetW* concentrations in their faeces compared to calves from farms using both soaking agents and disinfectants. This is consistent with some previous studies in veal calves. In the study of Dorado-Garcia *et al.* [15], a specific cleaning and disinfecting program was not effective to reduce MRSA prevalence in veal calf farms, while in pig farms a positive association between internal biosecurity (e.g. cleaning and disinfecting) and higher faecal AMR loads in pigs was observed [9]. It is hard to explain the effect of cleaning agents on AMR against the background of significant differences in AMU in farms at which different cleaning agents are being used. One hypothesis to explain this is that application and residual action of biocides may contribute to co-selection of biocide resistant genes and ARGs [20]. These results are in itself no evidence against the use of disinfectants while cleaning livestock stables. But, further studies to optimize cleaning and disinfection protocols in farms are advised.

Strengths and limitations

With 59 farms (405 samples included) included, our study is, to our knowledge, the largest cross-country study on faecal AMR loads in veal calves. Despite this, bias and errors could have been introduced in our study. In general, non-differential exposure misclassification might lead to attenuation of associations. Another limitation is whether unknown historical AMU may have had a potential effect on AMR in the non-use farms. Finally, possible false-positive chance findings might not be completely avoided.

Conclusions

We found a positive association between trimethoprim/sulfonamide use and the level of *sul2* abundance in faeces from veal calves. A higher weight of calves at arrival at the farm was negatively associated with *aph(3')-III* and *ermB,* respectively. Using only water for cleaning stables compared to using both soaking agents and disinfectants showed a negative association with AMR abundance in veal calves.

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Transparency declarations

None to declare.

Ethical Approval

Not required

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Chapter 4

Risk factors for the abundance of antimicrobial resistance genes *aph(3')-III*, *erm*(B), *sul2* and *tet*(W) in pig and broiler faeces in nine European countries

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Abstract

Objectives: The occurrence and zoonotic potential of antimicrobial resistance (AMR) in pigs and broilers has been studied intensively in the past decades. Here, we describe AMR levels of European pig and broiler farms and determine the potential risk factors.

Methods: We collected faeces from 181 pig farms and 181 broiler farms in nine European countries. Real-time PCR (qPCR) was used to quantify the relative abundance of four antimicrobial resistance genes (ARGs) (*aph(3')-III, erm*(B)*, sul2, tet*(W)) in these faeces samples. Information on antimicrobial use (AMU) and other farm characteristics such as farm size, animal age, and biosecurity measures were collected through a questionnaire. A mixed model using country and farm as random effects was performed to evaluate the relationship of AMR with AMU and other farm characteristics. The correlation between individual qPCR data and previously published pooled metagenomic data was evaluated. Variance component analysis was conducted to assess the variance contribution of all factors.

Results: The highest abundance of ARG was *tet*(W) in pig faeces and *erm*(B) in broiler faeces. In addition to the significant positive association between corresponding ARG and AMU levels, we also found on-farm biosecurity measures were associated with relative ARG abundance in both pigs and broilers. Between-country and between-farm variation can partially be explained by AMU. Different ARG targets may have different sample size requirements to represent the overall farm level precisely.

Conclusions: qPCR is an efficient tool for a targeted assessment of AMR in livestock-related samples. The AMR variation between samples was mainly contributed by between-country, between-farm, and within-farm differences, and then by on-farm AMU.

Introduction

Antimicrobial resistance (AMR) in farm animals is of increasing concern as it may be linked to human AMR.[1, 2] Identifying AMR determinants in farm animals may contribute to reducing AMR exposure at the animal-human interface and through the environment.

As the major food-producing animals in Europe, pigs and broilers are of special importance regarding the occurrence of AMR and related farm determinants.[3] On-farm antimicrobial use (AMU) has been identified as a major determinant influencing AMR levels in farm animals.[4, 5] Recently, Van Gompel et al. reported significant positive associations between AMU and corresponding AMR abundance (macrolides and tetracyclines) in pigs.[6] Similar associations were reported by Luiken et al. between tetracycline use in broiler farms and tetracycline resistance in broiler faeces.[7] In addition to on-farm AMU, other relevant farm characteristics may also influence AMR abundance in pigs and broilers. For example, biosecurity subcategories such as 'cleaning & disinfection', and 'measures between compartments & use of equipment' in pig farms were related to the significant increase in the relative abundance of all macrolide resistance genes (fragments per kilobase reference per million bacterial fragments (FPKM) were generated and aggregated) in the finisher faeces.[6] Moreover, a significant negative association was reported between manure storage at broiler farms and the prevalence of simultaneous resistance to amoxicillin-clavulanic acid, ceftiofur and cefoxitin in *E. coli* isolates from broiler faeces.[8]

As part of the cross-sectional project 'Ecology from Farm to Fork Of microbial drug Resistance and Transmission' (EFFORT), we previously reported on risk factors for AMR in pig and broiler farms based on metagenomic analysis of DNA isolated from pooled faecal samples. In the present study, we investigated whether risk factors for AMR abundance can also be found using selected antimicrobial resistance gene (ARG) targets analyzed by Real-time PCR (qPCR) in individual faecal samples. In addition, we aimed to study the effects of a sampling depth of 5-7 individual samples per farm on risk factors analysis and variability in ARG abundances within and between farms.

Methods and materials

Study population and sampling procedure

Between May 2014 and June 2016, 181 pig farms and 181 broiler farms were visited in nine European countries (Belgium, Bulgaria, Germany, Denmark, Spain, France, Italy, the Netherlands, and Poland). Countries were anonymized to letters 'A-I' in line with previous EFFORT publications.[6, 7, 9] In each country, 25 fresh faecal samples were randomly collected on each of 20 conventional farms (or 21 pig farms and 19 broiler farms in country E, 21 broiler farms in country A and country B) complying with the previously described inclusion criteria (e.g. non-mixed, as close to slaughter as possible).[6, 7, 10] Data from 179 pig farms and 180 broiler farms remained for the present analysis, excluding three farms that cannot be linked to AMU data. In agreement with local farming organizations, farms were selected based on inclusion criteria and partly based on convenience (e.g. distance to the farm). Also considering the limited sample numbers per country, the selected farms cannot be regarded as representing the livestock sector of the participating countries. Faecal samples were collected without floor contact (sterile spoons were used). Within 24 hours, all individual faecal samples were transported to the laboratory at 4°C and stored at -80°C until DNA extraction.[6, 7]

Questionnaire, AMU and biosecurity measurement

General herd characteristics, AMU (group treatment and purchased) and biosecurity information were retrieved from a standardized questionnaire (Table S1) completed by farmers in each participating farm together with the visiting researchers.[6, 7] Group treatment AMU was defined as any treatment simultaneously applied to all animals present in, at least, the smallest housing unit (i.e. pen in pigs, barn in broilers) of each farm. Purchased AMU was defined as the antimicrobials purchased for the entire farm one year before sampling. AMU was expressed as Treatment Incidences [TIs based on Defined Daily Dose (DDDvet)] as previously described.[6, 7] While one TI was provided for broilers, TIs calculated for pigs includes separate TIs for sucklers, weaners, fatteners, and a TI adjusted for a lifespan of 200 days (TI 200). Biosecurity in this study was calculated using the Biocheck.UGent™ scoring system, based on 108 questions related to farm biosecurity.[11] The internal biosecurity subcategories (e.g. cleaning and disinfection) were gathered by questions related to counteracting the pathogen spread within the farm, while the external biosecurity subcategories (e.g. location of the farm) were gathered by questions related to preventing pathogens from entering the farm. The mean of internal and external biosecurity was defined as total biosecurity. More information and one example of the questionnaire could be found in supplementary materials ('*Standardized questionnaire'*).

DNA extraction, qPCR and sequencing

Individual faecal DNA (7 samples per pig farm and 5 samples per broiler farm) and pooled faecal DNA (25 samples pooled together per farm) were extracted in one central lab using the modified QIAmp Fast DNA Stool Mini Kit (Cat. No. 51604; Qiagen, The Netherlands) as described before.[7, 12] Following DNA extraction, qPCR was performed to quantify the abundance of four ARGs (*aph(3')-III, erm*(B)*, sul2, tet*(W)) along with 16S rRNA gene used for the normalization of ARG copies. These gene targets represent four different antimicrobial classes and were chosen based on the results of metagenomic analyses,[10] showing that these genes are of sufficient abundance to be detected in the majority of faecal samples, and these genes are only moderately correlated, hence different aspects of the total resistome can be captured by a limited number of assays.[13] The qPCR analyses of 16S, *erm*(B) and *tet*(W) was previously described by Van Gompel et al.[12] Briefly, the DNA template was diluted with TE-buffer (1:100) (Thermo Fisher Scientific, USA) to overcome possible inhibition. For all DNA samples, the PCR reaction (10 µl) consisted of 5 µl supermix (IQ SYBR Green Supermix (16S), SsoAdvanced™ Universal Probes Supermix (*erm*(B)), or IQ supermix (*tet*(W)) (Bio-Rad, USA)), 3 µl DNA template, as well as primers (16S: 200 nM each, *tet*(W): 600 nM each, *erm*(B): 400 nM each) and a probe (*tet*(W): 200 nM, *erm*(B): 250 nM).

The qPCR process of *aph(3')-III* and *sul2* were previously described by Yang et al.[14] Briefly, the DNA template was diluted with TE-buffer (1:100) (Thermo Fisher Scientific, USA). For all DNA samples, the PCR reaction (10 µl) consisted of 5 µl supermix (SsoAdvanced™ Universal Probes Supermix, Bio-Rad, USA), 3 µl DNA template, as well as primers (*aph(3')-III*: 400 nM, *sul2*: 100 nM) and a probe (*aph(3')-III*: 250nM, *sul2*: 100nM).

In addition, synthetic DNA encoding blue fluorescence protein (bfp) was used as an internal amplification control (IAC). Bfp primers (*aph(3')-III*: 400 nM, *erm*(B): 400 nM, *sul2*: 100 nM, *tet*(W): 600 nM) and probes (*aph(3')-III*: 250nM, *erm*(B): 250 nM, *sul2*: 100nM, *tet*(W): 200 nM) were added. A total of 4 positive and 8 negative control samples (TE buffer pH8, Thermo Fisher Scientific, USA) were used per PCR plate. All samples and controls were run in two technical PCR duplicates. For each specific gene, qPCR was exerted in only one laboratory. More details regarding the qPCR assays, quality control procedures, and 16S normalization have been described previously.[12, 14]

DNA of pooled faecal samples from pigs and broilers was extracted at the Technical University of Denmark (DTU) and shipped on dry ice to the Oklahoma Medical Research Foundation (OMRF; Oklahoma City, OK, USA) for shotgun metagenomic sequencing. In total, faecal DNA from 181 pigs and 178 broilers were sequenced on the HiSeq3000 platform (Illumina), yielding >36 billion sequences (18 billion paired-end reads).

More details about the subsequent processing of metagenomic data were described in our previous research.[6, 7, 10]

Statistical analysis

All gene abundances were expressed as $\log_{10}$ gene copies. The relative abundance of ARGs in this study was calculated by $\log_{10}$ (ARG copies/16S copies) to normalize for different amounts of bacterial DNA per sample.

Overall, differences were compared by performing a classic or Welch's analysis of variance (ANOVA) depending on the homogeneity of variance.[15, 16] In case of a significant difference, post-hoc tests (i.e. respectively a Tukey's Honest Significant Difference test (Tukey HSD)[17] or Games-Howell Post-Hoc test[18]) were carried out. Unless otherwise specified, appropriate post-hoc test p-values are reported.

R version 4.0.3 was used for all statistical analyses.[19] Before running the mixed model, potential farm characteristics (age, weight, farm size, etc.) were selected based on the opinions of experts in the EFFORT group and the published literature on farm animal risk factor analysis.[6, 7, 14, 20, 21] A linear mixed model with both country and farm as random effects was applied to take the between-country and between-farm variation into account. Changes in estimates and significance of associations with or without AMU in the model were determined.

Firstly, we ran the mixed model for AMR and selected farm characteristics other than AMU. Associations were selected by univariable analysis ($p<0.2$) and subsequently, an automatic backward analysis using univariably selected variables was conducted using the 'step' function in the 'R' package *lmerTest*.[22] The multivariable model without AMU was adjusted after the fixed parts were eliminated step by step ($p>0.05$). Considering the high level and limited variance of biosecurity score in country I, we performed a sensitivity analysis between ARG abundances and farm characteristics without country I.

Secondly, the same procedure was applied, but with adjustment for AMU in all mixed models. Due to the right-skewed distribution, AMU was log10 transformed after adding a pseudo-count of 1. The associations were selected again by the univariable analysis ($p<0.2$) and subsequently by the automatic backward selection of variables using the step function in the 'R' package lmerTest[22] to obtain the multivariable model with AMU. Considering the number of broiler farms without AMU, a sensitivity analysis was performed between ARG abundances and binary AMU (1/0 meaning using antimicrobials or not).

After checking the distribution of datasets, Spearman's rank correlation was used to evaluate the correlation of relative ARG abundances between individual qPCR data and previously published pooled metagenomic data for pigs and broilers.[6, 7, 10] The median of 5-7 individual qPCR results per farm was calculated before correlation analysis. To match the ARG targets of qPCR, all downstream gene abundances of *aph(3')-III*, *erm*(B), *sul2* and *tet*(W) were collected from the metagenomic data (FPKM) and summed per gene target. FPKM was log10 transformed after adding a pseudo-count of 1.

To determine the variance contribution of all risk factors, variance component analysis (VCA) was conducted. First, we determined variance in the null model (AMR ~ country + farm) using the 'R' package *VCA*.[23] In addition, significant risk factors (AMU and other farm characteristics) in the multivariable model with AMU determined in previous step were included in the VCA null model. The variance components were inspected for each ARG target.

Results

The relative abundance of four ARGs varied highly between countries and farms in pigs and broilers (Figures 1-2). Among four ARGs (*aph(3')-III*, *erm*(B), *sul2*, *tet*(W)), the highest mean relative abundance was observed in *tet*(W) ($P<0.01$) in pigs and *erm*(B) ($P<0.01$) in broilers, while the lowest mean relative abundance was found in *sul2* ($P<0.01$) in pigs and *aph(3')-III* ($P<0.01$) in broilers (Tables S2-S3). Similar variation was also found in AMU data (Figures S1-S3), which showed that tetracyclines were most frequently used among all antimicrobial classes in pigs, while aminoglycosides use was generally lower than the other antimicrobial classes in broilers. In addition, we found that the main biosecurity scores (external, internal, total) showed large between- and within-country variation (Figures S4-S5).

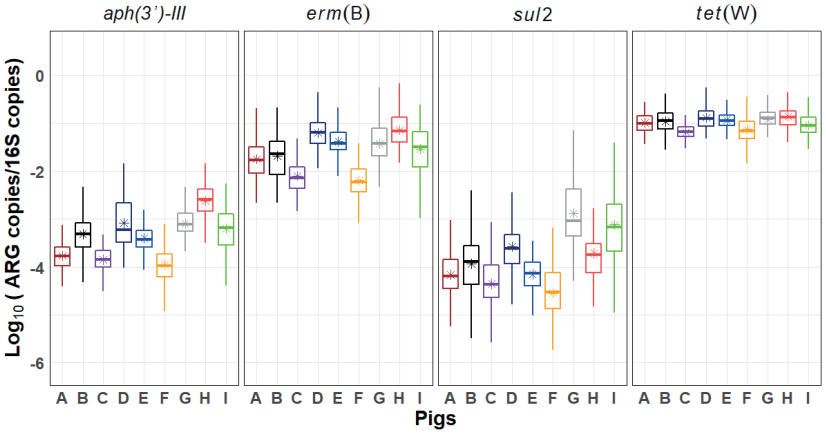

Figure 1. Relative abundance of four ARGs per country in pigs.

The whisker represents the interquartile range, and the centre line represents the median. The asterisk shows the mean in each country. Letters A–I represent the nine countries.

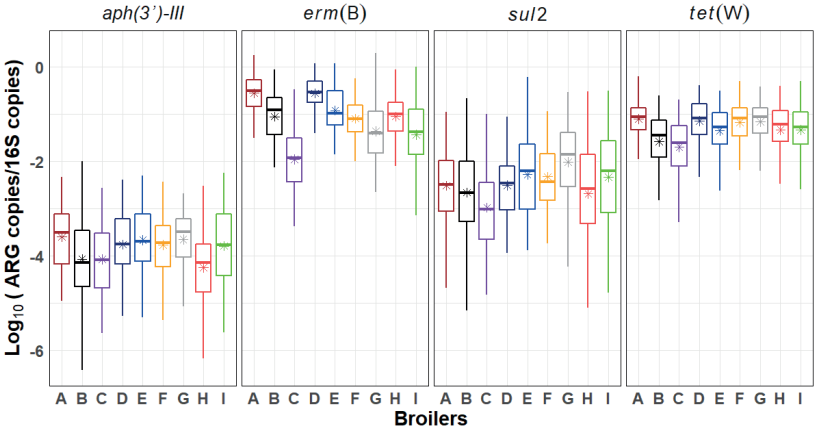

Figure 2. Relative abundance of four ARGs per country in broilers.

The whisker represents the interquartile range, and the centre line represents the median. The asterisk shows the mean in each country. Letters A–I represent the nine countries.

Association of AMR and farm characteristics other than AMU

The results of univariable analysis are presented in the supplementary materials ('*Univariable analysis between AMR and farm characteristics other than AMU*'). In pigs, all significant ($p<0.05$) farm characteristics (weaning age of piglets, current age of fatteners, biosecurity subcategories 'feeding and equipment supply' and 'location of the farm') in the univariable analysis were significant ($p<0.05$) in the multivariable model without AMU (Tables S4-S5), except for *erm*(B), where only the biosecurity sub-category 'cleaning and disinfection' remained significant ($\beta=0.004$, $p=0.02$) (Figure 3, Table S5). In both univariable analysis and multivariable model without AMU of broilers, we found significant ($p<0.05$) associations between relative ARG abundances with number of farmworkers (*erm*(B), *tet*(W)), weight of broilers at set-up (*erm*(B)), average number of rounds per year (*sul2*), biosecurity subcategories 'disease management' *tet*(W) and 'removal of manure and carcasses' (*aph(3')-III, erm*(B), *tet*(W)) (Tables S6-S7). The sensitivity analysis without biosecurity score in country I gave the same results as the analysis including biosecurity score in country I.

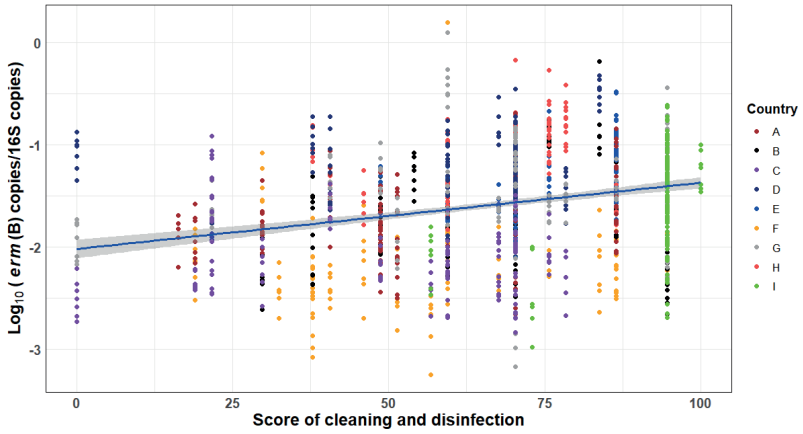

Figure 3. Associations between cleaning and disinfection level and relative *erm*(B) abundance in pigs in nine countries.

Cleaning and disinfection: One of the subcategories of internal biosecurity. The blue line represents the linear relationship between ARG abundance and the score of cleaning and disinfection, the grey area around the line demonstrates the 95% confidence interval. Letters A–I represent the nine countries.

In contrast, several significant ($p<0.05$) farm characteristics in the univariable models were dropped out from the multivariable model without AMU. For example in pigs, biosecurity subcategories 'vermin and bird control' and 'materials between compartments and equipment use' were non-significant ($p>0.05$) with relative *erm*(B) abundance in the multivariable model without AMU (Tables S4-S5). Similar results were found in broilers, where fewer variables (biosecurity categories 'cleaning and disinfection' and 'visitors and farmworkers') were left in the multivariable model without AMU for *aph(3')-III* and *erm*(B) (number of broilers at set-up, weight of broilers at sampling) compared with the results of univariable analysis (Tables S6-S7). Only one biosecurity sub-category 'removal of manure and carcasses' showed a significant association with the relative *aph(3')-III* abundance ($\beta=0.008$, $p<0.01$) in the multivariable model without AMU in broilers. Interestingly in broilers, the biosecurity score of 'cleaning and disinfection' showed no significant ($p>0.05$) association with relative *aph(3')-III* abundance as in the univariable analysis, but a significant ($\beta=-0.004$, $p=0.02$) negative association with the relative abundance of *tet*(W) in the multivariable model without AMU (Figure 4, Tables S6-S7).

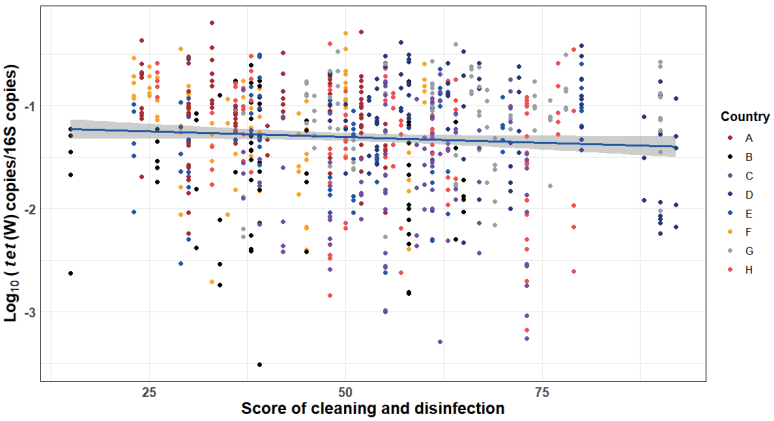

Figure 4. Associations between cleaning and disinfection level and relative *tet*(W) abundance in broilers in nine countries.

Cleaning and disinfection: One of the subcategories of internal biosecurity. The blue line represents the linear relationship between ARG abundance and the score of cleaning and disinfection, the grey area around the line demonstrates the 95% confidence interval. Letters A–I represent the nine countries.

Association of AMR, AMU and other farm characteristics

We found a significant positive association between lincosamide and macrolides use with relative *erm*(B) abundance in both pigs and broilers ($p<0.01$) (Tables 1-2, Figures S6-S7), and between tetracyclines use during suckler period with relative *tet*(W) abundance in pigs ($\beta=0.16$, $p=0.05$) (Table 1). Total AMU showed a significant association with relative *tet*(W) abundance in both pigs ($\beta=0.09$, $p=0.02$) and broilers ($\beta=0.17$, $p<0.01$) (Tables 1-2). The sensitivity analysis using dichotomized AMU data gave the same results as the analysis using continuous AMU data.

Adjustment for AMU led to some changes in the outcomes of risk factor analyses. In the multivariable model with AMU of pigs, 'current age of fatteners' and 'location of the farm' were omitted and 'current number of fatteners' ($\beta=0.00007$, $p=0.02$) was added for *aph(3')-III*; 'farrowing and suckling period' ($\beta=0.004$, $p=0.02$) was added for *sul2*; 'feeding and equipment supply' was omitted for *tet*(W). 'Cleaning and disinfection' retained its significant positive association with the relative *erm*(B) abundance ($\beta=0.004$, $p=0.02$) (Table 1, Table S5). In the multivariable model with AMU in broilers, almost all farm characteristics with significant ($p<0.05$) associations with ARG abundances remained present except for *tet*(W), in which 'number of farmworkers' was not significant anymore ($p>0.05$). In addition, in broilers we found a significant positive association ($p<0.01$) between the relative abundance of all ARG targets except for *sul2* and the biosecurity score of 'removal of manure and carcasses' (Table 2, Table S7).

Table 1. Multivariable linear mixed model with AMU in pigs.

	aph(3')-III			*erm*(B)			*sul2*			*tet*(W)		
	Beta	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**
AMU:												
TI_{DDDvet} tetracyclines (log_{10}) suckler							0.38	0.03	[0.04,0.73]	0.16	0.05	[0,0.32]
TI_{DDDvet} lincosamide & macrolide (log_{10}) fattener				0.40	<0.01	[0.19,0.6]						
TI_{DDDvet} total (log_{10}) fattener	0.34	<0.01	[0.23,0.46]				0.23	<0.01	[0.07,0.39]			
TI_{DDDvet} total (log_{10}) 200										0.09	0.02	[0.02,0.16]
Herd characteristics:												
Weaning age of piglets (days)	-0.02	0.01	[-0.04,-0.01]									
Current number of fatteners	7.70E-05	0.02	[0,0.0001]									
Internal biosecurity:												
Farrowing and suckling period							0.004	0.03	[0,0.008]			
Cleaning and disinfection				0.004	0.02	[0,0.01]						

The multivariable model with AMU was automatically adjusted using the 'step' function in the 'R' package *lmerTest*.[22] Only associations with a p-value less than 0.05 are involved.

Lincosamide & macrolide: Macrolide + lincosamide + lincomycin/spectinomycin use.

Table 2. Multivariable linear mixed model with AMU in broilers.

	aph(3')-III			*erm*(B)			*sul2*			*tet*(W)		
	Beta	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**
AMU:												
Group TI_{DDDvet} lincosamide & macrolide (log_{10})				0.47	<0.01	[0.27,0.67]						
Group TI_{DDDvet} trimethoprim & sulfonamide (log_{10})	-0.24	0.03	[-0.46,-0.02]									
Group TI_{DDDvet} total (log_{10})										0.17	<0.01	[0.08,0.26]
Purchase TI_{DDDvet} aminoglycosides (log_{10})							0.80	0.01	[0.18,1.41]	-0.34	0.04	[-0.68,-0.01]
Herd characteristics:												
Number of farmworkers				0.03	0.04	[0.001,0.06]						
Weight of broilers at set-up (g)				0.02	0.01	[0.004,0.04]						
Average number of rounds/year							0.17	<0.01	[0.07,0.26]			
Internal biosecurity:												
Disease management										-0.006	<0.01	[-0.01,-0.003]
Cleaning and disinfection										-0.004	0.03	[-0.008,-0.001]
External biosecurity:												
Removal of manure and carcasses	0.008	<0.01	[0,0.01]	0.006	0.01	[0.001,0.012]				0.007	<0.01	[0.003,0.01]

The multivariable model with AMU was automatically adjusted using the 'step' function in the 'R' package *lmerTest*.[22] Only associations with a p-value less than 0.05 are involved.

Lincosamide & macrolide: Macrolide + lincosamide + spectinomycin use. Trimethoprim & sulfonamide: Sulfonamide + trimethoprim/sulfonamide use.

Correlation analysis between the median of individual qPCR data and pooled metagenomic data

In the correlation assessment of median-individual qPCR data and pooled metagenomic data from pigs and broilers, we only found a moderate correlation (Figure 5). *Erm*(B) always showed a high correlation ($\rho>0.7$, $p<0.05$) of the four ARG targets.

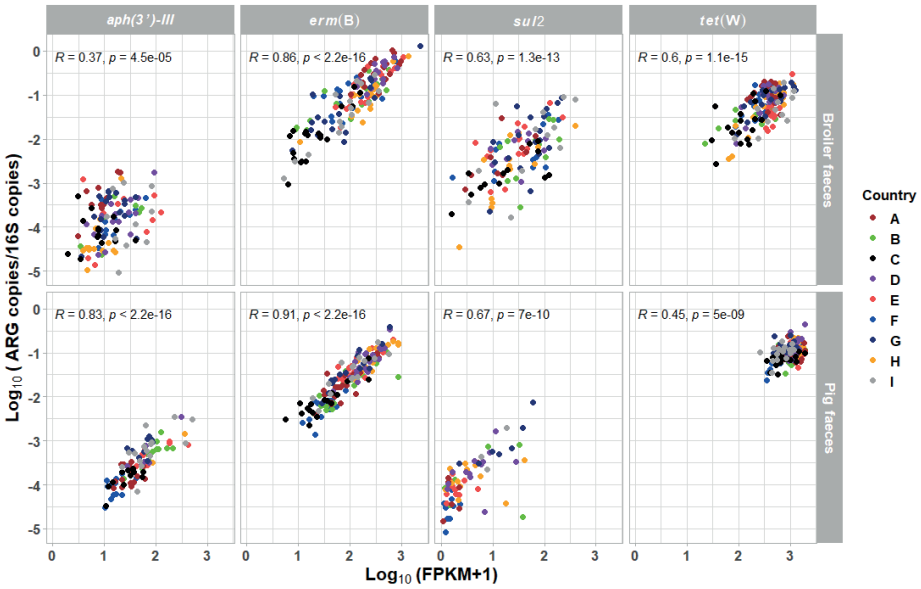

Figure 5. Correlation of AMR between median-individual qPCR data and pooled metagenomic data in pigs and broilers.

FPKM: Fragments per kilobase reference per million bacterial fragments.[10] ARG targets: *aph(3')-III, erm*(B)*, sul2, tet*(W).

The median of 5-7 individual qPCR results was calculated per farm before correlation analysis.

Letters A–I represent the nine countries.

Variance component analysis of multivariable linear mixed model

In the VCA null model of pigs (Table S8), variance contributions from country and farm were higher than the other variables, while in broilers (Table S9) most variances were due to within-farm variation. In both pigs and broilers, the within-farm variance was lowest for *erm*(B) compared to other components.

After farm characteristics were adjusted into the VCA null model, we found a shift of variance contribution from between-country or between-farm variation to farm characteristics, especially to AMU. In pigs, after farm

characteristics were adjusted, we found AMU contributed 12.60% to the total variation of relative *aph(3')-III* abundance, while the variance contribution percentages of country (53.83% to 46.73%) and farm (31.99% to 24.55%) decreased (Table 3, Table S8). In broilers, between-farm variation changed for all ARG targets. E.g. after adjustment, the contribution percentage of between-farm variation changed from 42.79% to 28.95%, while lincosamide and macrolides use contributed 10.59% to the total variation of relative *erm*(B) abundance (Table 4, Table S9).

Discussion

To find potential risk factors that contributed to AMR abundance in pigs and broilers, we assessed the relationship between on-farm AMR levels with AMU and other farm characteristics using a linear mixed model. The results showed that in addition to AMU, risk factors such as age and weight of animals, and biosecurity measures, were also significantly associated with AMR levels in pig and broiler faeces. A moderate correlation was observed between median-individual qPCR data and previously published pooled metagenomic data. The between-country and between-farm variation could partially be explained by AMU. Different ARG targets seem to have different sample size requirements to accurately represent their overall farm-level abundance.

It has been well documented that many farm factors other than AMU can affect the AMR levels in farm animals.[6, 8, 14, 21] In our study, different ARG targets were associated with different risk factors. This could be explained by the ARG target selection criterion that only a moderate correlation with other ARGs is allowed. In pigs, we found that piglets weaned at an older age have significantly lower *aph(3')-III* levels in their faeces before slaughter. We assume that the immune system of piglets weaned at an older age is more mature[24, 25] and therefore these animals require less antimicrobial treatment,[26] which results in lower AMR levels later in the rearing process.[27] The negative association ($p>0.05$) we found between weaning age of pigs and total AMU in the fattening period (data not shown) can also provide evidence for this. In broilers, we found a significant positive association between farm staff number and relative abundance of *erm*(B) and *tet*(W). This may suggest that farmworkers act as a source of ARGs for farm animals as was described for MRSA CC398 in pig farms in Norway.[28] However, there have been only occasional reports on the introduction of specific resistant bacteria into animal farms - mostly, the transmission was documented from animals to workers.

Table 3: Variance component analysis of the multivariable model with AMU in pigs.

	aph(3')-III			*erm*(B)			*sul2*			*tet*(W)		
	VC	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**
AMU:												
TI_{DDDvet} tetracyclines (log_{10}) suckler							0.003	*0.45*	0.05	0.01	*6.56*	0.07
TI_{DDDvet} lincosamide & macrolide (log_{10}) fattener				0.02	5.24	0.13						
TI_{DDDvet} total (log_{10}) fattener	0.04	12.60	0.21				NA*	0.00	0.00			
TI_{DDDvet} total (log_{10}) 200										NA*	0.00	0.00
Herd characteristics:												
Weaning age of piglets (days)	0.01	2.07										
Current number of fatteners	0.00	0.00	0.00									
Internal biosecurity:												
Farrowing and suckling period							0.01	1.64	0.10			
Cleaning and disinfection				NA*	0.00	0.00						
Others:												
Country	0.17	**46.73**	0.41	0.10	32.53	0.32	0.30	**44.03**	0.54	0.01	9.33	0.09
Farm	0.08	24.55	0.29	0.15	**49.32**	0.39	0.18	26.77	0.42	0.04	**47.46**	0.20
Residual	0.05	14.05	0.22	0.04	12.91	0.20	0.18	27.11	0.43	0.03	36.65	0.17

VC: Variance component. %Total: The percentage of the variance component. SD: Standard deviation. Lincosamide & macrolide: Macrolide + lincosamide + spectinomycin use. NA*: Too small value that was automatically displayed as NA by the '*VCA*' package.[23]

Values in bold indicate the highest VC percentage for each model. All values are rounded at two digits behind the comma unless rounding would lead to the misinterpretation of an effect.

Table 4: Variance component analysis of the multivariable model with AMU in broilers.

	aph(3')-III			*erm*(B)			*sul2*			*tet*(W)		
	VC	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**
AMU:												
Group TI_{DDDvet} lincosamide & macrolide (log_{10})				0.05	10.59	0.23						
Group TI_{DDDvet} trimethoprim & sulfonamide (log_{10})	0.02	3.02	0.13									
Group TI_{DDDvet} total (log_{10})										0.04	14.23	0.20
Purchase TI_{DDDvet} aminoglycosides (log_{10})							0.08	9.19	0.29	0.04	13.37	0.20
Herd characteristics:												
Number of farmworkers				0.02	3.64	0.14						
Weight of broilers at set-up (g)				0.00	0.00	0.00						
Average number of rounds/year							0.02	2.17	0.14			
Internal biosecurity:												
Disease management										NA	0.00	0.00
Cleaning and disinfection										NA	0.00	0.00
External biosecurity:												
Removal of manure and carcasses	0.01	0.95	0.07	0.004	0.87	0.07				0.01	4.04	0.11
Others:												
Country	0.04	7.51	0.21	0.17	**33.68**	0.42	0.07	8.03	0.27	0.01	2.37	0.08
Farm	0.11	18.15	0.32	0.15	28.95	0.39	0.25	27.77	0.50	0.03	11.17	0.18
Residual	0.41	**70.38**	0.64	0.11	22.28	0.34	0.47	**52.84**	0.69	0.16	**54.82**	0.40

VC: Variance component. %Total: The percentage of the variance component. SD: Standard deviation.

Lincosamide & macrolide: Macrolide + lincosamide + spectinomycin use. Trimethoprim & sulfonamide: Sulfonamide + trimethoprim/sulfonamide use.

Values in bold indicate the highest VC percentage for each model. NA*: Too small value that was automatically displayed as NA by the '*VCA*' package.[23] All values are rounded at two digits behind the comma unless rounding would lead to the misinterpretation of an effect.

Biosecurity is increasingly valued by farmers, several studies have shown that a high biosecurity index (high hygiene level,[29, 30] good management,[31] good feeding practices[32]) has a positive effect on the control of disease and AMR levels in farm animals. In the present study, we found similar results in broiler farms, where 'disease management' and 'cleaning and disinfection' as biosecurity subcategories were significantly associated with a lower relative *tet*(W) abundance. In contrast, biosecurity was also reported to be positively associated with AMR levels in pig faeces from the same farms as described here, particularly for the biosecurity sub-category 'cleaning and disinfection'.[6] Similar results were reported in previous veal calf studies.[14] Furthermore, in this study we found that broiler farms with higher biosecurity scores of 'transfer of faeces and carcasses' have higher AMR levels than other broiler farms. This is probably related to one biosecurity measure (removal of farm manure) included for this biosecurity sub-category. Similar results were reported in a previous study in broilers, in which manure storage on farms was shown to be negatively associated with the prevalence of β-lactam resistance in flocks.[8] These results indicate a complex relationship between on-farm biosecurity and AMR levels in farm animals. More in-depth and specific analyses of AMR and farm biosecurity are necessary in the future to understand the impact of possible interventions to reduce AMR in farm animals.

After the multivariable model was adjusted for AMU, we expected that fewer farm characteristics would be associated with AMR (compared to the model without AMU), because of assumed interlinkages between AMU and other farm characteristics. Generally, our results were in agreement with these expectations. However, compared to the model without AMU, there was one additional factor (biosecurity sub-category 'farrowing and suckling period') showed a significant positive association with relative *sul2* abundance in the AMU adjusted model in pigs. One explanation is that at the same AMU level, pigs with a longer farrowing and suckling period are at a higher risk of acquiring resistance. Several studies have reported a possible bacteria spread[33] and ARG transmission[34, 35] between sows and piglets, especially around parturition. Therefore, it is necessary to take co-varying factors into account when establishing potential risk factors of AMR.

Consistent with previous risk factor analysis reports of metagenomic data in pigs,[6] we found significant positive associations between relative *erm*(B) abundance with lincosamide and macrolides use, and between total AMU during the fattening phase and relative abundance of *aph(3')-III* and *sul2.* When comparing our results with the meta-analysis of metagenomic data in broilers,[7] we did not find comparable significant ARG-AMU associations in this study, which may be due to the fact that the selected specific gene targets for qPCR approach do not necessarily represent the whole ARG group linked with a specific antimicrobial class.

In the correlation analysis, we only found a moderate correlation of AMR between the median of 5-7 individual qPCR readouts and the previously published metagenomic data assessed in pooled samples.[10] In addition to the not completely reproduced risk factor analysis results of these two datasets, we speculate that collecting 5 or 7 individual faecal samples per farm probably do not represent the farm-level AMR as accurately as pooling 25 individual faecal samples together per farm. Meanwhile, we found consistent results with previous metagenomic data in risk factor analysis of *erm*(B), and we observed a high correlation of *erm*(B) abundance between median-individual qPCR data and pooled metagenomic data in both pigs and broilers. This may indicate that ARGs have different sample size requirements per farm to accurately represent their overall farm level. The low within-farm variance for *erm*(B) in VCA results of both pigs and broilers may further provide evidence to our speculation.

In addition, the VCA results in the multivariable model with AMU showed that AMU is the most important variance component in comparison to other farm characteristics. Compared to the null VCA model, the between-country and between-farm variation in pigs (*aph(3')-III*) and the between-farm variation in broilers (all ARG targets) decreased considerably, mainly shifted to AMU. This suggests that the between-country and between-farm variation can partially be explained by AMU. Furthermore, it appeared that the farm characteristics included in our study only explained a limited part of the observed total AMR variation. This indicates that there are likely unidentified/unstudied determinants (e.g. historical AMU, farm management factors) need to be evaluated and considered in future studies.

Conclusions

This study shows that qPCR is an efficient tool for a targeted assessment of AMR in livestock-related samples. The AMR variation between samples was first and foremost caused by between-country, between-farm, and within-farm differences, and secondly by AMU. In addition, there are other farm characteristics that have a low but significant impact on AMR levels in farm animals, which requires further research. More attention needs to be paid to sample size in future epidemiological studies of ARGs.

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Transparency declarations

None to declare.

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Supplementary Materials

Risk factors for the abundance of antimicrobial resistance genes *aph(3')-III, erm*(B), *sul2* and *tet*(W) in pig and broiler faeces in nine European countries

Methods and materials

Standardized questionnaire

A standardized questionnaire including information on AMU, farm management, and animal welfare was developed based on the algorithm of the Biocheck.UGent™ scoring system.[1] The questionnaire included items on farm characteristics (Table S1). EpiData 3.1 was used for data entry, potential factors in the questionnaire were used for analysis only when more than 95% of the questions were answered.

Table S1: ***Questionnaire on antimicrobial consumption, management, biosecurity, health and welfare in broiler farms***

Confidentiality statement	
Date	
Name	
Signature	
Part I. General information	
Farm code	
Farm name	
Farm address	
GPS location	
Farm manger name	
Farm manger telephone	
Farm manger email address	
Completion date	
Farm characteristics	
Bqq001	Are there any other animals present at the farm? 0 - no 1 - yes, which animals
Bqq002	How many people are working at the broiler farm in total?
Characteristics of sampled house	
Bqq003	How many chicks were actually set-up in the current round in the sampled house?
Bqq004	What was the average weight of the chicks at set-up?
Bqq005	How many chickens are currently present in the sampled house?
.	.
.	.
.	.
Part II. Technical data	
Bqq010	Average number of rounds/year?
Bqq011	Average number of chicks set up / round?
Bqq012	Number of chickens delivered to the slaughterhouse/year?
.	.
.	.
.	.
Part III. Biosecurity check	
Bqq016	Do the day-old chicks come from the same hatchery or different hatcheries? 1-Same, 2-Different.
Bqq017	Are day-old chicks delivered to the own farm before other broiler farms are supplied by the same transport vehicle? 0-No, 1-Yes, 2-Sometimes, 3-I don't know.
Bqq018	Are hygienic criteria posed on the transport vehicle that brings the day-old chicks to the farm, according to the farmer? 0-No, 1-Yes, 2-I don't know.
.	.
.	.
.	.
Part IV. Antimicrobial usage	
A	Group treatments Table of antimicrobials used in the treatments.
B	List of purchased antimicrobials Table of antimicrobials purchased in the first year before the study.
Part V. Welfare indicators	
The form 'welfare indicators' is included in the field forms. Don't forget to score the welfare and fill in the form!	

Results

Table S2. Overview and summary statistics of relative ARG abundances in pigs.

			Final sample number No. (%)				Mean*				10-90th percentile*			
	Countries	**Farms**	***aph(3')-III***	***erm*(B)**	***sul2***	***tet*(W)**	***aph(3')-III***	***erm*(B)**	***sul2***	***tet*(W)**	***aph(3')-III***	***erm*(B)**	***sul2***	***tet*(W)**
Pigs	A	20	129 (92)	135 (96)	121 (86)	137 (98)	-3,77	-1,75	-4,17	-0,99	-4.13--3.40	-2.19--1.22	-4.83--3.51	-1.27--0.72
	B	20	133 (95)	138 (99)	113 (81)	137 (98)	-3,32	-1,69	-3,94	-0,96	-3.85--2.87	-2.33--0.98	-4.86--3.14	-1.28--0.68
	C	20	121 (86)	136 (97)	71 (51)	136 (97)	-3,85	-2,10	-4,35	-1,19	-4.16--3.57	-2.54--1.57	-5.01--3.79	-1.44--0.96
	D	20	96 (69)	140 (100)	130 (93)	140 (100)	-3,08	-1,20	-3,58	-0,88	-3.71--2.19	-1.64--0.77	-4.13--2.92	-1.16--0.58
	E	20	130 (93)	139 (99)	124 (89)	139 (99)	-3,40	-1,38	-4,15	-0,94	-3.70--3.01	-1.75--0.93	-4.67--3.67	-1.14--0.69
	F	20	120 (86)	131 (94)	117 (84)	131 (94)	-3,95	-2,19	-4,55	-1,12	-4.38--3.5	-2.60--1.76	-5.30--3.93	-1.50--0.77
	G	20	114 (81)	133 (95)	111 (79)	134 (96)	-3,10	-1,43	-2,88	-0,93	-3.47--2.75	-2.11--0.85	-3.66--1.96	-1.16--0.61
	H	20	77 (55)	134 (96)	131 (94)	134 (96)	-2,62	-1,14	-3,72	-0,85	-3.17--2.21	-1.56--0.74	-4.34--2.94	-1.11--0.59
	I	19	126 (95)	127 (95)	85 (64)	128 (96)	-3,20	-1,54	-3,12	-1,04	-3.76--2.52	-2.19--0.93	-3.91--1.93	-1.37--0.73
Overall	A-I	179	1046 (83)	1213 (97)	1003 (80)	1216 (97)	-3.41	-1.60	-3.83	-0.99	-4.05--2.65	-2.35--0.91	-4.76--2.84	-1.31--0.67

*The relative ARG abundance was calculated by $\log_{10}$ (ARG copies/16S copies), which is rounded at two digits behind the decimal point in this table. Letters A–I represent the nine countries. Farms: Only farms that can be linked to questionnaire data were included. Final sample number: All samples left after a quality check (technical standard, limit of detection (LOD) and limit of quantification (LOQ)).

Table S3. Overview and summary statistics of relative ARG abundances in broilers.

			Final sample number No. (%)				Mean*				10-90th percentile*			
	Countries	**Farms**	***aph(3')-III***	***erm*(B)**	***sul2***	***tet*(W)**	***aph(3')-III***	***erm*(B)**	***sul2***	***tet*(W)**	***aph(3')-III***	***erm*(B)**	***sul2***	***tet*(W)**
Broilers	A	21	94 (90)	102 (97)	90 (86)	102 (97)	-3,56	-0,54	-2,49	-1,09	-4.5--2.65	-1.14--0.06	-3.33--1.47	-1.64--0.67
	B	20	86 (86)	96 (96)	92 (92)	93 (93)	-4,08	-1,09	-2,59	-1,56	-5.2--3.08	-1.81--0.42	-3.9--1.34	-2.38--0.93
	C	20	93 (93)	99 (99)	92 (92)	98 (98)	-4,08	-1,95	-2,98	-1,69	-5.06--3.11	-2.76--1.07	-4.00--1.85	-2.46--0.95
	D	20	94 (94)	96 (96)	87 (87)	96 (96)	-3,76	-0,56	-2,50	-1,14	-4.66--2.75	-0.98--0.16	-3.32--1.80	-1.92--0.59
	E	19	89 (93)	95 (99)	88 (92)	95 (99)	-3,67	-0,91	-2,30	-1,34	-4.72--2.77	-1.53--0.34	-3.53--1.23	-2.04--0.80
	F	20	88 (88)	94 (94)	90 (90)	93 (93)	-3,77	-1,09	-2,32	-1,17	-4.56--2.99	-1.62--0.51	-3.10--1.41	-1.83--0.72
	G	20	98 (98)	97 (97)	98 (98)	99 (99)	-3,65	-1,36	-2,01	-1,15	-4.4--2.91	-2.04--0.60	-3.14--1.09	-1.78--0.67
	H	20	91 (91)	98 (98)	88 (88)	100 (100)	-4,25	-1,05	-2,69	-1,33	-5.24--3.36	-1.68--0.39	-4.26--1.44	-2.19--0.75
	I	19	88 (86)	96 (94)	89 (87)	93 (91)	-3,76	-1,48	-2,35	-1,33	-4.74--2.70	-2.51--0.68	-3.71--1.09	-2.08--0.73
Overall	A-I	180	821 (91)	873 (97)	814(90)	869 (96)	-3.84	-1.11	-2.47	-1.31	-4.79--2.88	-2.00--0.30	-3.68--1.30	-2.09--0.72

*The relative ARG abundance was calculated by $\log_{10}$ (ARG copies/16S copies), which is rounded at two digits behind the decimal point in this table. Letters A–I represent the nine countries. Farms: Only farms that can be linked to questionnaire data were included. Final sample number: All samples left after a quality check (technical standard, limit of detection (LOD) and limit of quantification (LOQ)).

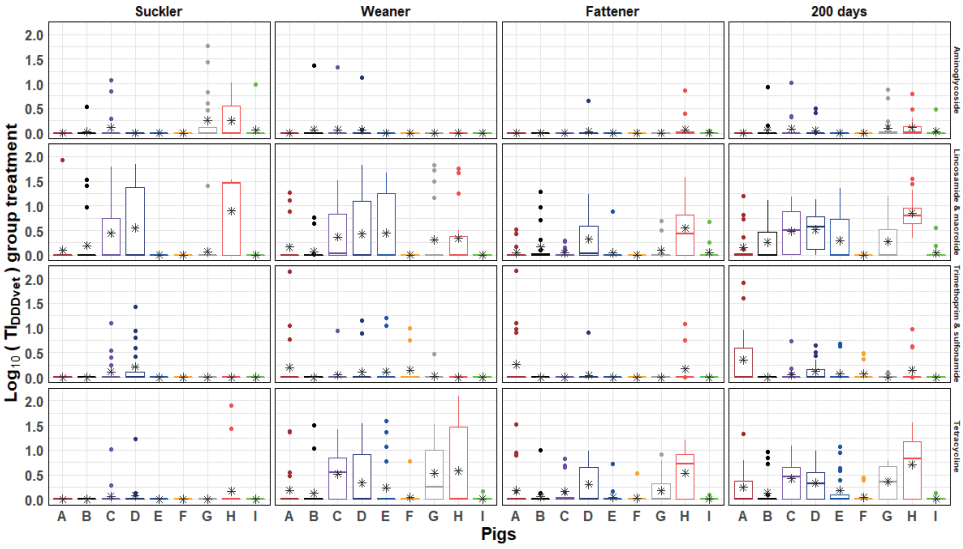

Figure S1. Treatment incidence along the whole life of pigs per country.

Lincosamide & macrolide: Macrolide + lincosamide + spectinomycin use. Trimethoprim & sulfonamide: Sulfonamide + trimethoprim/sulfonamide use.

The whisker represents the interquartile range, and the centre line represents the median. The asterisk shows the mean in each country. Letters A–I represent the nine countries.

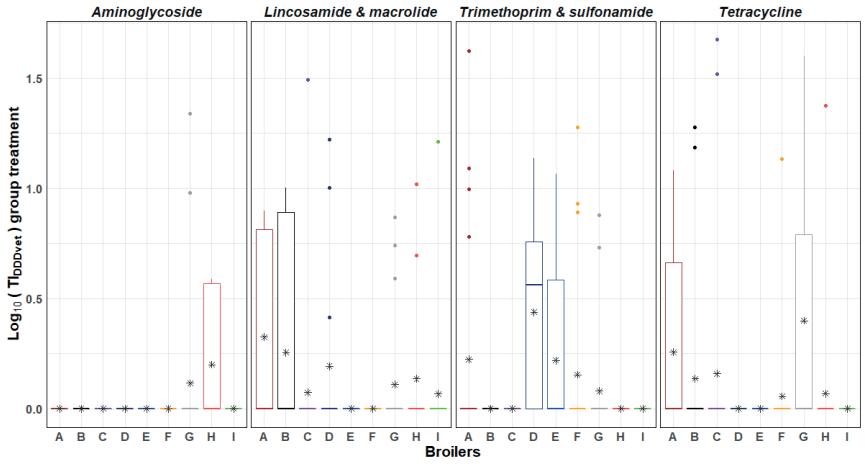

Figure S2. Treatment incidence in broilers per country.

Lincosamide & macrolide: Macrolide + lincosamide + spectinomycin use. Trimethoprim & sulfonamide: Sulfonamide + trimethoprim/sulfonamide use. The whisker represents the interquartile range, and the centre line represents the median. The asterisk shows the mean in each country. Letters A–I represent the nine countries.

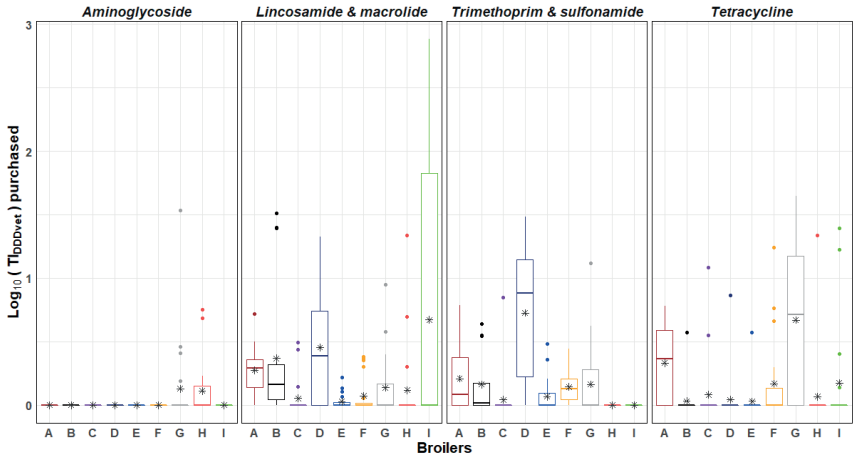

Figure S3. Treatment incidence in broilers per country, based on purchased data.

Lincosamide & macrolide: Macrolide + lincosamide + spectinomycin use. Trimethoprim & sulfonamide: Sulfonamide + trimethoprim/sulfonamide use. The whisker represents the interquartile range, and the centre line represents the median. The asterisk shows the mean in each country. Letters A–I represent the nine countries.

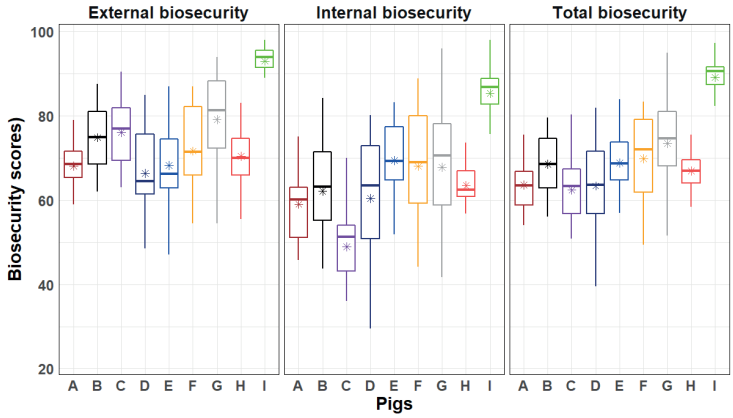

Figure S4. Three main biosecurity scores (external, internal and total) in pig farms.

The whisker represents the interquartile range, and the centre line represents the median. The asterisk shows the mean in each country. Letters A–I represent the nine countries.

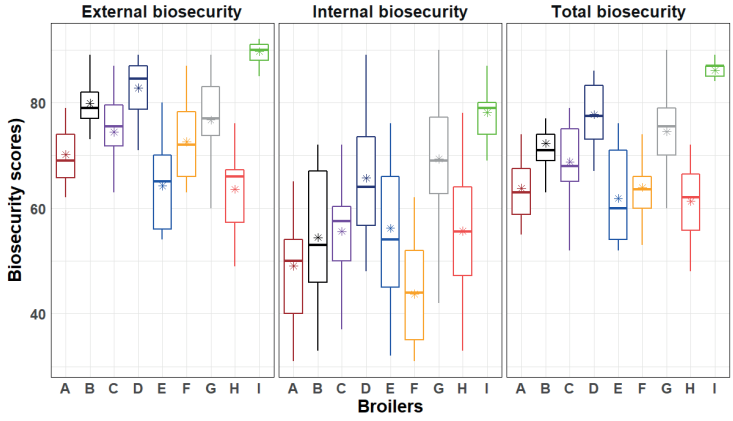

Figure S5. Three main biosecurity scores (external, internal and total) in broiler farms.

The whisker represents the interquartile range, and the centre line represents the median. The asterisk shows the mean in each country. Letters A–I represent the nine countries.

Univariable analysis between AMR and farm characteristics other than AMU

In the univariable analysis of pigs (linear mixed model, random effects for country and farm), weaning age of piglets (β=-0.02, p=0.01), current age of fatteners (β=-0.003, p=0.01), and biosecurity sub-category 'location of the farm' (β=-0.002, p=0.03) showed a significant negative association with relative *aph(3')-III* abundance. All

the biosecurity subcategories showed a positive association with the relative *erm*(B) abundance, including significant associations for 'materials between compartments and equipment use' (β=0.004, p=0.03), 'cleaning and disinfection' (β=0.004, p=0.02), and 'vermin and bird control' (β=0.003, p=0.05). The sub-category 'feeding and equipment supply' showed a significant association with relative *tet*(W) abundance (β=-0.002, p=0.04). No significant association was found in the univariable analysis for *sul2* in pigs (Table S4).

In the univariable analysis of broilers (linear mixed model, random effects for country and farm), the biosecurity sub-category 'cleaning and disinfection' showed a significant association (β=0.006, p=0.01) with the relative abundance of *aph(3')-III,* but a negative association with the relative abundance of the other three targets (*erm*(B), *sul2*, *tet*(W)). The number of farmworkers showed a significant ($p<0.05$) positive association with the relative abundance of both *erm*(B) and *tet*(W). In addition, for *erm*(B), we found a significant ($p<0.05$) higher abundance in farms with more broilers at set-up, with higher weight of broilers at set-up, and with higher weight of broilers at sampling. The average number of rounds per year showed a significant positive association with the relative abundance of *sul2* (β=0.15, $p<0.01$). The biosecurity sub-category 'disease management' was found negatively associated with the relative abundance of *tet*(W) (β=-0.006, $p<0.01$). The biosecurity sub-category 'removal of manure and carcasses' showed a significant positive association with the relative abundance of all antimicrobial resistance genes (ARGs), except for *sul2* (Table S6).

Table S4. Univariable association of farm characteristics and relative abundance of *aph(3')-III*, *erm*(B), *sul2* and *tet*(W) resistance genes in pigs.

	aph(3')-III			*erm*(B)			*sul2*			*tet*(W)		
	Beta	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**
Herd characteristics:												
Other animals present at the farm (no: ref)	-0.05	0.39	[-0.18,0.07]	-0.09	0.18	[-0.23,0.04]	0.04	0.60	[-0.12,0.2]	0.04	0.29	[-0.03,0.11]
Number of farmworkers	0.00	0.28	[-0.004,0.01]	0.005	0.34	[-0.005,0.01]	-0.01	0.16	[-0.02,0.003]	-0.003	0.17	[-0.008,0.001]
Weaning age of piglets (days)	**-0.02**	**0.01**	**[-0.04,-0.005]**	-0.02	0.11	[-0.04,0.004]	-0.02	0.16	[-0.04,0.007]	-0.01	0.11	[-0.02,0.002]
Weaning weight of piglets (kg)	-0.01	0.82	[-0.076,0.060]	-0.001	0.97	[-0.08,0.08]	0.03	0.50	[-0.06,0.12]	0.01	0.59	[-0.03,0.05]
Age of weaners at transfer to the growing-finishing barns (days)	-0.003	0.29	[-0.008,0.002]	-0.003	0.34	[-0.009,0.003]	-0.002	0.57	[-0.009,0.005]	0.001	0.63	[-0.002,0.004]
Current age of fatteners (days)	**-0.003**	**0.01**	**[-0.005,-0.001]**	-0.002	0.13	[-0.005,0.001]	0.00	0.42	[-0.004,0.002]	0.0002	0.74	[-0.001,0.001]
Current number of fatteners	4.21E-05	0.13	[-0.0001,0.0001]	0.005	0.55	[-0.0001,0.0001]	0.005	0.86	[-0.0001,0.0001]	0.005	0.82	[-0.0001,0.0001]
Current weight of fatteners (kg)	0.00	0.15	[-0.01,0.002]	-0.002	0.56	[-0.009,0.005]	-0.004	0.30	[-0.01,0.004]	0.001	0.41	[-0.002,0.004]
Season of sampling:												
Spring	-0.03	0.75	[-0.24,0.17]	-0.04	0.75	[-0.19,0.22]	-0.06	0.68	[-0.32,0.21]	-0.06	0.29	[-0.18,0.05]
Summer	-0.05	0.64	[-0.24,0.15]	-0.01	0.94	[-0.27,0.19]	0.02	0.85	[-0.23,0.28]	-0.03	0.55	[-0.14,0.08]
Autumn	-0.11	0.23	[-0.3,0.07]	0.02	0.87	[-0.23,0.21]	0.11	0.37	[-0.13,0.35]	-0.04	0.50	[-0.14,0.07]
Winter	ref	.	.	ref	.	.	ref	.	.	ref	.	.
Biosecurity:												
Internal biosecurity	-0.0004	0.88	[-0.005,0.004]	**0.006**	**0.01**	**[0.001,0.01]**	0.004	0.22	[-0.002,0.010]	-0.001	0.56	[-0.003,0.002]
External biosecurity	-0.001	0.66	[-0.007,0.004]	0.005	0.10	[-0.001,0.011]	0.003	0.45	[-0.005,0.010]	-0.003	0.08	[-0.006,0.00001]
Total biosecurity	-0.001	0.73	[-0.007,0.005]	**0.008**	**0.01**	**[0.002,0.01]**	0.005	0.25	[-0.003,0.012]	-0.002	0.20	[-0.005,0.001]
Internal biosecurity:												
Disease management	0.002	0.15	[-0.001,0.005]	0.001	0.69	[-0.002,0.004]	0.002	0.33	[-0.002,0.005]	-0.0001	0.91	[-0.002,0.001]
Farrowing and suckling period	-0.002	0.15	[-0.005,0.001]	0.003	0.10	[-0.001,0.006]	0.003	0.09	[-0.001,0.007]	0.0003	0.67	[-0.001,0.002]
Nursery unit	-0.0002	0.90	[-0.003,0.003]	0.0005	0.79	[-0.003,0.004]	0.002	0.42	[-0.002,0.006]	0.0004	0.67	[-0.001,0.002]
Finishing unit	-0.001	0.37	[-0.004,0.002]	0.002	0.30	[-0.002,0.005]	-0.0005	0.82	[-0.004,0.003]	-0.001	0.25	[-0.003,0.001]
Materials between compartments and equipment use	-0.0003	0.83	[-0.003,0.003]	**0.004**	**0.03**	**[0.00001,0.007]**	0.001	0.68	[-0.003,0.005]	-0.001	0.29	[-0.003,0.001]
Cleaning and disinfection	0.001	0.40	[-0.002,0.004]	**0.004**	**0.02**	**[0.001,0.007]**	0.002	0.27	[-0.002,0.006]	-0.0001	0.91	[-0.002,0.001]
External biosecurity:												
Purchase of breeding pigs, piglets and semen	-0.002	0.44	[-0.008,0.003]	0.001	0.68	[-0.005,0.008]	0.001	0.84	[-0.007,0.008]	-0.001	0.67	[-0.004,0.003]
Transport of animals, removal of carcasses and manure	0.0001	0.97	[-0.004,0.004]	0.002	0.34	[-0.002,0.007]	0.002	0.50	[-0.003,0.007]	-0.001	0.27	[-0.004,0.001]
Feeding and equipment supply	0.0003	0.85	[-0.003,0.003]	0.002	0.17	[-0.001,0.006]	0.001	0.67	[-0.003,0.005]	**-0.002**	**0.04**	**[-0.004,-0.000]**
Visitors and farmworkers	4.98E-05	0.97	[-0.003,0.003]	0.003	0.09	[-0.000,0.006]	0.002	0.43	[-0.002,0.005]	-0.001	0.24	[-0.003,0.001]
Vermin and bird control	0.003	0.06	[-0.00001,0.006]	**0.003**	**0.05**	**[-0.000,0.006]**	0.0003	0.86	[-0.003,0.004]	-0.0004	0.60	[-0.002,0.001]
Location of the farm	**-0.002**	**0.03**	**[-0.004,-0.000]**	0.000	0.84	[-0.002,0.002]	0.0002	0.83	[-0.002,0.003]	-0.0004	0.40	[-0.001,0.001]

Selection of variables from the univariable analysis used in the mixed model with a random effect for both country and farm. The bold type indicates associations with $p<0.05$.

Table S5. Multivariable linear mixed model without AMU in pigs.

	aph(3')-III			*erm*(B)			*sul2*			*tet*(W)		
	Beta	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**
Herd characteristics:												
Weaning age of piglets (days)	-0.02	0.03	[-0.04,-0.002]									
Current age of fatteners (days)	-0.003	0.01	[-0.005,-0.001]									
Internal biosecurity:												
Cleaning and disinfection				0.004	0.02	[0.001,0.007]						
External biosecurity:												
Feeding and equipment supply										-0.002	0.04	[-0.004,-0.0001]
Location of the farm	-0.002	0.03	[-0.004,-0.001]									

The multivariable model without AMU was automatically adjusted using the 'step' function in the 'R' package *lmerTest*.[2] Only associations with a p-value less than 0.05 were involved.

Table S6. Univariable association of farm characteristics and relative abundance of *aph(3')-III*, *erm*(B), *sul2* and *tet*(W) resistance genes in broilers.

	aph(3')-III			*erm*(B)			*sul2*			*tet*(W)		
	Beta	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**
Herd characteristics:												
Other animals present at the farm (no: ref)	-0.01	0.87	[-0.17,0.14]"	-0.01	0.86	[-0.18,0.15]	0.09	0.42	[-0.13,0.3]	-0.03	0.60	[-0.16,0.09]
Number of farmworkers	-0.01	0.39	[-0.04,0.02]	**0.03**	**0.04**	**[0,0.06]**	-0.004	0.85	[-0.05,0.04]	**0.026**	**0.02**	**[0,0.05]**
Number of broilers at set-up	0.00E+00	0.16	[-0.002,0.001]	**0.00E+00**	**0.04**	**[0,0.001]**	0.00E+00	0.57	[-0.008,0.001]	0.00E+00	0.26	[-0.002,0.001]
Weight of broilers at set-up (g)	-0.01	0.17	[-0.03,0.01]	**0.02**	**0.04**	**[0,0.04]**	-0.014	0.27	[-0.04,0.01]	0.01	0.09	[0,0.03]
Number of broilers at sampling	0.00E+00	0.20	[-0.002,0.001]	0.00E+00	0.12	[-0.002,0.001]	0.00E+00	0.27	[-0.004,0.001]	0.00E+00	0.33	[-0.003,0.001]
Age of broilers at sampling (days)	-0.001	0.93	[-0.01,0.01]	0.01	0.10	[0,0.02]	-0.008	0.30	[-0.02,0.01]	0.005	0.30	[-0.001,0.01]
Weight of broilers at sampling (g)	-1.90E-05	0.80	[-0.002,0.001]	**0.00E+00**	**0.01**	**[0,0.001]**	-6.45E-05	0.52	[-0.002,0.001]	0.00E+00	0.13	[-0.002,0.002]
Average number of rounds / year	-0.02	0.55	[-0.09,0.05]	-0.009	0.83	[-0.09,0.07]	**0.15**	**0.002**	**[0.06,0.25]**	-0.03	0.29	[-0.08,0.03]
Season of sampling:												
Spring	0.03	0.81	[-0.2,0.25]	-0.07	0.58	[-0.3,0.16]	0.10	0.52	[-0.2,0.41]	0.03	0.78	[-0.13,0.16]
Summer	-0.13	0.21	[-0.34,0.07]	-0.04	0.68	[-0.25,0.17]	0.009	0.95	[-0.27,0.29]	0.007	0.93	[-0.15,0.2]
Autumn	-0.15	0.13	[-0.34,0.04]	-0.13	0.20	[-0.32,0.07]	-0.05	0.69	[-0.32,0.21]	0.02	0.84	[-0.15,0.17]
Winter	ref	.	.	ref	.	.	ref	.	.	ref	.	.
Biosecurity:												
Internal biosecurity	**0.007**	**0.01**	**[0,0.01]**	-0.002	0.46	[-0.01,0.001]	0.0002	0.96	[-0.01,0.01]	**-0.006**	**0.01**	**[-0.01,0]**
External biosecurity	**0.01**	**0.03**	**[0,0.02]**	0.002	0.73	[-0.01,0.01]	0.005	0.40	[-0.01,0.02]	-0.001	0.73	[-0.01,0.01]
Total biosecurity	**0.013**	**0.01**	**[0,0.02]**	0	0.96	[-0.01,0.01]	0.003	0.59	[-0.01,0.02]	-0.005	0.18	[-0.01,0.001]
Internal biosecurity:												
Disease management	0.001	0.60	[-0.001,0.01]	-5.11E+01	0.98	[-0.01,0.01]	0.005	0.09	[-0.001,0.01]	**-0.006**	**0.001**	**[-0.01,0.001]**
Cleaning and disinfection	**0.006**	**0.01**	**[0,0.01]**	-0.002	0.55	[-0.01,0.001]	-0.001	0.80	[-0.01,0.01]	-0.003	0.11	[-0.01,0.001]
Materials and measures between compartments	0.002	0.21	[-0.001,0.005]	-0.001	0.37	[-0.004,0.002]	-0.001	0.64	[-0.005,0.003]	-0.002	0.05	[-0.004,0.001]
External biosecurity:												
Purchase of one-day-old chicks	-0.002	0.48	[-0.006,0.003]	-0.002	0.29	[-0.007,0.002]	-0.002	0.44	[-0.008,0.004]	-0.001	0.56	[-0.004,0.002]
Depopulation of broilers	0.002	0.48	[-0.004,0.008]	-0.004	0.20	[-0.011,0.002]	-0.004	0.29	[-0.012,0.004]	-0.002	0.40	[-0.007,0.003]
Feed and water	0.001	0.69	[-0.003,0.005]	0.00E+00	0.82	[-0.004,0.005]	0.002	0.41	[-0.003,0.008]	-0.001	0.42	[-0.004,0.002]
Removal of manure and carcasses	**0.008**	**0.002**	**[0.003,0.013]**	**0.006**	**0.05**	**[0,0.01]**	0.002	0.51	[-0.005,0.009]	**0.006**	**0.004**	**[0.002,0.01]**
Visitors and farmworkers	**0.006**	**0.03**	**[0.001,0.011]**	0.001	0.65	[-0.005,0.007]	0.005	0.14	[-0.002,0.012]	-0.003	0.17	[-0.007,0.001]
Material supply	0.002	0.18	[-0.001,0.005]	-0.001	0.34	[-0.004,0.001]	-0.001	0.78	[-0.004,0.003]	-0.001	0.43	[-0.003,0.001]
Infrastructure and biological vectors	0.004	0.25	[-0.003,0.01]	0.003	0.43	[-0.004,0.009]	0.001	0.77	[-0.007,0.01]	0.00E+00	0.96	[-0.005,0.005]
Location of the farm	0.001	0.71	[-0.003,0.005]	0.00E+00	0.88	[-0.004,0.004]	0.003	0.22	[-0.002,0.009]	0.001	0.68	[-0.002,0.004]

Selection of variables from the univariable analysis used in the mixed model with a random effect for both country and farm. The bold type indicates associations with $p<0.05$.

Table S7. Multivariable linear mixed model without AMU in broilers.

	aph(3')-III			*erm*(B)			*sul2*			*tet*(W)		
	Beta	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**	**Beta**	***P***	**95%CI**
Herd characteristics:												
Number of farmworkers				0.03	0.04	[0,0.06]				0.02	0.05	[0.001,0.04]
Weight of broilers at set-up (g)				0.02	0.01	[0.01,0.04]						
Average number of rounds/year							0.15	<0.01	[0.06,0.25]			
Internal biosecurity:												
Disease management										-0.005	<0.01	[-0.009,-0.001]
Cleaning and disinfection										-0.004	0.02	[-0.008,-0.001]
External biosecurity:												
Removal of manure and carcasses	0.008	<0.01	[0.003,0.01]	0.007	0.01	[0,0.01]				0.007	<0.01	[0.003,0.01]

The multivariable model without AMU was automatically adjusted using the 'step' function in the 'R' package *lmerTest*.[2] Only associations with a p-value less than 0.05 were involved.

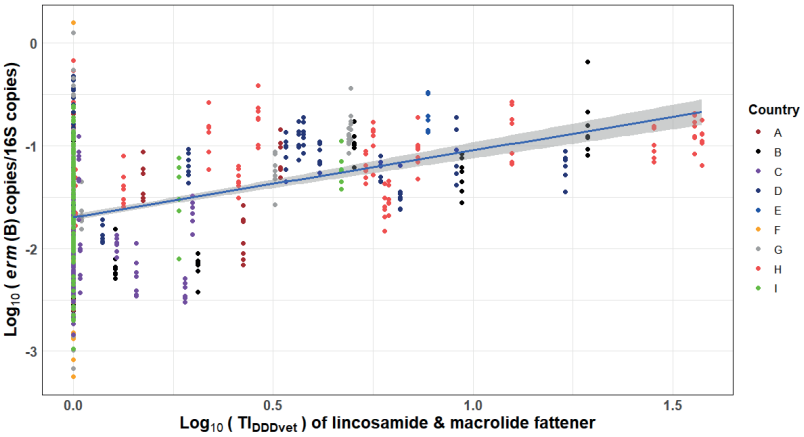

Figure S6. Associations between lincosamide & macrolide use during fattening phase and relative *erm*(B) abundance in pigs in nine countries.

Lincosamide & macrolide: Macrolide + lincosamide + spectinomycin use. The blue line represents the linear relationship between ARG abundance and the score of cleaning and disinfection, the grey area around the line demonstrates the 95% confidence interval. Letters A–I represent the nine countries.

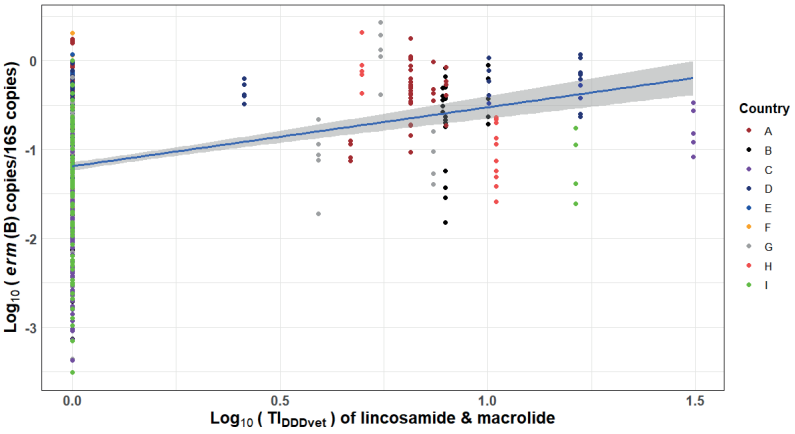

Figure S7. Associations between lincosamide & macrolide use and relative *erm*(B) abundance in broilers in nine countries.

Lincosamide & macrolide: Macrolide + lincosamide + spectinomycin use. The blue line represents the linear relationship between ARG abundance and the score of cleaning and disinfection, the grey area around the line demonstrates the 95% confidence interval. Letters A–I represent the nine countries.

Table S8: Variance component analysis of the null mixed model in pigs.

	aph(3')-III			*erm*(B)			*sul2*			*tet*(W)		
	VC	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**
Between-country	0.20	**53.83**	0.44	0.12	36.33	0.34	0.28	**42.36**	0.53	0.01	10.63	0.09
Between-farm	0.12	31.99	0.34	0.16	**50.94**	0.40	0.20	30.00	0.44	0.04	**50.59**	0.20
Within-farm	0.05	14.17	0.23	0.04	12.73	0.20	0.18	27.64	0.43	0.03	38.78	0.17

VC: Variance component. %Total: The percentage of the variance component. SD: Standard deviation.

All values are rounded at two digits behind the comma. Values in bold indicate the highest VC percentage for each model.

Table S9: Variance component analysis of the null mixed model in broilers.

	aph(3')-III			*erm*(B)			*sul2*			*tet*(W)		
	VC	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**	**VC**	***%Total***	**SD**
Between-country	0.04	6.50	0.19	0.18	**37.85**	0.43	0.06	7.32	0.19	0.04	11.98	0.19
Between-farm	0.19	34.49	0.44	0.21	42.79	0.45	0.36	45.03	0.44	0.12	38.98	0.34
Within-farm	0.33	**59.01**	0.58	0.09	19.36	0.30	0.38	**47.66**	0.58	0.15	**49.04**	0.38

VC: Variance component. %Total: The percentage of the variance component. SD: Standard deviation.

All values are rounded at two digits behind the comma. Values in bold indicate the highest VC percentage for each model.

References

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Chapter 5

Risk Factors for the antimicrobial resistome in European livestock: An Updated Analysis of Metagenomic Data

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Manuscript in preparation

Abstract

Metagenomic sequencing has been proved to be a powerful tool in the quantification of antimicrobial resistance (AMR). The aim of this study is to examine the impact of utilizing an updated AMR reference database and alternative bioinformatics/computational approaches on AMR abundance and the outcome of the risk factor analyses in European pigs and broilers. We processed previously published metagenomic data by an updated bioinformatics workflow for faecal samples of 181 pig and 178 broiler farms from nine countries to assess the correlation with previously published data and to analyze the impact on identified potential risk factors from pig and broiler resistomes, which include farm management practices and antimicrobial usage. In addition, faecal resistomes of 61 veal calf farms from three countries were also included in a risk factor analysis. In pigs and broilers, we found a high correlation ($\rho>0.7$) of AMR abundance between previous and updated metagenomic data per antimicrobial resistance gene class. The updated bioinformatics pipeline of AMR has produced comparable but not completely reproducible associations between AMR and farm risk factors, which is most likely caused by the updated reference databases and the higher resolution metagenomic alignment methods.

Introduction

Antimicrobial resistance (AMR) is an important factor threatening the health of animals and humans.[1] Multiple previous studies[2-5] have reported clonal spread of resistant bacteria and the transmission of antimicrobial resistance genes (ARGs) between humans and farm animals within the One Health domain. To reduce the risk of human exposure to livestock AMR, more attention needs to be paid to risk factors for AMR in farm animals.

Precise and comprehensive AMR global surveillance has become the critical first step in the One Health approach[6, 7] proposed in recent years to control AMR. Metagenomic shotgun sequencing (MG-SS) as an emerging AMR quantification method has been shown to capture antimicrobial use (AMU)-induced AMR more broadly than traditional methods that are mainly based on individual indicator organisms/pathogens.[8, 9] In addition, the convenience of performing random and large-scale targeting of the entire microbial community[10, 11] and the declining deep-sequencing costs of MG-SS in recent years[12] have made MG-SS increasingly popular in quantitative AMR research.[13-17]

In the 'Ecology from Farm to Fork Of microbial drug Resistance and Transmission' (EFFORT) European project we previously conducted resistome risk factor analyses in pigs and broilers (Van Gompel et al., 2019, Luiken et al., 2019).[13, 14] In the current study, we re-used the primary sequencing data but with updated ResFinder[18] (February 2019 compared to November 2016) and bacterial genomes databases. Furthermore, the metagenomic mapping strategy using the conclave algorithm implemented in the novel *k*-mer alignment software KMA[19] performs well when mapping against redundant databases such as the ones we used in this study. In addition to data from pigs and broilers, new metagenomic data generated from faecal samples of fish, turkeys, and veal calves are described in a manuscript of Munk et al. (in preparation),[20] while the resistome data in relation to risk factors on turkey farms have been published by Horie et al.[21]

A meta-analysis with random effects by country was performed for the updated AMR data with a matching questionnaire (e.g. AMU, biosecurity) in pigs, broilers and veal calves as described before.[13, 14] In addition, the impact of computational approaches (MGmapper vs KMA alignment method; AMR measure with fragments per kilobase reference per million bacterial fragments (FPKM) vs additive log-ratios (ALR); zero imputation with pseudo-counts vs Square Root Bayesian multiplicative treatment) on the outcome of risk factor analyses was examined in pigs and broilers by comparing these with earlier findings.

Methods

Sampling Procedure

Livestock herds from regionally dispersed conventional pig and broiler farms in nine European countries and veal calf farms in three European countries were sampled as previously described.[13-15, 22] For each species, twenty herds per country were sampled.

After 25 randomly distributed faecal samples (pigs, broilers, and veal calves) were collected at each farm close to the date of slaughter, one pooled sample representing each herd was prepared: 25 individual samples from each herd were pooled, with individual samples all contributing equal mass. The pooled samples were stored at -80°C locally by EFFORT partners and sent to the Technical University of Denmark (DTU) in batches on dry ice.[15]

DNA extraction and sequencing

The DNA extraction and sequencing procedures for veal calf samples were designed to be as similar as possible to the procedures used by Munk et al. 2018[15] to evaluate the pig and broiler resistomes. Briefly, DNA was extracted from farm-level faecal pools using a previously published bead-beating-optimized standard operating procedure (SOP) based on the QIAamp Fast DNA Stool Mini Kit (51604, Qiagen).[23] Two batches of DNA extracted from pooled veal calf faecal samples were shipped on dry ice to Admera Health (South Plainfield, New Jersey, USA) who performed library preparation and shotgun metagenomic sequencing. After DNA fragmentation (Covaris LE220), sequencing libraries were prepared and multiplexed using the PCR-free KAPA HyperPrep kit (Kapa Biosystems). The generated libraries were sequenced on the NovaSeq 6000 platform (Illumina), using a 2 x 150-bp paired-end (PE) approach targeting 40M read clusters per sample.

Updated processing and computational methods

After trimming low-quality nucleotides and adaptor sequences as described before,[15] the trimmed reads of each sample were aligned to the updated ResFinder database[18] (13 February 2019). In order to filter out low-coverage alignments before the downstream analysis of AMR genes, we required that the read consensus sequence covered at least 20% of its ResFinder reference sequence.

The reads were also mapped and aligned against an updated bacterial genomic database using the *k*-mer alignment software KMA(v1.2.8).[19] The genomic database was created by merging genome sub-databases

(bacteria_20190205, archaea_20190213, MetaHitAssembly_20140701, HumanMicrobiome_20140702, bacteria_draft_20190205, plasmid_20190205). More details of this process have been described previously.[21]

Metagenomic studies often use the so-called 'log ratio' for downstream analyses [e.g. ALR or centred log-ratios (CLR)] accompanied by zero imputation methods to handle non-positive values.[24] In this study, 'R' package *zCompositions* [function 'cmultRepl', method square root Bayesian multiplication (Bayesian SQ)][25] was used to impute zeros before log transformation to retrieve zero-corrected values.[24, 26] The zero replacement method is based on a Square Root Bayesian multiplicative treatment (Bayesian SQ), which not only replaces zero gene counts but also performs a multiplicative adjustment on the non-zero gene counts according to their compositional nature.[27]

Comparison of livestock resistome computational methods in pigs and broilers

Combining originally applied and updated metagenomic methods and computational strategies, we aimed to compare several versions of our AMR dataset in a risk factor analysis in pigs and broilers using the same 'R' package *metaphor* (v2.4.0).[28] An overview of the resulting pigs' and broilers' AMR datasets is provided in Table 1. AMR in pigs and broilers was both expressed as FPKM[15] (Formula 1) and as ALR[24, 29] (Formula 2).

Table 1. Overview table of quantified ARG datasets used in the analysis

Data type	Metagenomic read mapping software/method	Access date ResFinder and genomic databases	Zero-replacement method	Transformation method	References to previous risk factor analyses
v1-FPKM	MGmapper, tweaked BWA-MEM algorithm	17 November 2016	None	Log10 (FPKM + 1)	Van Gompel et al., 2019 (pigs), Luiken et al., 2019 (broilers), NA (veal calves)
v2-FPKM	KMA	13 February 2019	None	Log10 (FPKM + 1)	Horie et al., 2021 (turkeys).
v2-FPKM$_{zc}$	KMA	13 February 2019	Square root Bayesian multiplicative treatment	Log10 (FPKM)	NA
v2-ALR$_{zc}$	KMA	13 February 2019	Square root Bayesian multiplicative treatment	Log2	NA

NA: Unless a specific reference was provided, all data was generated and described for the first time in this paper. FPKM: Fragments per kilobase reference per million bacterial fragments. ALR: Resistome additive log-ratios. KMA: K-mer alignment. Formulas for FPKM and ALR are provided in Formula 1-2.

Formula 1. Calculation of FPKM values

$$\text{FPKM} = \frac{ARG\ fragments}{ARG\ length\ x\ total\ bacterial\ fragments}\ x\ 10^9$$

FPKM: Fragments per kilobase reference per million bacterial fragments.[13-15] ARG: Antimicrobial resistance gene.

Formula 2. Calculation of resistome ALR values

$$\text{ALR} = log_2(\frac{ARG\ fragments}{ARG\ length\ x\ total\ bacterial\ fragments})$$

ALR: Resistome additive log-ratios.[24] ARG: Antimicrobial resistance gene.

Descriptive resistome analysis

The mean ARG abundances (ALR) were visualized per country and species in a box plot. Overall, per species, country resistome differences were compared by performing a classic or Welch's analysis of variance (ANOVA) depending on the homogeneity of variance.[30, 31] In case of a significant difference, post-hoc tests (i.e. respectively a Tukey's Honest Significant Difference test (Tukey HSD)[32] or a Games-Howell Post-Hoc test[33]) were carried out. Unless otherwise specified, appropriate post-hoc test p-values are reported. Subsequently, considering there were nine countries included for both pigs and broilers, a multiple testing correction was performed [Benjamini-Hochberg false discovery rate (FDR $p < 0.1$)] in the comparison between countries.

The resistome dataset was aggregated at the ARG class level. Subsequently, some ARG class clusters [e.g. MLS (macrolide, lincosamide, spectinomycin)] were combined consistently with previous analyses for pigs[13] and broilers[14] or as described in Table 2 (veal calves).

Table 2. Overview table of all outcomes and potential risk factors included in the meta-analysis of veal calves.

Outcome variables	Potential risk factors		
ARG classes	**AMU classes**[22, 34]	**Biosecurity variables**[35]	**Other farm characteristics**[22]
Aminoglycosides	Aminoglycosides	**Internal biosecurity**	Sampling season
Polymyxins	Polymyxins	Health management	Other animals present at the farm (yes/no)
MLS	MLS	Calving management	Agents used for stable cleaning (no cleaning/only-water/soaking agents/disinfectants/soaking agents and disinfectants)
Phenicols*	Phenicols	Calf management	
Quinolones	Quinolones	Dairy management	Mean weight at arrival (kg)
Sulfonamides	Trimethoprim & sulfonamide	Adult cattle management	Mean age of at arrival (days)
Tetracyclines	Tetracyclines	Working organisation and equipment	Mean current age (days)
Trimethoprim	Trimethoprim & sulfonamide	**External biosecurity**	Total current Number of calves
β-lactams	β-lactams	Purchase and reproduction	
Total AMR	Total AMU	Transport and carcass removal	
		Feed and water	
		Visitors and farmworkers	
		Vermin control and other animals	
		Total biosecurity	

ARG classes and AMU classes combinations were listed correspondingly in the first two columns.

MLS: Macrolide + lincosamide + spectinomycin. Phenicols*: Phenicol, oxazolidinone and phenicol resistance. Quinolones: Fluoroquinolones and other quinolones. Trimethoprim & sulfonamide: Sulfonamide + trimethoprim/sulfonamide use. β-lactams: Penicillins + aminopenicillins + cephalosporines. Total AMR: Sum of all AMR resistance (Except for the listed classes in the table, fosfomycin, glycopeptides, nitroimidazoles, oxazolidinone and rifampicin were also included).

Correlations between resistome levels obtained by different computational methods in pigs and broilers

Spearman rank correlations were computed to assess the similarity of old (v1) and updated (v2) AMR datasets in pigs and broilers. To evaluate the effects of different metagenomic and data processing methods, correlations between v1-FPKM, v2-FPKM, v2-$FPKM_{zc}$, and v2-ALR_{zc} were analyzed.

Questionnaire data

General farm characteristics, AMU group treatments and biosecurity information were retrieved from a standardized questionnaire completed by farmers, as published previously.[13, 14, 22] The usage of antimicrobials on farms for pigs, broilers and veal calves has been elaborately described elsewhere.[34, 36, 37]

Aggregated biosecurity scores per farm were retrieved from previous studies (pigs,[13] broilers[14]) or newly calculated from the questionnaire based on the algorithm of the Biocheck.UGent™ scoring system (veal calves)[38].

Risk factor analysis: Random-effects meta-analyses in pigs, broilers and veal calves

For pigs, broilers and veal calves, there were 176, 176 and 59 farm datasets with matching questionnaires (e.g. AMU, biosecurity) and updated ARG available for analysis, respectively.[13, 14, 22] For pigs and broilers, three updated datasets (v2-FPKM, v2-$FPKM_{zc}$, and v2-ALR_{zc}) were included in the risk factor analyses to compare with the previously published associations[13, 14] in v1-FPKM data. Since there is no published information of v1-FPKM data in veal calves to compare with the updated datasets, only v2-FPKM data was included in the risk factor analysis of veal calves. We used R version 3.6.3 for all risk factor analyses.[39] A meta-analysis with random effects by country was run using the 'R' package *metaphor*[28] (v2.4.0) as described previously in pigs and broilers.[13, 14]

Briefly, for each country, ARG classes were separately linearly regressed on AMU, biosecurity and other farm variables before estimates were combined in a meta-analysis by country. Prior to running a meta-analysis, AMR data was standardized (mean=0, sd=1) by country to avoid a large influence of country on the analysis weights. The latter also produces better comparable FPKM/ALR datasets, since the effect of a particular kind of log transformation (i.e. $\log_{10}$ vs $\log_2$) on the estimates arising from a meta-analysis is minimized. For pigs, all possible combinations of AMU vs AMR were tested, while for broilers and veal calves only the established

drug-class-resistance combinations were evaluated due to low levels of on-farm AMU in line with previous analyses.[13, 14] Subsequently, a multiple testing correction was performed [Benjamini-Hochberg false discovery rate (FDR $p < 0.1$)]. For pigs, summary estimates were only preserved when at least four countries were incorporated in the association for consistency with the previous analysis which focused on the most robust associations only.[13] For broilers and veal calves, all associations were included due to low levels of AMU (broilers)[14] or a limited number of sampled countries (veal calves, n=3). Assumptions of all statistically significant regression analyses (FDR $p < 0.1$) were checked using diagnostic tests, and consequently spurious associations were removed if they didn't meet the assumptions. In case more than one determinant was found to be associated with an outcome, the regression analysis was repeated including all statistically significantly associated (FDR $p < 0.1$) determinants.

Availability of data and code

DNA sequences corresponding to metagenomic samples obtained from 181 pig herds and 178 broiler herds are available in the European Nucleotide Archive (ENA) via project accession PRJEB22062. DNA sequences corresponding to the 61 veal calf samples are available via ENA project accession PRJEB39685.

An example of how to run a meta-analysis in 'R' using resistome data can be found at https://zenodo.org/record/5707425#.YZurRfnMKUk

Results

Acquired resistome characterization

On average, irrespective of which annotated dataset was used, the highest relative AMR loads were observed in pigs, followed by veal calves and broilers (Figure 1). Large differences between farms were observed. The total AMR load varied to a lesser extent between countries, which was most visible in broilers, e.g. after multiple corrections, the mean AMR abundance in broilers sampled in country I was found to be statistically significantly higher than in countries C, E, and F (FDR $p < 0.1$) (Figure 1). For pigs, there was no statistically significant difference in mean AMR abundances between countries (FDR $p > 0.1$). There was no significant difference in mean AMR abundances between countries for veal calves ($p>0.05$, One-way ANOVA).

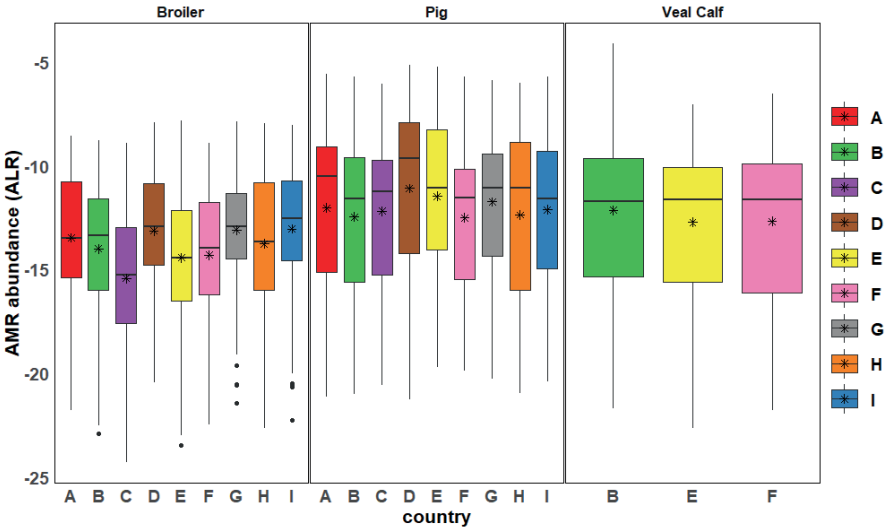

Figure 1. Total AMR abundances among countries and species (ALR). The horizontal box lines depict the first quartile, the median and the third quartile. Dots represent the outliers. Asterisk shows the mean by country. AMR indicates the additive log-ratios of AMR without zero replacement. Letters A-I represent the nine European countries.

Correlations between resistome levels obtained by different computational methods

Most ARG classes (e.g. MLS in pigs and broilers) were highly correlated ($\rho>0.7$) between different datasets in both pigs (Figure 2, Tables 3) and broilers (Figure 3, Tables 4). A low correlation ($\rho<0.7$) was generally found in less abundant ARG classes (e.g. polymyxins in pigs, rifampicin in broilers, fosfomycin in both pigs and broilers).

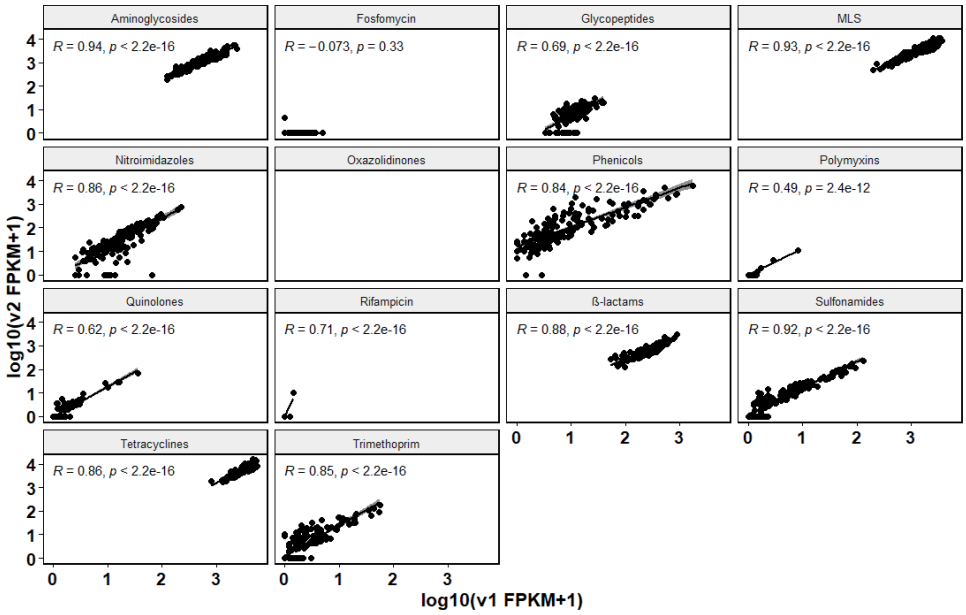

Figure 2. Spearman's rank correlation between v1-FPKM and v2-FPKM per antimicrobial class in pigs. MLS: Macrolide, lincosamide and spectinomycin resistance. Oxazolidinones: Oxazolidinone, oxazolidinone and phenicol resistance. Phenicols: Phenicol, oxazolidinone and phenicol resistance. β-lactams: Penicillins, aminopenicillins and cephalosporines resistance.

Table 3. Comparison of pig AMR datasets - Spearman correlation analysis

ARG classes	v1-FPKM/v2-ALR$_{zc}$	v1-FPKM/v2-FPKM	v2-FPKM/v2-FPKM$_{zc}$	v2-FPKM/v2-ALR$_{zc}$
Oxazolidinones	-*	-*	0.66	0.66
Fosfomycin	0.01	0.07	0.18	0.18
Rifampicin	0.1	0.71	0.13	0.13
Polymyxins	0.22	0.49	0.25	0.25
Quinolones	0.54	0.62	0.25	0.61
Glycopeptides	0.69	0.69	1	1
Trimethoprim	0.83	0.85	0.95	0.95
Phenicols	0.84	0.84	1	1
Nitroimidazoles	0.86	0.86	1	1
Tetracyclines	0.86	0.86	1	1
β-lactams	0.88	0.88	1	1
Sulfonamides	0.91	0.92	0.99	0.99
MLS	0.93	0.93	1	1
Aminoglycosides	0.94	0.94	1	1

All values are rounded at two digits behind the comma. Values in 'gray' represent non-significant correlations ($p>0.05$). *: No correlation is shown when ‘oxazolidinone’ is not present in the v1-FPKM pig dataset. Oxazolidinones: Oxazolidinone, oxazolidinone and phenicol resistance. Phenicols: Phenicol, oxazolidinone and phenicol resistance. β-lactams: Penicillins, aminopenicillins and cephalosporines resistance. MLS: Macrolide, lincosamide and spectinomycin resistance.

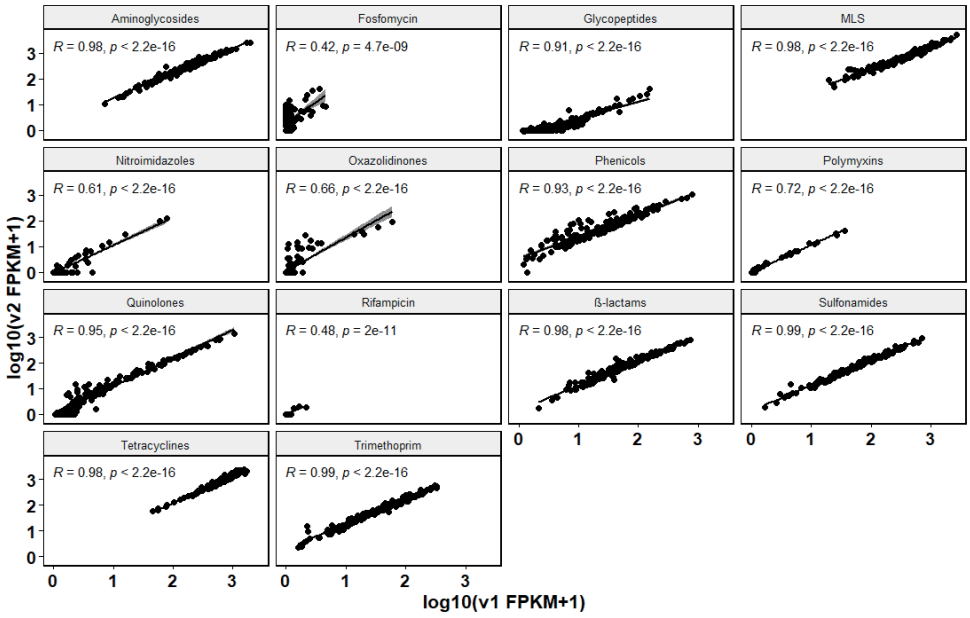

Figure 3. Spearman's rank correlation between v1-FPKM and v2-FPKM per antimicrobial class in broilers. MLS: Macrolide, lincosamide and spectinomycin resistance. Oxazolidinones: Oxazolidinone, oxazolidinone and phenicol resistance. Phenicols: Phenicol, oxazolidinone and phenicol resistance. β-lactams: Penicillins, aminopenicillins and cephalosporines resistance.

Table 4. Comparison of broiler datasets - Spearman correlation analysis

ARG classes	v1-FPKM/v2-ALR$_{zc}$	v1-FPKM/v2-FPKM	v2-FPKM/v2-FPKM$_{zc}$	v2-FPKM/v2-ALR$_{zc}$
Rifampicin	0.07	0.48	0.22	0.22
Fosfomycin	0.42	0.42	0.98	0.98
Nitroimidazoles	0.43	0.61	0.59	0.59
Polymyxins	0.51	0.72	0.6	0.6
Oxazolidinones	0.63	0.66	0.68	0.68
Glycopeptides	0.9	0.91	0.97	0.97
Phenicols	0.93	0.93	1	1
Quinolones	0.95	0.95	1	1
Aminoglycosides	0.98	0.98	1	1
β-lactams	0.98	0.98	1	1
MLS	0.98	0.98	1	1
Tetracyclines	0.98	0.98	1	1
Sulfonamides	0.99	0.99	1	1
Trimethoprim	0.99	0.99	1	1

All values are rounded at two digits behind the comma. Values in 'gray' represent non-significant correlations ($p>0.05$).

Oxazolidinones: Oxazolidinone, oxazolidinone and phenicol resistance. Phenicols: Phenicol, oxazolidinone and phenicol resistance. β-lactams: Penicillins, aminopenicillins and cephalosporines resistance. MLS: Macrolide, lincosamide and spectinomycin resistance.

Risk factor analyses in pigs, broilers and veal calves

For pigs, all previously significant AMU-AMR associations (v1-FPKM) were confirmed ($p<0.05$) in the analyses using the updated datasets (v2-FPKM, v2-$FPKM_{zc}$, v2-ALR_{zc}), as well as the associations between biosecurity and resistance (Table 5, ALR data), apart from the association between tetracyclines use (standardized 200-day lifespan), MLS use (standardized 200-day lifespan) and respectively tetracyclines and macrolides resistance. However, only associations between MLS use in fatteners and macrolides/MLS resistance were statistically significant (FDR $p < 0.1$) taking multiple testing into account. Compared to the previous analyses, we also found a slight decrease of all estimates (β coefficients) when analyzing the updated datasets. The β estimates of associations using updated datasets did not differ substantially amongst each other (data not shown).

For broilers, all previously statistically significant associations between AMU and AMR could be reproduced (FDR $p<0.1$) (v2-FPKM, v2-$FPKM_{zc}$, v2-ALR_{zc}), except for the statistically significant associations with internal biosecurity (Table 6, ALR data). Furthermore, β estimates of analyses (v2-FPKM, v2-$FPKM_{zc}$, v2-ALR_{zc}) differed only slightly from previous estimates (v1-FPKM), whereas estimates of analyses using the updated data (v2) were generally similar (data not shown).

For veal calves, no previous resistome risk factor analysis had been carried out. A negative association was observed between polymyxins use and polymyxins resistance (v2-FPKM). Interestingly, we found that veal calves sampled on farms without a specific cleaning regime for the stables (compared to farms that used soaking agents and disinfectants) carried lower phenicols resistance levels in their faeces (v2-FPKM, FDR $p < 0.1$).

Table 5. Meta-analysis in pigs [v2-ALR$_{zc}$]

AMU and farm characteristics	ARG classes	Estimate (β)	95% CI	P-value	FDR	Countries and number of farms*
Macrolide use (fatteners)	**Macrolide**	**0.99**	**[0.50, 1.49]**	**<0.01**	**0.04**	**A-2, B-4, C-2, D-7, E-1, G-3, H-12**
Macrolide use (fatteners)	**MLS**	**0.99**	**[0.50, 1.49]**	**<0.01**	**0.04**	**A-2, B-4, C-2, D-7, E-1, G-3, H-13**
IB: Cleaning & disinfection	Macrolide	0.01	[0.005, 0.02]	<0.01	0.18	A-20, B-20, C-20, D-20, E-20, F-19, G-20, H-20
IB: Cleaning & disinfection	MLS	0.01	[0.005, 0.02]	<0.01	0.18	A-20, B-20, C-20, D-20, E-20, F-19, G-20, H-20
MLS use (fatteners)	Macrolide	0.80	[0.30, 1.30]	<0.01	0.19	A-3, B-5, C-6, D-10, E-1, G-4, H-14, I-2
MLS use (200 days)	Macrolide	0.66	[0.22, 1.09]	<0.01	0.25	A-5, B-10, C-16, D-16, E-6, G-9, H-20, I-2
MLS use (200 days)	MLS	0.66	[0.22, 1.09]	<0.01	0.25	A-5, B-10, C-16, D-16, E-6, G-9, H-20, I-2
IB: Measures comp. & equipment use	Glycopeptides	0.01	[0.004, 0.02]	0.01	0.34	A-20, B-20, C-20, D-20, E-20, F-19, G-20, H-20
IB: Measures comp. & equipment use	Macrolide	0.01	[0.003, 0.02]	0.01	0.36	A-20, B-20, C-20, D-20, E-20, F-19, G-20, H-20
IB: Measures comp. & equipment use	MLS	0.01	[0.003, 0.02]	0.01	0.36	A-20, B-20, C-20, D-20, E-20, F-19, G-20, H-20
Total biosecurity	Macrolide	0.03	[0.006, 0.05]	0.01	0.43	A-20, B-20, C-20, D-20, E-20, F-19, G-20, H-20
Total biosecurity	MLS	0.03	[0.006, 0.05]	0.01	0.43	A-20, B-20, C-20, D-20, E-20, F-19, G-20, H-20
Internal biosecurity	MLS	0.02	[0.004, 0.04]	0.02	0.44	A-20, B-20, C-20, D-20, E-20, F-19, G-20, H-20
Internal biosecurity	Macrolide	0.02	[0.004, 0.04]	0.02	0.44	A-20, B-20, C-20, D-20, E-20, F-19, G-20, H-20
β-lactams use (fatteners)*	Phenicols	0.96	[0.03, 1.89]	0.04	0.65	A-2, B-6, C-8, D-6, G-3, H-14, I-3
β-lactams use (fatteners)**	Phenicols	0.92	[-0.02, 1.87]	0.05	0,68	A-2, B-5, C-8, D-5, G-3, H-12, I-2
Tetracyclines use (200 days)	Tetracyclines	0.34	[-0.10, 0.77]	0.13	0,94	A-8, B-4, C-14, D-13, E-5, F-2, G-14, H-14, I-1
Lincosamide use (200 days)	Macrolide	0.5	[-0.40, 1.39]	0.37	0,99	A-2, B-1, C-8, D-5, E-1, G-1, H-6, I-2

All figures are rounded at two digits behind the comma unless rounding would lead to the misinterpretation of an effect. ARG classes clusters in this table represent resistome additive log-ratios (ALR). *: Countries were excluded if no AMU was recorded on all farms from the respective country. All biosecurity analyses included eight countries. Associations in bold have an FDR p value < 0.1.

Table 6. Meta-analysis in broilers [v2-ALR$_{zc}$]

AMU and farm characteristics	ARG classes	Estimate (β)	95% CI	P-value	FDR	Countries and number of farms*
MLS	**MLS**	**1.15**	**[0.67, 1.63]**	**<0.01**	**<0.01**	**A-8, B-5, C-1, D-4, G-3, H-3, I-1**
Tetracyclines	**Tetracyclines**	**0.96**	**[0.55, 1.36]**	**<0.01**	**<0.01**	**A-6, B-2, C-2, F-1, G-8, H-1**
Trimethoprim & sulfonamide	**Trimethoprim**	**0.89**	**[0.23, 1.56]**	**0.01**	**0.03**	**A-4, D-11, E-5, F-3, G-2**
Aminoglycosides	**Aminoglycosides**	**1.14**	**[0.15, 2.13]**	**0.02**	**0.05**	**G-2, H-7**
Beta-lactam	**Beta-lactam**	**0.38**	**[0.05, 0.72]**	**0.03**	**0.05**	**A-10, B-3, D-15, E-9, F-1, G-10, H-6, I-1**
Summer (ref: winter)	**Quinolones**	**0.68**	**[0.24, 1.11]**	**<0.01**	**0.07**	**A-4, B-4, C-2, D-6, E-5, F-6, G-4, H-8, I-3**
Total AMU	Total ALR	0.37	[-0.12-0.86]	0.13	0.22	A-18,B-11,C-3,D-19,E-16,F-5,G-19,H-16,I-3
Phenicols	Phenicols	1.23	[-0.69-3.15]	0.21	0.26	G-1
Total biosecurity	Tetracyclines	-0.03	[-0.06--0.00]	0.03	0.43	A-20, B-18, C-20, D-20, E-20, F-20, G-20, H-20, I-18
Quinolones	Quinolones	0.08	[-0.31-0.47]	0.69	0.77	A-7,B-1,D-6,E-10,F-1,G-16,H-7,I-2
Polymyxins	Polymyxins	0.04	[-0.31-0.39]	0.83	0.83	B-2,D-9,E-3,G-6,H-4
Internal biosecurity	Oxazolidinones	0.01	[-0.02, 0.03]	0.54	0.86	A-20, B-18, C-20, D-20, E-20, F-20, G-20, H-20, I-18

All values are rounded at two digits behind the comma. ARG classes clusters in this table represent resistome additive log-ratios (ALR). *: Countries were excluded if no AMU was recorded on all farms from the respective country. All biosecurity analyses included eight countries. Associations in bold have an FDR p value < 0.1. MLS: Macrolide + lincosamide + spectinomycin. Oxazolidinones: Oxazolidinone, oxazolidinone and phenicol resistance. Trimethoprim & sulfonamide: Sulfonamide + trimethoprim/sulfonamide use.

Table 7. Meta-analysis of FPKM data in veal calves [v2-FPKM]

AMU and farm characteristics	ARG classes	Estimate (β)	95% CI	P-value	FDR	Country and number of farms*
Polymyxins	**Polymyxins**	**-1.94**	**[-3.39, -0.48]**	**<0.01**	**0.05**	**B-3, E-19, F-2**
On-farm cleaning of stables:						
No cleaning	**Phenicols***	**-3.30**	**[-5.01, -1.58]**	**<0.01**	**0.01**	**B-1, F-4**
Only water		-0.71	[-1.43, 0.01]	0.05	0.7	B-5, E-1, F-7
Soaking agents		-0.87	[-1.90, 0.17]	0.10	0.7	B-3, F-1
Disinfectants		-0.30	[-0.93, 0.32]	0.34	0.99	B-5, E-15, F-8
Soaking agents and disinfectants		ref	-	-	-	B-5, E-4

All values are rounded at two digits behind the comma. ARG in this table represents fragments per kilobase reference per million bacterial fragments (FPKM). *: Countries were excluded if no AMU was recorded on all farms from the respective country. Associations in bold have an FDR p value < 0.1.

Phenicols: Phenicol, oxazolidinone and phenicol resistance.

Discussion

We conducted a comprehensive analysis of the European livestock resistome by metagenomic shotgun sequencing of faecal samples from pigs, broilers and veal calves. In total, we sampled 420 herds, spanning nine European countries. The present study assessed the robustness of previously demonstrated AMR risk factor estimates by comparing old and updated livestock resistome datasets and repeating earlier risk factor analyses using updated pig and broiler resistome data, while also performing an additional risk factor analysis in veal calves.

The pig and broiler datasets using different computational annotation methods were shown to correlate highly for most ARG classes. It is therefore not surprising that previous statistically significant risk factors (FDR $p<0.1$) were confirmed when analyzing the updated AMR data (e.g. MLS use versus MLS resistance in pigs and broilers, tetracyclines use versus tetracyclines resistance in broilers).

However, we also observed that using an updated version of a reference database (February 2019 versus November 2016) combined with a different sequence alignment method affects class-level resistance abundances, resulting in low correlation when comparing the data at low ARG class counts (e.g. fosfomycin in pigs and broilers). For those ARG classes, there are no statistically significant associations with potential risk factors (FDR > 0.1), both in the previous and updated analyses. An explanation is that the precision of low abundance ARG classes is limited, which may be due to the high noise level in quantification.

For veal calves, we found that farms without cleaning stables showed lower phenicols resistance abundance than farms cleaning stables with soaking agents and disinfectants. One possible explanation is that the antibiotic susceptibility of phenicols can be reduced with extensive exposure to quaternary ammonium compounds (a major ingredient in disinfectants).[40] A similar association was previously described for the same animal population,[22] in which calf farms using only water for cleaning showed a lower faecal abundance of *ermB* and *tetW* (quantified using qPCR) than farms using soaking agents and disinfectants. This might potentially be explained by co- and cross-selection of ARGs and genes coding for biocide resistance on farms using disinfectants.[13, 41, 42] However, these results should not be interpreted as an argument against application of cleaning or disinfection procedures. In addition to the negative association between polymyxins use and polymyxins resistance in this study, we conclude that the interpretation of association-studies should be done with caution.

Evaluation of the Bayesian SQ zero replacement method

Before log transformation (e.g. to satisfy assumptions of regression analysis) of AMR abundances, zero values need to be imputed with a pseudo count[13, 14, 22] or an estimated value.[27, 43, 44] Some previous studies have proposed the use of a zero-replacement technique.[43, 44] To explore the impact of zero-replacement techniques, we used a Bayesian SQ method to impute zeros[27] in our study. After log transformation, we found that the zero corrected datasets (zc) were more normally distributed compared with the datasets at which a pseudo count of '1' was added before log transformation, and might subsequently be more suited to the assumptions of the applied regression analysis.

Nevertheless, the Bayesian SQ zero replacement method assumes the data follows a multinomial likelihood with random vector π_{ij} of probabilities that need to be estimated, where $\Sigma_{ij}\ \pi_{ij} = 1$. This means the Bayesian SQ method should only be performed on data that is compositional,[27, 45] a characteristic that applies for microbiome data, but not necessarily for normalized resistome data. Therefore, we believe this Bayesian SQ method should not be recommended for dealing with zero problems in this study. This Bayesian SQ method assumes that in the metagenome all ARGs have been sequenced ($\Sigma_{ij}\ \pi_{ij} = 1$), it will automatically ignore the unsequenced ARG targets (due to the limited sequencing depth) and overestimate the imputation of zero values in our dataset. In the future, further analyses of resistome and other metagenome data are needed to conclude which method is most appropriate for dealing with zero values in datasets that include many zeros.

Strengths and limitations of this study

The strengths of our study include the diversity of included countries and animal species, the relatively large number of samples for metagenomic sequencing, and the homogeneity of protocols used across different samples so that reliable comparative analysis can be carried out.

Despite the high quality of data collection, interpretation in our study is limited by the cross-sectional study design. Collecting only one pooled sample per farm close to slaughter may impede causal inferences about the associations between farm-level risk factors and ARG abundances. In addition, although samples from pig and broiler farms were collected in nine countries, veal calf farms were only collected from three countries, which prevented direct cross-species comparisons for each country.

Conclusion

In summary, previous risk factor analysis results of the FPKM dataset could be confirmed but not completely reproduced in the updated FPKM/ALR datasets. The small differences are mainly due to the updated reference database and metagenomic alignment methods. Updated FPKM dataset (v2-FPKM) without Bayesian SQ zero replacement is recommended to use.

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Transparency declarations

None to declare.

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Chapter 6

Risk Factors for Antimicrobial Resistance in Turkey Farms: A Cross-Sectional Study in Three European Countries

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Abstract

Food-producing animals are an important reservoir and potential source of transmission of antimicrobial resistance (AMR) to humans. However, research on AMR in turkey farms is limited. This study aimed to identify risk factors for AMR in turkey farms in three European countries (Germany, France and Spain). Between 2014 and 2016, faecal samples, antimicrobial usage (AMU) and biosecurity information were collected from 60 farms. The level of AMR in faecal samples was quantified in three ways: by measuring the abundance of AMR genes through (i) shotgun metagenomics sequencing (n=60) and (ii) quantitative real-time polymerase chain reaction (qPCR) targeting *ermB*, *tetW*, *sul2* and *aph3'-III*; (n=304), and (iii) by identifying the phenotypic prevalence of AMR in *Escherichia coli* isolates by minimum inhibitory concentrations (MIC) (n=600). The association between AMU or biosecurity and AMR was explored. Significant positive associations were detected between AMU and both genotypic and phenotypic AMR for specific antimicrobial classes. Beta-lactam and colistin resistance (metagenomics sequencing); ampicillin and ciprofloxacin resistance (MIC) were associated with AMU. However, no robust AMU-AMR association was detected by analysing qPCR targets. Also, no evidence was found that lower biosecurity increases AMR abundance. Using multiple complementary AMR detection methods added insights into AMU-AMR associations at turkey farms.

Keywords: antimicrobial use; antimicrobial resistance; turkeys; poultry; farm; antimicrobial resistance genes; biosecurity; risk factor; metagenomics; qPCR; isolates

Introduction

Antimicrobial resistance (AMR) is a global public health concern causing a substantial health and economic burden [1]. The types of antimicrobials used in food-producing animals are often the same or closely related to those used in human medicine [2]. Besides, resistance can spread rapidly and unpredictably through various environments. Therefore, AMR developed in animals can also be transferred to humans. To combat this, AMR is being addressed as part of a One Health approach [3,4].

Turkeys and turkey meat are possible sources for transmission of AMR [5]. Within the European poultry sector, turkey fattening is the second biggest meat production sector after broiler production, accounting for around 14 % of the overall poultry meat production [6]. Recently, monitoring data in European countries has shown that a substantial proportion of isolates from turkeys are resistant to several classes of antimicrobials [7].

Farm-level risk factors for AMR in turkeys, such as antimicrobial usage (AMU) and biosecurity measures, have been examined in specific countries [8–13]. For example, antimicrobial usage (AMU) in the flock and evidence of mice were reported as risk factors for ciprofloxacin resistance in *Escherichia coli* (*E. coli*) in Great Britain [8]. In Germany, the floor design of the turkey house did not affect the development of resistance to enrofloxacin and ampicillin in *E. coli* isolates from turkeys [12,13]. However, it is unclear if these risk factors are country specific or not, because large variation exists between countries and farms in terms of the amount and type of antimicrobials used [14]. Furthermore, farming practices, including biosecurity measures, vary between countries and farms. Therefore, risk factors for AMR at a regional level may not be predictive for other regions or countries.

So far, all studies in turkeys have focused on the prevalence and characteristics of phenotypic resistance. Bacterial species such as *E. coli*, *Salmonella enterica* and *Campylobactor* spp. were isolated from faeces and Minimum Inhibitory Concentrations (MIC) were determined for fixed panels of antimicrobials [8–15]. There are many mechanisms by which these specific bacteria acquire resistance to antimicrobials. For example, there are multiple gene families encoding Extended Spectrum Beta-Lactamases (ESBL) or plasmid-mediated AmpC beta-lactamases. Enterobacteriaceae producing these enzymes are resistant to antibiotics such as penicillins and 3rd and 4th generation cephalosporins. These isolates can then transfer ESBL or AmpC genes to other bacteria in the gut environment or through the food chain. In poultry production pyramids, ESBLs are frequently found [16]. Therefore, culture-dependent methods may underestimate AMR in unculturable gut microbiota. Genotypic methods enable faecal AMR gene detection. When using metagenomics or quantitative real-time polymerase chain reaction (qPCR), the abundance and diversity of AMR genes present in samples can be measured without

culturing bacteria. Combining this kind of AMR data with data on AMU and other potential on-farm risk factors, allows for exposure-response relationships to be explored [17–19]. Comparing AMR detection methods provides a better understanding of the complex mechanisms behind AMR occurrence in food-producing animals.

As part of the Ecology from Farm to Fork of Microbial Drug Resistance and Transmission (EFFORT) project (http://www.effort-against-amr.eu/), the present study aimed to explore AMR in turkeys from 60 farms in three European countries. The objectives of this paper were to (i) quantify the abundance and diversity of AMR genes in turkey faeces by applying metagenomics and qPCR, and to (ii) determine risk factors for AMR such as AMU as well as other potential farm-level risk factors. In addition, the used AMR quantification methods were compared.

Results

Overview of the Sampled Farms and Flocks

General characteristics of the sampled farms (n=60) are shown in Table 1. The total number of turkeys per farm varied considerably (median ten thousand turkeys per farm, range: 2950-56850). We carried out sampling across all seasons: spring (n=21), summer (n=8), autumn (n=16) and winter (n=15). All farms in country H were sampled in spring and summer. The weight of turkeys at set up differed substantially between the three countries, and within country B. In country H, all the farms followed an integrated fattening process where the turkeys were introduced to the fattening farms after 28 days of life in breeding, resulting in a small variation in set up weights.

The median age of turkeys at sampling was 115 days. Flocks were separated by sex in country B and H, with the exception of country E where both cocks and hens were usually housed together with a mobile fence. Therefore, some of the hens within those flocks had been removed from the house prior to sampling of the cocks. The overall expected slaughter age was 118 days. For some flocks we could not exactly determine how many days before slaughter sampling was performed, since these included several groups with a different expected slaughter date. Consequently, we calculated the average expected slaughter age per flock.

Biosecurity status at the farm was reduced to two levels. Due to a large number of questions, the questions that were significantly related with AMR in the applied models were shown in Table 1 with the number of farms that answered yes. The proportion of farms that answered yes differed between countries for several biosecurity

statuses. For instance, farms where turkeys had outdoor access were only included in country B (70% of the farms in country B).

Table 1. Characteristics of the sampled turkey farms and flocks by country and overall countries.

Characteristics	Country			Overall
	B	E	H	
Farm information				
Included farms, n	20	20	20	60
No. of turkeys present on the farm, median (Min-Max)	12683 (5000 - 46500)	7275 (2950 - 38000)	12609 (4404 - 56850)	10055 (2950 - 56850)
Farms where other livestock is present, n (%)	4 (20)	11 (55)	4 (20)	19 (32)
No. of people working at the farm, median (Min-Max)	2 (1 - 28)	1.5 (1 - 3)	1 (1 - 4)	1.5 (1 - 28)
Farms sampled in spring and summer, n (%)	4 (20)	5 (25)	20 (100)	29 (48)
Flock information				
Number of turkeys at sampling, median (Min-Max) [a]	4213 (2050 - 11660)	4140 (450 - 9155)	6422 (302 - 21356)	4710 (450 - 21356)
No. of turkeys at set-up in the current round in the sampled house, median (Min-Max) [b]	5040 (2997 - 13000)	9180 (4240 - 22000)	7020 (3000 - 21794)	7850 (2997 - 22000)
Weight of turkeys at set-up, kg, median (Min-Max) [c]	1.5 (0.1 – 6.4)	0.1 (0.1 – 0.5)	1.1 (0.9 – 1.3)	1.1 (0.1 – 6.4)
Age of turkeys at sampling, days, median (Min-Max) [b]	134 (96 - 147)	116 (74 - 140)	101 (86 - 118)	115 (74 - 147)
Average expected age at delivery to slaughter, days, median (Min-Max) [b]	146 (106 - 154)	109 (79-138)	117 (95-127)	118 (79-154)
Biosecurity at the farm				
Visitor access more than once a month (family members, technicians, etc), n (%)	8 (40)	20 (100)	16 (80)	44 (73)
Outdoor access possible for turkeys, n (%)	14 (70)	0 (0)	0 (0)	14 (23)
Different age categories of turkeys present, n (%)	10 (50)	5 (25)	0 (0)	15 (25)
Bird- and vermin proof grids placed on the air inlets, n (%)	20 (100)	15 (75)	18 (90)	53 (88)
Staff keeps turkeys or birds at home, n (%)	2 (10)	7 (35)	1 (5)	10 (17)
Disinfecting footbaths present on the farm, n (%)	14 (70)	10 (50)	10 (50)	34 (57)
The nearest turkey farm within 500m, n (%)	4 (20)	5 (25)	4 (20)	13 (22)
Other livestock farm present within 500m, n (%)	12 (60)	18 (90)	7 (35)	37 (62)
Wild birds can enter the stables, n (%)	1 (5)	6 (30)	8 (40)	15 (25)

Missing observations were excluded to calculate the average. a,b,c The number of farms with missing observations: a 2, b 1, c 10. Biosecurity status displayed in the table are those significantly associated with the AMR in the applied models.

Antimicrobial Usage

Antimicrobial group treatments applied during the entire rearing period of the sampled flock were quantified using treatment incidence (TI) as unit of measurement.

There were differences in amounts and types of antimicrobials used between countries (Figure 1). The mean TI per farm was 8.03, 9.95 and 18.4, in country B, E and H, respectively. Aminoglycosides and spectinomycins, and macrolides and lincomycins were grouped together because they have the common resistance mechanism. The most frequently used antimicrobial groups across all the farms were beta-lactams, polymyxins and quinolones. The sum of TI at 60 farms is shown in Figure S2. Across all farms, 7 (11.7%) did not use any antimicrobials (country B:3, E:3 and H:1).

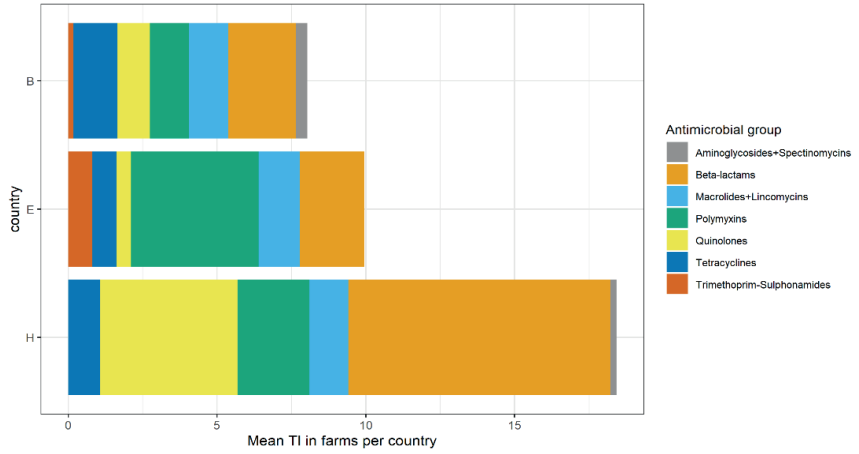

Figure 1. Average antimicrobial usage on farm level in 60 turkey farms in three countries.

Mean treatment incidence (TI) shows the average number of treatment-days per 100 days. Antimicrobials were grouped after TI was calculated for lincomycin-spectinomycin combination product and subsequently divided and added to macrolides and aminoglycosides, respectively. Beta-lactams included aminopenicillins and penicillins. Quinolones included fluoroquinolones and other quinolones (flumequine). Countries were anonymized as B, E and H.

AMR Genes Identified by Metagenomics

The Abundance and Composition of AMR Genes

In total 573 different AMR genes were identified in samples from 60 turkey farms using ResFinder as a reference database [20]. The abundance of AMR genes was quantified using normalized Fragments Per Kilobase reference per Million bacterial fragments (FPKM) values. The FPKM values for the different AMR genes were summed for each antimicrobial class. In general, the composition of AMR genes appeared rather homogenous across farms despite the difference in AMU, and even when comparing farms that did or did not use antimicrobials (Figure 2). The clusters of AMR genes encoding for resistance to tetracyclines, macrolides and aminoglycosides were most abundant. Moreover, AMR gene clusters encoding for resistance to aminoglycosides, beta-lactams, macrolides, phenicols, sulphonamides, tetracyclines and trimethoprim classes were detected on all farms. A stacked bar chart showing FPKM values (i.e. not proportional) is shown in Figure S3.

The total abundance of AMR genes, expressed as the summed FPKM values differed between the three countries. The mean total abundance on the farms in country E was significantly lower than that of country H (One-way ANOVA, Tukey HSD, $p<0.01$) (Figure S4).

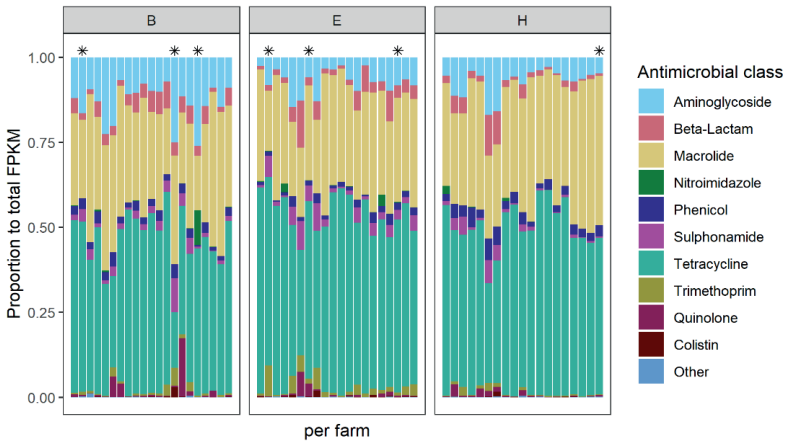

Figure 2. Relative abundance of antimicrobial (AMR) genes expressed as a proportion of total fragments per kilobase reference per million bacterial fragments (FPKM).

Columns represent 60 samples from 60 farms from three countries (B: n=21, E: n=20, H: n=19). One additional farm was visited in country B due to incomplete questionnaire data in one of the farms, resulting in twenty-one samples in total. One sample in country H was removed due to errors during processing. The AMR genes were aggregated to antimicrobial classes. Seven farms where no antimicrobial use was reported in the sampled flock are indicated with an asterisk above the columns.

Factors Associated With the Abundance of AMR Gene Clusters

Factors associated with the abundance of AMR gene clusters of eight antimicrobial classes were investigated for 57 farms with complete data (country B: n=18, E: n=20, H: n=19). Using a random-effects meta-analysis by country, Table 2 presents the associations between AMU and the abundance of AMR gene cluster of the corresponding antimicrobial class. Three significant associations between AMU and the corresponding AMR gene cluster were detected: beta-lactam use (penicillin and aminopenicillins) and beta-lactam resistance, polymyxin use and colistin resistance, and aminoglycosides or spectinomycin use (binary variable) and aminoglycoside resistance (p value < 0.1 adjusted for multiple testing). At farms that reported a higher TI of beta-lactam and polymyxins, a higher faecal abundance of the corresponding AMR gene clusters was observed. Farms with reported aminoglycosides or spectinomycin use had a higher faecal abundance of aminoglycoside resistance genes compared to the farms that did not use these antimicrobial classes. However, only one and five farms reported usage of aminoglycoside and lincomycin-spectinomycin, respectively.

None of the other farm characteristics than AMU was significantly associated with the abundance of AMR gene clusters after Benjamini-Hochberg multiple testing correction (adjusted p value ≥0.1).

Table 2. Associations between antimicrobial usage (AMU) and relative abundance of the corresponding antimicrobial resistance (AMR) genes detected by metagenomics, obtained from a random-effects meta-analysis by country.

AMU	AMR gene cluster [a]	Estimate	Adjusted p value [b]	95% CI	Country and number of farms with reported AMU
Log_{10} TI beta-lactam	Beta-lactam	**1.06**	**0.033**	**[0.29-1.84]**	B-15, E-14, H-18
Log_{10} TI polymixin	Colistin	**0.99**	**0.033**	**[0.29-1.69]**	B-4, E-11, H-5
Aminoglycosides or spectinomycin used (ref:no)	Aminoglycoside	**0.92**	**0.097**	**[0.08-1.76]**	B-3, H-3
Trimethoprim-sulphonamides used (ref:no)	Trimethoprim	0.78	0.221	[-0.15-1.71]	B-2, E-3
Trimethoprim-sulphonamides used (ref:no)	Sulphonamide	0.68	0.282	[-0.26-1.61]	B-2, E-3
Log_{10} TI quinolone	Quinolone	0.69	0.338	[-0.43-1.81]	B-5, E-4, H-12
Log_{10} TI tetracyclines	Tetracycline	0.09	0.948	[-0.82-1.00]	B-6, E-6, H-9
Log_{10} TI macrolides + lincomycin	Macrolide	-0.17	0.948	[-1.35-1.01]	B-6, E-12, H-7
Log_{10} TI total AMU	Total FPKM	-0.02	0.948	[-0.62-0.58]	B-15, E-17, H-18

AMU=Antimicrobial usage; AMR=Antimicrobial resistance; 95%CI=95% Confidence Interval; TI=Treatment incidence; In the models, 57 farms with complete data were included (country B: n=18, E: n=20, H: n=19).

a: Relative abundance of AMR genes were clustered per antimicrobial class and calculated as sum of fragments per kilobase reference per million bacterial fragments. b: P values were adjusted with Benjamini-Hochberg correction with a false discovery rate set to 10%. Associations in bold have adjusted p<0.1.

ermB, tetW, sul2 and aph3'-III Identified by qPCR

Abundance of *ermB*, *tetW*, *sul2* and *aph3'-III*

In total 304 samples were analyzed by qPCR. Across all samples, the number of 16S rRNA gene copies varied (log_{10} copies median=10.8, min=7.73, max=12.8). The number of 16S rRNA copies were used subsequently to calculate relative concentrations of the AMR gene copies. After the qPCR quality check, in order to include samples with low concentration of *sul2* (11 samples) and *aph3'-III* (20 samples) that were below the limit of detection or limit of quantification, the following values were assigned: *sul2*: 5.10; *aph3'-III*: 3.62. The unit was the number of gene copies (log_{10} copies) before normalization with 16S rRNA. Of those, two *aph3'-III* samples were removed due to low abundance of 16S rRNA (log_{10} 16S rRNA copies < 8.51). As a result, 283 (93.1%), 287 (94.4%), 262 (86.1%) and 269 (88.5%) samples for *ermB*, *tetW*, *sul2* and *aph3'-III*, respectively, were available for analysis. The abundance of the four genes relative to bacterial DNA (16S rRNA), stratified per country and gene is shown in Figure 3.

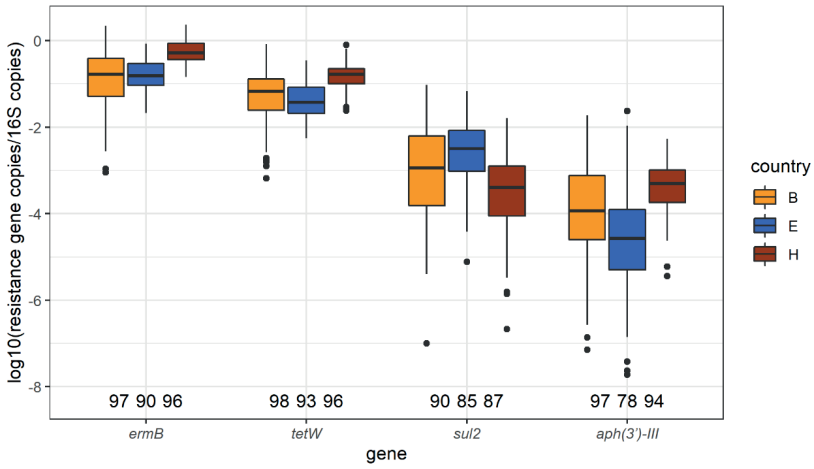

Figure 3. Relative abundance of *ermB, tetW, sul2 and aph3'-III* in turkey faeces sampled in three countries, detected by qPCR. Resistance gene log_{10} copies were normalized using 16S rRNA abundances. The numbers displayed above the horizontal axis are the number of the samples eligible for analysis.

Chapter 6

Factors Associated with the Abundance of *ermB*, *tetW*, *sul2* and *aph3'-III*

In the univariate analysis, total AMU (summed TI of all the antimicrobial classes at farm level) was positively associated with the abundance of *ermB* (Geometric Mean Ratio, GMR=1.86) and *tetW* (GMR=1.81). No significant association between AMU and the corresponding resistance gene abundances were detected (Table S1).

Table 3 presents GMR estimates and 95% confidence intervals of the final multivariable models mutually adjusted for technical farm characteristics and biosecurity. None of the biosecurity variables was associated with the abundance of *sul2*. Linear mixed models with random effect for country were fitted for all the genes, however, there was no variance between countries in the final *sul2* model.

Trimethoprim-sulphonamide treatment in flocks was positively associated with the abundance of *sul2* in turkey faeces, when adjusted for sampling season and the presence of other livestock at the farm (GMR=7.38). No association was detected between the abundance of *ermB, tetW* and *aph3'-III* and the use of corresponding AMU in multivariable models. Three biosecurity variables remained in the final *ermB* model, and two in the final *tetW* and *aph3'-III* models. The abundance of *ermB* and *tetW* in faeces was significantly lower at the farms where access of visitors was granted more than once a month, and at the farms where turkeys had outdoor access. The concentration of *ermB* in faeces was lower if there were different age categories of turkeys present on the farm. For the abundance of *aph3'-III*, having wild bird- and vermin proof grids placed on the air inlets was positively associated while having a permanent staff that keeps turkeys or birds at home was negatively associated.

Table 3. Multivariable model associations between antimicrobial usage (AMU), characteristics, biosecurity measures of the turkey farms and the median relative faecal abundance of *ermB*, *tetW*, *sul2* and *aph3'-III* per farm.

Model variables	*ermB*		*tetW*		*sul2*		*aph3'-III*	
	GMR	[95%CI]	GMR	[95%CI]	GMR	[95%CI]	GMR	[95%CI]
AMU								
Log_{10} TI macrolides + lincomycin [a]	1.57	[0.77,3.23]						
Log_{10} TI tetracyclines [a]			1.54	[0.80,2.97]				
Trimethoprim-sulphonamides used (ref:no)					**7.38**	**[1.61,33.8]**		
Aminoglycosides or spectinomycin used (ref:no)							1.47	[0.42,5.14]
Technical farm characteristics								
Age of turkeys at sampling (standardized) [a]	**0.73**	**[0.54,0.98]**						
Other livestock present (ref:no)					**2.89**	**[1.17,7.14]**	**0.38**	**[0.15,0.95]**
Sampling season (ref: autumn, winter)					**0.21**	**[0.09,0.48]**		
Biosecurity								
Visitor access more than once a month (ref:no)	**0.41**	**[0.22,0.75]**	**0.36**	**[0.21,0.60]**				
Outdoor access possible for turkeys (ref:no)	**0.35**	**[0.17,0.75]**	**0.37**	**[0.19,0.74]**				
Different age categories of turkeys present (ref:no)	**0.45**	**[0.25,0.83]**						
Bird- and vermin proof grids placed on the air inlets (ref:no)							**6.32**	**[1.76,22.73]**
Staff keeps turkeys or birds at home (ref:no)							**0.27**	**[0.09,0.83]**

AMU=Antimicrobial usage; GMR=Geometric mean ratio; 95%CI=95% Confidence Interval; TI=Treatment incidence. Technical farm characteristics and biosecurity variables displayed in the table are those significantly associated with the abundance of each gene in the final models. Significant associations are marked in bold (P<0.05).

Phenotypic Resistance Identified by Minimum Inhibitory Concentrations

E. coli Resistance to Antimicrobials

Ceccarelli *et al*., previously described the MIC values derived from the turkey faeces collected in this study [21]. *E. coli* was successfully isolated from 596 out of 600 samples, and MIC values were determined by broth microdilution for a fixed panel of 14 antimicrobials for those isolates. Epidemiological cut-off values were used to determine non-wild type susceptible (≈ microbiological resistant) isolates. However, misinterpretation of

sulphamethoxazole MIC-endpoints (overestimation of resistance) for country B led to the exclusion of these data from the analysis.

The proportions of resistant *E. coli* isolates differed between countries and between antimicrobials [21]. The proportion of isolates resistant to ampicillin and tetracycline was higher than 70% in all three countries. The proportion of isolates resistant to ciprofloxacin, nalidixic acid and chloramphenicol was higher than 55% in country H, whereas those in both country B and E were less than 35 %. Less than 10 % of all the isolates were resistant to cefotaxime, ceftazidime, meropenem, azithromycin, gentamicin and tigecycline. All meropenem-resistant isolates were confirmed to be negative for known carbapenemases by PCR.

Factors Associated with E. coli Resistance

The univariate association between potential risk factors and the occurrence of *E. coli* resistant to ampicillin, tetracycline and ciprofloxacin from the mixed effect logistic models are presented in Table S2. These three antimicrobials were selected for this analysis because both (i) the number of the farms on which corresponding antimicrobial classes were used and (ii) the prevalence of isolates resistant to the antimicrobials were higher than 10%. Significant positive associations were detected between AMU and the occurrence of *E. coli* resistant to ampicillin, tetracycline and ciprofloxacin. The total amount of AMU was also positively related to resistance to all three antimicrobials. In addition to these three antimicrobials, a univariate association between polymyxin use and resistance to colistin was detected (p=0.001). However, because of model convergence failure, the multivariable model for colistin resistance could not be investigated. A random intercept for farm was included in all the models and country intercept was also added to the ciprofloxacin model because it significantly improved the model fit.

Table 4 shows that there was a significant positive association between AMU at the farm and resistance of *E. coli* isolates for ampicillin and ciprofloxacin when mutually adjusted for other farm characteristics. The presence of a turkey farm within 500m was negatively associated with ciprofloxacin resistance of *E. coli* isolates. Other associations between biosecurity and resistance of *E. coli* isolates were not statistically significant after mutual adjustment for potential other determinants identified in the univariate analysis.

Correlations Between AMR Genes Abundances Detected by Metagenomics and qPCR

The correlation between abundance of *ermB, tetW, sul2* and *aph3'-III* detected by metagenomics and qPCR is shown in Figure S5. Metagenomics samples were pooled at farm level and the median of the qPCR

samples per farm were used. A significant but modest correlation was observed for all four genes ($p < 0.001$, Spearman rho=0.47-0.74). The highest correlation was observed for *ermB* (rho=0.74).

The abundance of metagenomically derived AMR genes clustered at the 90% identity level and present within the macrolide, tetracycline, sulphonamide and aminoglycoside class clusters were shown in Table S3. The abundance of *ermB, tetW, sul2* and *aph3'-III* accounted for 69.0%, 42.3%, 42.6% and 25.3% of the macrolide, tetracycline, sulphonamide and aminoglycoside resistance class clusters, respectively.

Table 4. Multivariable associations between antimicrobial usage (AMU) and characteristics and biosecurity measures of the turkey farms and the occurrence of *E. coli* isolates from turkey faeces resistant to ampicillin, tetracycline and ciprofloxacin.

Model variables	AMP		TET		CIP	
	OR	[95%CI]	OR	[95%CI]	OR	[95%CI]
AMU						
Log_{10} TI aminopenicillins	**4.10**	**[1.37,12.30]**				
Log_{10} TI tetracyclines			3.32	[0.75,14.7]		
Log_{10} TI quinolones					**12.85**	**[4.00,41.2]**
Technical farm characteristics						
Age of turkeys at sampling (standardized)	0.83	[0.53,1.31]	0.74	[0.48,1.13]		
Sampling season (ref: autumn, winter)			2.13	[0.85,5.31]		
Biosecurity						
Other livestock farms present within 500m (ref: no)	0.48	[0.19,1.18]				
Wild birds can enter the stables (ref: no)			2.67	[0.90,7.87]		
Different age categories of turkeys present (ref: no)			0.48	[0.19,1.20]		
The nearest turkey farm within 500m (ref: no)					**0.28**	**[0.11,0.69]**

AMU=Antimicrobial usage; OR=Odds Ratio; 95%CI=95% Confidence Interval; AMP=ampicillin; TET=tetracycline; TI=Treatment incidence. Significant associations are marked in bold ($p < 0.05$). All OR shown in the table are mutually adjusted for class specific AMU and farm characteristics/biosecurity variables for the specific column.

Discussion

In this multi-country risk factor study on 60 turkey farms, we investigated risk factors for the faecal abundance of AMR genes in turkeys detected by both metagenomics and qPCR, as well as the prevalence of resistance in *E. coli* isolates in turkey faeces collected in Germany, France and Spain. We detected positive

associations between AMU and both genotypic and phenotypic AMR, specifically for beta-lactam and colistin resistance (metagenomics) and ampicillin and ciprofloxacin resistance (MIC).

Substantial differences in AMU were observed between farms and countries. The most frequently used antimicrobial groups were beta-lactams (aminopenicillins and penicillins), followed by polymyxins and quinolones (fluoroquinolones and other quinolones). A previous study on Italian turkey farms reported that polymyxins, penicillins (including aminopenicillins) and sulphonamides were widely used [22]. A substantial variation in use of antimicrobial classes within and between countries is expected since there are many possible explanations such as differences in antimicrobial stewardship of veterinarians, differences in availability of pharmaceutical products and national legislations [23]. A similar high variation in AMU was observed on broiler farms from nine European countries [24].

The relative AMR gene composition detected by metagenomics was similar across the 60 included farms, including flocks that did not receive any antimicrobial treatment. This was in accordance with European broiler studies, where the faecal AMR genes composition appeared to be roughly similar between farms, despite the absence of AMU in many flocks [18,25]. Genes encoding for resistance to tetracyclines were the most dominant cluster, followed by macrolides and aminoglycosides, when clustered at the antimicrobial class level. This is consistent with previously published gut microbiome data in Polish turkeys [26]. These classes, however, did not correspond with the most frequently used antimicrobials in our study. The presence of these AMR gene classes in faeces of other animal species is reported in multiple countries, regardless of AMU [25–27]. These AMR genes may be present in the various bacterial species in the gut of turkeys. It suggests that there are other factors that affect the composition of AMR genes in the gut environment, in addition to direct AMU. This could include co-selection of resistance by AMU in the production round or in previous rounds at the farm, through which antimicrobial residues and resistant bacteria remained in the environment. Physical transfer of bacteria via movement of animals may have contributed as well [28].

Significant positive associations were detected between AMU and the abundance of corresponding AMR genes for some antimicrobial classes. The result of the random effects meta-analyses using metagenomics data showed that flocks which received more beta-lactam and colistin antimicrobials had a higher abundance of the corresponding AMR genes. Horizontal gene transfer plays a role in the acquisition of beta-lactam and colistin resistance in addition to chromosomal mutations [29,30]. Therefore, AMU may select for and thus accelerate such transmission.

Fluoroquinolone use has previously been identified as a risk factor for increased fluoroquinolone resistance in *E. coli* [9,12,13]. These studies also reported an increased prevalence of ampicillin resistant isolates in trials in absence of ampicillin use [12,13]. In line with these studies, we also observed that increased fluoroquinolone use was related to higher proportions of *E. coli* isolates being resistant to ciprofloxacin. In addition, we observed an AMU-AMR association for ampicillin in *E. coli* isolates. Boulianne *et al*. reported associations between tetracycline use and the occurrence of tetracycline resistance in *E. coli* isolates in Canadian turkey flocks [11]. We also observed positive phenotypic AMU-AMR associations on tetracycline in our study, which were statistically significant in the univariate analysis, but with a wide confidence interval. To study more phenotypic AMU-AMR associations, susceptibility testing in gram positive bacteria such as *Enterococcus* spp. could be considered [11].

We found no evidence that good biosecurity measures were related to lower faecal AMR abundance in turkeys. Our results differ from earlier findings on association between biosecurity measures and fluoroquinolone resistance in *E. coli* in turkey faeces in Great Britain [8]. They reported on-farm presence of mice was risk factor, while disinfection of floors and walls at depopulation appeared protective. However, information on the quantity of AMU in the sampled flock was not included in their study, so it may be possible that AMU was correlated with the biosecurity factors. In our study, we could not verify if the presence of mice increases the risk, but we observed that bird- and vermin proof grids placed on the air inlets were associated with a higher risk for *aph3'-III* detected by qPCR. Additionally, the fact that all the farms provided the same answer for "there is a preventive vermin control program" and "stables are disinfected after every round" in our study may suggest that these measures are not associated with variations of AMR on turkey farms. Chuppava *et al*. reported that the floor design of the turkey house did not correlate with the development of ampicillin and enrofloxacin resistant *E. coli* isolates [12,13]. Furthermore, there was little evidence for associations between farm biosecurity and the abundance of AMR genes in European broilers [18]. Interestingly, poor biosecurity such as staff having contact with other birds among others, were in fact related to a lower faecal abundance of *aph3'-III* detected by qPCR. In addition, the presence of a turkey farm within 500m was negatively associated with *E. coli* resistance to ciprofloxacin. However, we cannot explain this phenomenon biologically. Therefore, the relationship between biosecurity and AMR on turkey farms remains uncertain.

Three different AMR detection methods were used in this study. We observed modest correlations between the abundance of AMR genes quantified by metagenomics and qPCR. A possible reason may be the difference in sample selection. For metagenomic sequencing, the samples were pooled per farm before DNA extraction to

represent the farm, whereas DNA was extracted from five to six samples individually for qPCR analysis to detect variations within farms. Pooled samples provide a composition representative of the common AMR genes at the farm [31], whereas the abundance of particular genes may vary between individual samples. Additionally, low correlation could be due to the low concentration of the target genes or inhibition of gene expression [32]. We chose multiple genes in metagenomic sequencing based on 90% identity level and summed to compare with qPCR, but we can also speculate that there might have been more genes that qPCR detects. On the other hand, the agreement between the abundance of genotypic resistance and phenotypic resistance was not tested. This is because genotypic resistance in this study represents the abundance in the total faecal bacterial community whereas phenotypic resistance is specific to *E. coli*. To compare and predict phenotypic resistance in specific isolates, whole genome sequencing studies could be performed [33].

Detecting total genotypic resistance in samples, rather than isolating specific bacteria, is a good choice to find risk factors for AMR genes associated with horizontal gene transfer. Genotypic detection methods in our study enabled to confirm that AMR genes were widely present in turkey faeces for some antimicrobial classes such as macrolides and aminoglycosides, despite low phenotypic resistance to specific antimicrobials expressed in *E. coli*. The strength of metagenomic sequencing was that it showed the composition of AMR genes in the resistome (the collection of all resistance genes in a sample). Moreover, AMR genes could be analyzed at several grouping levels, such as gene and antimicrobial class level. On the other hand, qPCR may be a better choice for detecting specific genes of interest because of lower costs and simple procedures over metagenomic sequencing. However, the selection of the most appropriate target gene may be difficult. Our qPCR target genes were the most abundant gene clusters within the respective antimicrobial classes. However, such information may not always be available beforehand. Limitations for both metagenomic sequencing and qPCR lie in the difficulty to compare the results with other studies since genotypic AMR data in turkeys is still scarce and methods can vary between studies. In contrast, phenotypic AMR in specific bacteria has been studied in a standardized manner for monitoring purposes, making it easier to compare results between studies or to monitor trends over time. However, using dichotomized outcomes by epidemiological cut-off in our study hampered data analysis for antimicrobial classes in which the resistant proportion of isolates were low. In summary, we showed that these methods are complementary and the choice depends on the research question.

Our study is unique considering that farms were included from three European countries using standardized sampling, which enabled the identification of risk factors that are not country-specific. We also related AMU and multiple farm-level factors to both genotypic and phenotypic AMR. However, information on

purchased AMU at the farm level was not available in all countries and could therefore not be studied as an alternative to group treatments. This could explain the on-farm background levels of AMR in absence of reported usage. Moreover, although we included group treatments data at the breeding farms, farm characteristics of those farms were not collected. Both AMU and biosecurity information of the sampled farms was from farmers' reports rather than registered data. Therefore, underreporting of AMU and misclassification or missing biosecurity answers could have led to social desirability bias. We quantified the 16S rRNA gene to normalize AMR gene results detected by qPCR, but many bacterial species have more than one copy of the 16S rRNA gene. There is no suitable approach to correct for copy numbers in microbiome data [34,35]. Although gut bacterial composition between turkeys may differ, we expect that this taxonomic difference will not have a large effect on the between-group comparisons. Error in quantification of the 16S rRNA gene that we used to normalize the AMR genes would lead to a less precise estimate of AMR, resulting in attenuation of risk estimates (e.g. AMU-AMR associations). Despite these limitations, our study shows association between AMU and AMR on turkey farms which is a potential exposure route to humans.

Materials and Methods

Selection of farms

Between October 2014 and October 2016, 60 conventional fattening turkey farms were visited in three countries (Germany, France and Spain, 20 farms per country). German farms were geographically spread over the country, while all French and Spanish farms were concentrated in Brittany and Andalusia, respectively, which both are the major turkey production sites in these countries (Figure S1). The preferable selection of farms was based on the following criteria: conventional farms with an all-in-all-out system, and with the size 3000 - 15000 birds per farm. However, the size criteria were not always met. Farms included in the study were unrelated. Both farms and countries were anonymized (country B, E and H) to ensure that the results cannot be traced back, consistent with previous EFFORT publications in which data from nine countries (A to I) was analysed. The selected farms cannot be considered representative for the respective countries.

Each farm was visited once to collect faecal samples. On each farm, the unit for sampling was a turkey house with a flock that had not been moved or mixed with other flocks except the removal of individual birds before the sampling time. In the flocks, all animals have received the same group treatments by water, medicated feed or injection during their lifetime. The sampling was intended at maximally one week before the

final slaughter date of the hens, but samples were collected randomly regardless of sex. Farms were visited across all seasons.

Questionnaire data: antimicrobial usage, farm characteristics and biosecurity

Information on all antimicrobials administered as group treatments to the sampled flock during their whole lifetime was documented by the farmers with supervision of the researchers or veterinarians. Before introducing the sampled flock, researchers informed the farmers on how to document the antimicrobial treatments. Group treatment data included not only those administered in the sampled farms but also in previous breeding farms if applicable. Technical farm characteristics and biosecurity status were obtained by a questionnaire filled out by the participating farmers. Answers in the questionnaire were entered into EpiData version 3.1 Software (EpiData Association, Odense, Denmark).

Quantification of AMU

To quantify AMU, TI was calculated based on antimicrobials administered to the sampled flock, as previously described [21,24]. Defined Daily Dose for turkeys (DDD_{turkey}) was assigned for all the antimicrobials used on the included farms. Therefore, TI is expressed as the number of DDD_{turkey} administered per 100 turkey-days at risk or the number of days per 100 turkey-days that the flock received a standardized dose of antimicrobials (1). The latter can also be interpreted as the percentage of time that a turkey is treated with antimicrobials in its life.

$$TI = \frac{Total\ amount\ of\ active\ substance\ administered\ (mg)}{DDD\ turkey\ (mg/kg/day) \times number\ of\ days\ at\ risk \times kg\ turkey\ at\ risk} \times 100\ turkeys\ at\ risk \quad (1)$$

For determining “kg turkey at risk”, a standard weight of 6kg was used according to the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) guidelines [36]. Then, the standard weight was multiplied by the number of turkeys at setup. “Number of days at risk” was equal to the expected age of slaughter at each farm. When there were a few different age group of slaughter batches within the sampled flock, the average age within the sampled flock was used. From this formula, TI was calculated for each antimicrobial class per farm. Total TI per farm was also calculated.

For the risk factor analyses, the sum of TI at farm level for each antimicrobial class was used. Furthermore, we grouped antimicrobials (TIs) that possessed similar mechanisms of resistance, i.e. macrolides and lincomycin, aminoglycosides and spectinomycin. Since lincomycin and spectinomycin were administered as

combination products with a fixed ratio (lincomycin:spectinomycin, 1:2) [37], TI was first calculated using DDD_{turkey} for lincomycin-spectinomycin and subsequently divided for each active substance. Aminopenicillin and penicillin were grouped as beta-lactam, fluoroquinolones and other quinolones (flumequine) were grouped together as quinolones.

Sampling and processing of faecal samples

Per farm, twenty-five fresh faecal droppings were collected from the floor of one turkey house. After collection, each sample was refrigerated at 4 °C and transported to the laboratories within 24 hours.

On arrival at the laboratory of each sampling country, samples for *E. coli* isolation were processed. Simultaneously, samples for metagenomics and qPCR were prepared and stored at -80 °C until shipment. Frozen samples were shipped on dry ice to the Institute for Risk Assessment Sciences (IRAS, Utrecht, the Netherlands).

Metagenomic sequencing and processing data

Metagenomic sequencing and processing were performed as described previously, with modifications [25,38]. The reads are available in the European Nucleotide Archive, under project accession number PRJEB39685.

At the laboratory, the individual faecal samples were homogenized by stirring thoroughly with a tongue depressor or a spoon for a few minutes. Twenty-five individual samples from the same farm were pooled with 0.5g faeces from each sample and stirred for a few minutes. DNA extraction was centrally performed at Technical University of Denmark (The National Food institute, Kgs. Lyngby, Denmark). From a 0.2g sample, DNA was extracted using a modified QIAmp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) [39]. The samples were sequenced on the NovaSeq 6000 platform (Illumina Inc, CA, USA) by Admera Health (South Plainfield, New Jersey, USA), using a 2 x 150-bp paired-end (PE) read approach and aiming for 35M PE reads per sample.

After removing low-quality nucleotides as well as adaptor sequences, trimmed read pairs corresponding to each farm-level sample were aligned to the ResFinder database and, separately, to a merged database of genomic sequences using the k-mer alignment software KMA (v1.2.8). The ResFinder database repository was accessed on 13 February, 2019 and contained 3,081 AMR genes. Read was aligned to the ResFinder database using the KMA parameters '-mem_mode -ef -1t1 –cge -nf -nc'. In order to filter out low-coverage alignments, alignments that were lower than 20% consensus of the corresponding reference were removed. The genomic sequence database was described previously [40]. Reads were assigned to the genomic database using KMA parameters '-mem_mode -ef -

1t1 -apm f -nf -nc'. The sum of sequencing fragments mapped to the bacteria, archaea, plasmid, bacteria_draft, HumanMicrobiome and MetaHitAssembly sub-databases was used as the sample size factor for the FPKM calculation.

As the unit of outcome, FPKM values were computed as previously described [25]. The values were aggregated at the antimicrobial class cluster level for risk factor analysis. Distribution was checked, and a pseudocount of one and $\log_{10}$ transformation was applied to FPKM values. Furthermore, the values were aggregated at the 90% identity clustering [41], to analyze the abundance of the specific AMR genes.

qPCR analysis

For qPCR analysis, five to six samples per farm were randomly selected, resulting in 304 samples. Five samples per farm were included to depict between-animal variation which is assumed to be small within one turkey house. From each sample, 0.5g faeces were transferred to a 2mL cryotube. From a 0.2g sample, DNA was extracted using a modified QIAmp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) [39]. For all the samples, DNA extraction was performed centrally at IRAS, in the Netherlands.

Four AMR genes, *ermB*, *tetW*, *sul2* and *aph3'-III,* were selected as qPCR targets. These genes encode resistance against macrolides, tetracyclines, sulphonamides and aminoglycosides, respectively. These antibiotic classes of public health relevance were chosen based on their abundance in metagenomic data of pooled pig and broiler faeces samples collected within the EFFORT project [25]. In addition, 16S rRNA was targeted for normalization of the AMR genes to bacterial DNA in each sample. Three gene targets of qPCR assay (16S rRNA, *sul2* and *aph3'-III*) were performed at the National Veterinary Institute (PIWet, Puławy, Poland), while the other two (*ermB* and *tetW*) were at IRAS. Overall results were centrally analyzed at IRAS.

A qPCR assay was performed as previously described [*ermB, tetW*, 16S rRNA [42]; *sul2* and *aph3'-III* [19]]. Briefly, all samples were run in two technical PCR duplicates with a non-competitive internal amplification control (IAC) to control quality. From raw amplification data, Ct values were derived by the R project package "chipPCR" [43]. For each gene, the number of copies derived from the Ct values was normalized to bacterial load ($\log_{10}$ (copies of AMR gene/copies 16S rRNA)).

Among the samples passing the qPCR quality criteria (IAC and replicate consistency), those without a quantifiable 16S rRNA concentration were excluded from further analysis (14 samples). Additionally, *sul2* (11 samples) and *aph3'-III* (20 samples) were below the limit of detection or limit of quantification. Those samples were assigned a value (in $\log_{10}$ copies) corresponding to the 1st percentile of the distribution when considering

all values of all samples together per gene (*sul2*: 5.10; *aph3'-III*: 3.62). Of those, the samples with low abundance of 16S rRNA (lower than the 1st percentile of the copy unit of all 16S rRNA concentrations) were excluded from data analyses because these present very high normalized values.

E. coli isolation and MIC determination

Isolation of *E. coli* and MIC determination was performed as previously described [21]. The individual samples were suspended in buffered peptone water 1/10 (w/v) with 20% glycerol in a 2mL cryotube and thoroughly mixed. Ten samples from each farm were selected (no 2, 4, 6, 8, 10, 12, 14, 16, 18, 20), resulting in 600 samples for *E. coli* isolation. Briefly, all samples were inoculated on MacConkey agar and after incubating 24 hours overnight, suspected colonies were isolated and confirmed as *E. coli*. Isolated samples were stored individually in buffered peptone water with 20% glycerol at -80°C. Next, MIC values by broth microdilution were determined for a fixed panel of antimicrobials by commercially available microtitre plates (Sensititre, EUVSEC, Themo Fisher Scientific UK Ltd., Loughborough, UK). European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values were used to differentiate between wild type and non-wild-type susceptibility.

Variable selection and statistical analysis

First, to examine association between AMR and farm-level factors, univariate models with AMR and the corresponding AMU were applied, as well as with other farm-level variables selected from the questionnaires. Next, according to the association observed in univariate models, multivariable models were built.

All statistical analyses were performed in R version 3.6.1 (https://www.R-project.org).

Explanatory variables

The distributions of continuous variables (i.e. AMU, "total number of turkeys at the farm", "age of turkeys at sampling") were explored and $\log_{10}$ transformed in case of skewness. Age of turkeys was standardized by subtracting the mean and dividing it by the standard deviation to avoid modelling errors due to scale differences between variables. As only a limited number of farms (<10) used trimethoprim-sulphonamide, aminoglycosides or spectinomycin, we dichotomized these variables. From the questionnaires, the most important farm characteristics variables were selected based on expert knowledge and prior studies [8,17,19,44,45].

In case of high correlation between technical farm characteristics and biosecurity variables (Spearman $\rho > 0.7$), technical farm characteristics variables were selected. Variables without contrast and those with missing

values were excluded. One missing value of age of turkeys in country B was replaced with the median age of the sampled birds in country B (134 days). All categorical variables were reduced to two levels to avoid convergence errors in modelling.

Four technical farm characteristics variables, namely, "total number of turkeys at the farm"," age of turkeys at sampling", "other livestock is present at the farm" and "season of the sampling" and 19 biosecurity variables fulfilling the above criteria were considered in the following models (Supplementary material part B).

Factors associated with AMR gene clusters identified by metagenomics sequencing

Three samples from farms for which the metagenomic data could not be matched with the questionnaire data were excluded in the risk factor analysis, resulting in 57 farms to be analyzed (country B: n=18, E: n=20, H: n=19). The abundance of AMR genes clustered at the antimicrobial class level were used as the outcome variable. Eight clusters with the reported corresponding AMU were chosen for the models. Random effects meta-analyses by country were performed as previously described [17,18]. First, linear regressions were calculated per country, after which the overall associations were calculated using a random effect for country to take the between-country variance into account. To prevent certain countries from largely influencing the estimates, the outcome variable was standardized (mean 0, SD 1) by country. R package Metafor was used [46].

Briefly, univariate associations between AMR gene clusters and corresponding AMU, technical farm characteristics and biosecurity variables were examined. Additionally, the association between the summed FPKM of all the clusters (total FPKM) and total AMU at the farm was analyzed. P-values were adjusted for multiple testing by the Benjamini-Hochberg procedure with the false discovery rate set to 10% [47].

Factors associated with *ermB*, *tetW*, *sul2* and *aph3'-III* identified by qPCR

The abundances of the four genes were averaged at the farm level using the median value of the five to six samples within each farm to remove correlation in farm (i.e. 60 samples in total), instead of adding a random effect for farm. Random effect for both farm and country resulted in convergence errors when modelling. Linear mixed models with random intercept for country were applied for both univariate and multivariable analyses.

First, univariate models were built for each gene to look for factors significantly influencing the AMR gene concentrations. Subsequently, we applied the step function of the R lmerTest package, which performs a backward elimination of non-significant effects in multivariable models [48]. We applied this to the fixed effects while keeping the random effect for country. The variables included in the full models were: (i) the corresponding AMU variable, (ii) the variables significantly related with AMR in the univariate analysis (Satterthwaite's degrees of freedom method, p value <0.05) and (iii) four technical farm characteristics variables

because these may be related with AMU and biosecurity variables. Fixed effect variables were eliminated backward from the full models according to the p value (alpha=0.05), while keeping the corresponding AMU variable. To make the model coefficients more interpretable, all estimates and their 95% CIs were expressed as GMR values by exponentiating with base 10 coefficients (Table 3, Table S1).

Factors associated with E. coli resistance

The occurrence of *E. coli* isolates resistant to ampicillin, tetracycline and ciprofloxacin were used as the outcome variables. These three antimicrobials were selected because there were more than six farms (i.e. 10% of all the farms) with reported corresponding AMU and there were more than 60 resistant isolates (i.e. 10% of all the isolates). Nalidixic acid was not selected but ciprofloxacin was selected for quinolone resistance. This is because when using the epidemiological cutoff to define non-wild type susceptible isolates, nalidixic acid and the fluoroquinolone ciprofloxacin show the same results in proportions of non-wild type strains. Corresponding AMU variables were aminopenicillin, tetracycline and quinolone use (fluoroquinolone and other quinolones). Penicillins were not included since *E. coli* is intrinsically resistant to penicillin. At first, it was intended to investigate the association between polymyxin use and colistin resistant *E. coli,* but many models failed to converge in univariate analysis, which made it impossible to further investigate risk factors. Mixed effects logistic models with random intercept for farm were applied. A country random intercept was added when it improved the fit in null models.

Following univariate analysis, the variables significantly related in univariate analysis (p value <0.05) were added in the multivariable models. Additionally, the effect of total AMU was examined by including this in a model instead of the corresponding AMU variable. All ORs and their 95% CIs are shown in the results (Table 4, Table S2).

Comparisons between metagenomics and qPCR

First, two genotypic resistance methods, namely metagenomics and qPCR samples were compared. Metagenomics samples were pooled at farm level while for qPCR samples, the median value of the five to six samples within each farm was used. Associations between the abundance of *ermB*, *tetW*, *sul2* and *aph3'-III* clusters as identified by metagenomics and the abundance of these genes by qPCR were examined by calculating the Spearman correlation coefficient (Figure S5). In addition, total abundance (i.e. summed FPKM of all the farms) per gene level cluster was calculated and the proportion of the respective gene within the according macrolide, tetracycline, sulphonamide and aminoglycoside class level cluster was calculated (Table S3).

Conclusions

We investigated risk factors for AMR in European turkey farms using three different AMR detection methods. Positive AMU-AMR associations were detected for both genotypic and phenotypic AMR: beta-lactam and colistin (metagenomic sequencing) and aminopenicillin and fluoroquinolone (MIC). No robust AMU-AMR association was detected by analyzing qPCR targets. No evidence was found that lower biosecurity increases AMR abundance. We showed AMR genes encoding for some antimicrobial classes were abundant in faeces despite low prevalence of phenotypic resistance in *E. coli* isolates. Since different AMR detection methods provide information on different aspects of AMR, the choice depends on availability of resources and research questions. We have shown that using multiple complementary AMR detection methods adds insights into AMU-AMR associations at turkey farms.

Supplementary Materials

The following are available online at www.mdpi.com/xxx/s1, Part A: Figure S1: Distribution of the 60 turkey farms across three countries; Figure S2: Antimicrobial usage in 60 farms in three countries, expressed as the sum of treatment incidence (TI); Figure S3: Abundance of antimicrobial resistance (AMR) genes detected by metagenomics per farm, expressed as fragments per kilobase reference per million bacterial fragments (FPKM); Figure S4: Total abundance of antimicrobial resistance (AMR) genes detected by metagenomics per country, expressed as the sum of fragments per kilobase reference per million bacterial fragments (FPKM); Figure S5: Correlations between the abundance of *ermB*, *tetW*, *sul2* and *aph3'-III* genes detected by metagenomics and those genes detected by qPCR; Table S1: Univariate associations between antimicrobial usage (AMU), technical farm characteristics, biosecurity measures of the turkey farms and the median relative faecal abundance of *ermB*, *tetW*, *sul2* and *aph3'-III* per farm; Table S2. Univariate associations between antimicrobial usage (AMU), characteristics, biosecurity measures of the turkey farms and the occurrence of *E. coli* isolates from turkey faeces resistant to ampicillin, tetracycline and ciprofloxacin; Table S3: Ten most abundant antimicrobial resistance (AMR) genes in turkey faeces quantified by metagenomics and their proportion within the macrolide, tetracycline, sulphonamide and aminoglycoside class clusters; PartB: Selected biosecurity check questions from the questionnaire used in risk factor analyses.

Author Contributions

All authors have read and agreed to the published version of the manuscript. Conceptualization, J.A.W., D.H, D.M, H.S, L.A.M.S and L.V.G.; methodology, D.M., H.S., L.A.M.S, and L.V.G.; software, D.Y., L.V.G.; formal analysis, M.H, D.Y.; investigation, P.M., K.W., C.C., G.M; resources, P.M., K.W., C.C., G.M; data curation, D.Y., P.J., P.M., M.S., H.S.; writing—original draft preparation, M.H.; writing—review and editing, all authors; visualization, M.H.; supervision, D.M., L.A.M.S., and L.V.G .; project administration, J.A.W.; funding acquisition, J.D., F.M.A., T.B., P.S., B.G.Z., D.Z., J.A.W., D.H., and D.M.

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Chapter 6

Conflicts of Interest

The authors declare no conflicts of interest. Author M.H was also employed by the Ministry of Health, Labour and Welfare, Japan during the time of the analyses and has no conflicts of interest to declare.

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Chapter 7

General Discussion

Main findings in this thesis

As part of the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project (http://www.effort-against-amr.eu/, accessed on 17 August 2021), the studies in this thesis aimed to provide a comprehensive overview of antimicrobial resistance (AMR) in animals, occupationally exposed humans (farmers, slaughterhouse workers) and the environment in Europe. In our studies, a large number of samples were collected from various sources in nine European countries. In the samples, AMR was quantified by three methodologies (real-time PCR (qPCR), next-generation sequencing (NGS), assessment of the minimum inhibitory concentration-value (MIC-value)). NGS was applied on pooled faecal samples which consisted of 25 individual samples per farm. qPCR was applied on these pooled samples, as well as on a sample of 5-7 individual samples out of the 25. Correlations between individual/median-individual/average-individual qPCR data, pooled qPCR data and pooled metagenomic data were assessed. Risk factor analyses were performed using both metagenomic and qPCR data for pigs, broilers, veal calves and turkeys to determine the potential farm characteristics related to farm animal AMR. In addition, the main factors contributing to AMR variability (between countries, between farms, within farm per country) were evaluated using individual qPCR data.

Overall, this thesis showed that AMR measured by both metagenomic and qPCR analysis of faecal samples varied highly between countries and farms in pigs, and within farms in broilers, veal calves and turkeys. The between-country and between-farm AMR variation clearly differs for different animal species and is associated with farm biosecurity, and differences in national antimicrobial use (AMU) as well as on-farm AMU. Comparison of pooled metagenomic data with pooled qPCR data for a limited number of genetic targets showed that qPCR with pooled data can be considered as an alternative to NGS for quantification of a selection of AMR genes in large population studies.

AMU and farm biosecurity measures were identified as the main determinants of AMR quantified by qPCR among multiple farm characteristics. However, it appeared that these factors only explained a limited part of the observed total variation in AMR, probably in part due to measurement error in these explanatory variables. In addition, there are likely unidentified/unstudied determinants (e.g. historical AMU, farm management factors) that need to be evaluated and considered in future studies. Furthermore, we found that average AMR abundance in livestock workers' stool samples was significantly higher if AMR levels in their working environments were higher (farmers > slaughterhouse workers > control subjects). A few topics related to the abovementioned results will be discussed further in this chapter.

Table 1. Main results of the association between AMR and AMU in pigs, broilers, veal calves and turkeys.

Animal species	Corresponding AMR vs AMU			
	Individual qPCR* (p<0.05)	**Average-individual qPCR** (p<0.05)**	**Pooled qPCR*** (p<0.05)**	**Pooled Metagenome*** (p<0.05)**
Pigs	**MLS use in fattener period and *ermB*, (chapter 4)** Tetracycline use in suckler period and *tetW*, (chapter 4) **Total AMU in fattener period and *aph(3')-III/sul2*, (chapter 4)**	**MLS use in fattener period/200 days and *ermB*** Tetracycline use in 200 days and *tetW* **Total AMU in fattener period and *aph(3')-III*/sul2**	**MLS use in fattener period and *ermB*, (chapter 2)** Trim & sulfa use (200 days) and *sul2* (chapter 2) **Total AMU in fattener period and *aph(3')-III/sul2/tetW*, (chapter 2)**	**MLS use in suckler/weaner/fattener period/200 days and MLS resistance, (chapter 5)** Trim & sulfa use in fattener period and sulfonamides resistance, (chapter 2) **Total AMU in fattener period and aminoglycosides resistance, (chapter 5)**
Broilers	**MLS use and *ermB*, (chapter 4)** **Total AMU and *tetW*, (chapter 4)**	**MLS use and *ermB*** **Total AMU and *tetW***	**MLS use and *ermB*, (chapter 2)** Aminoglycoside use and *aph(3')-III*, (chapter 2) Trim & sulfa use and *sul2* (chapter 2) Tetracycline use and *tetW*, (chapter 2)	**MLS use and MLS resistance, (chapter 5)** Aminoglycoside use and aminoglycosides resistance, (chapter 5) Tetracycline use and tetracyclines resistance, (chapter 5)
Veal calves	Trim & sulfa use and *sul2* (chapter 3)	Trim & sulfa use and *sul2*	n.a.	NS
Turkeys	Trim & sulfa use and sul2, (chapter 6)	Total AMU and *ermB*	n.a.	Aminoglycoside use and aminoglycosides resistance, (chapter 6)

AMR is characterized by using individual qPCR results, the average of the 5-7 qPCR results, the qPCR results of 25 pooled samples and the metagenomic results from the same 25 pooled samples. Only the significant (p<0.05) corresponding AMU-AMR associations for four ARG targets/AMR classes are shown. * Mixed model for qPCR data including all individual samples per farm. ** Meta-analysis including the average level of individual samples per farm. *** Meta-analysis including one pooled qPCR/metagenomic data per farm. n.a.: Data was not available; NS: No significant association was observed. In bold: Associations found by both qPCR and metagenomic analysis. MLS: Macrolide + lincosamide + spectinomycin. Trim & sulfa: Trimethoprim/sulfonamide. Total AMU: Sum of all antimicrobials used in group treatment.

Agreement between associations between AMR and determinants, based on qPCR and metagenomic data

As mentioned, associations between AMU and AMR were determined by using three types of AMR data:

- qPCR data on DNA isolated from pooled and 5-7 individual faecal samples per farm;
- metagenomic data on DNA isolated from 25 pooled faecal samples per farm;
- MIC data determined for one randomly isolated *E. coli* from ten individual samples collected per farm.

From the comparison of results in these AMU-AMR association analyses (Table 1) (MIC data not shown), we found that in many cases similar associations were observed using qPCR data and metagenomic data, especially between pooled qPCR data and metagenomic data. However, this was not the case when we compared associations observed for individual/average-individual qPCR data and metagenomic data. The fact that more and generally stronger (i.e. estimates were larger and p values were lower) associations are observed for the pooled qPCR data than individual qPCR data clearly follows theoretical expectations based on the fact that the more samples are used to characterize farm level AMR, the more reliable the AMR estimate. It is thus hypothesized that misclassification in AMR leads to weaker associations when fewer samples are taken. By comparing the risk factor analysis results of AMR and herd characteristics and biosecurity measures (see Appendix), we found similar or less pronounced patterns as for AMU. The less pronounced pattern may be due to the intricacies of the questions related to farm characteristics in our questionnaire. Several interesting relations were picked up, and more details will be further described.

For herd characteristics (see Appendix), we surprisingly found that when other animals were present at the farm abundance of *aph(3')-III* in veal calves (Chapter 3) and of *tetW* in turkeys was less abundant (Chapter 6). This contrasts with previous findings by Dorado-García et al., who reported that the presence of free-ranging farm cats on veal calf farms was significantly associated with a higher prevalence of livestock-associated-methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in veal calves.[1] One possible explanation for this is that the 'other animals' we discussed in our study included not only companion animals but also other farm animals (e.g. donkeys, sheep), which may not be free-ranging. In addition, another possible explanation is that the spread of ARGs in farms may vary for different ARG targets or strains dependent on the specific transmission routes.[2, 3] In any case, all these results indicate that the presence of other animals may be related to the dissemination of AMR on the farm. In the future, more specific research that encompasses the detailed information (e.g. species, numbers, free-ranging status) of other animals present on the farm will be necessary.

For farm biosecurity (see Appendix), we found a negative association between internal biosecurity and the AMR abundance in broilers (Chapter 4). However, in the other three animal species (pigs, veal calves and turkeys), we generally found a positive association between biosecurity level and AMR abundance. For example, we observed a positive association between AMR abundance with internal biosecurity measure (cleaning and disinfection) in veal calves (Chapter 3) and pigs (Chapter 4). In addition, in Chapter 6, we found that turkey farms with higher external biosecurity levels (e.g. staff had less contact with other birds than turkeys) showed a higher relative abundance of ARGs (*ermB*, *tetW* and *aph(3')-III*) quantified by qPCR. These different results in comparison among species may point to an intrinsic limitation of the studies in this thesis. The design of these studies is cross-sectional implicating that both the determinant and AMR are measured at one point at a time. As a result, it is not clear whether exposure to the determinant preceded a change in AMR making causal inferences impossible. Furthermore, determinants explored in this study may be correlated with (unmeasured) causally related farm management characteristics. In the future, appropriately designed longitudinal studies are needed and in particular intervention studies can be well-suited to obtain evidence of the causality between biosecurity measures and AMR abundance in farm animals.

The question remains which factors may explain the differences in outcomes in our studies for associations between different proxies of AMR and AMU and other determinants. With regard to the associations with other determinants, measurement errors in the explanatory variables may have resulted in attenuation of associations with farm characteristics, but the measurement error in independent variables would have played a role in all analyses, on the basis of qPCR, metagenomic analyses or MIC as an endpoint. Therefore, measurement error in the determinants does not seem to be a likely explanation for differences observed within this study. Several other explanations seem plausible as well:

- One explanation is that the qPCR method was limited to a few selected specific antimicrobial resistance genes (ARGs) which could not capture all genetic variation within a particular class of AMR genes.
- In addition, analytical differences (LOD, sensitivity, etc.) and the different sampling processes of individual qPCR data and pooled metagenomic data may also have an impact on the results in risk factor analysis.

We will discuss these issues further in the next section.

Analytical issues: the correlation between average-individual qPCR data and pooled metagenomic data

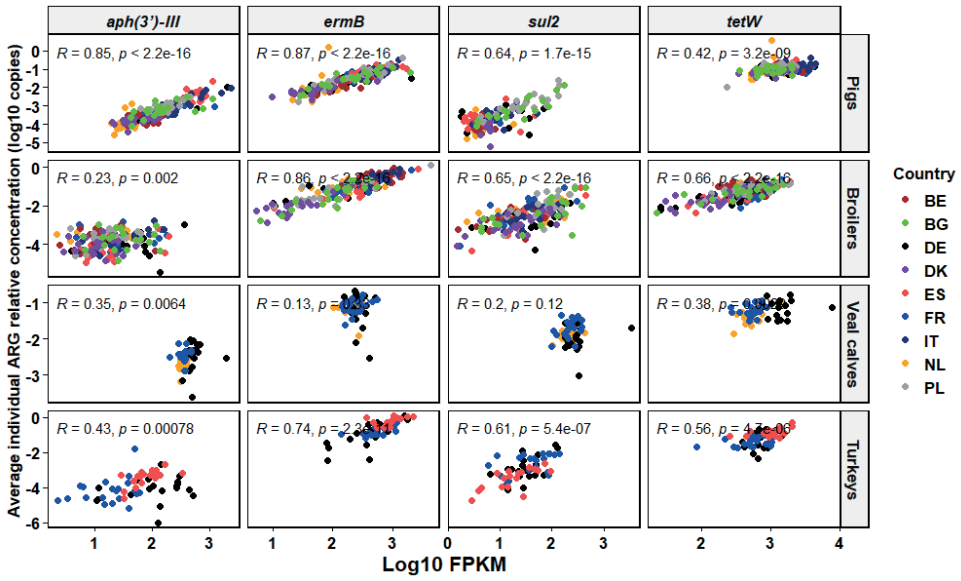

Figure 1. Correlation of average individual qPCR samples and pooled samples analysed using metagenomic analysis in pigs, broilers, veal calves and turkeys. AMR: The relative abundance of *aph(3')-III, ermB, sul2* and *tetW*. The average of 5-7 individual qPCR samples was calculated before correlation analysis.

We observed a moderate-to-high correlation between average-individual qPCR data and pooled metagenomic data in pigs, broilers, veal calves and turkeys (Figure 1). For the individual qPCR data, 5-7 individual faecal samples were only a part of the pooled sample that was analysed for metagenomics. It can be expected that ARG abundance from individual qPCR samples deviates from the ARG abundance of pooled on-farm samples. In addition, 5-7 individual samples per farm may not have been enough (at least for broilers, veal calves and turkeys) to cover the complete variation of 25 individual samples or represent AMR at the level of the entire farm. Furthermore, the correlation between pooled qPCR data and pooled metagenomic data was considerably higher (Chapter 2), as expected according to exposure misclassification theory.[4] In addition, in veal calves, the relatively low correlation between average-individual qPCR data and pooled metagenomic data may be a result of the limited contrast in ARG abundance.

Sampling strategy: pooled samples versus individual samples

The different sampling methods may lead to different variation structures in the ARG abundance per farm. Based on a higher number, it may lead to a more stable estimate of average AMR gene abundance. Although technically feasible, it was too costly to analyse all 25 samples per farm by qPCR.

In general, several studies have shown that collecting 5 samples per subgroup for pooling can obtain stable results,[8-10] and pooling has been proven to obtain high similarity and repeatability of outcomes in the previous study with the same dataset[11] through the triple sampling repeatability experiment. In addition, in Chapter 2, we found the relative ARG abundance between pooled qPCR data and pooled metagenomic data in both pigs and broilers was highly correlated. Therefore, combining the findings of previous studies[8-11] and results from this thesis when using aggregated data, it seems that pooling 5-25 samples for each subgroup (e.g. farms) is sufficient to maintain the stability and reproducibility of results. Furthermore, this high correlation between pooled qPCR data and pooled metagenomic data indicates that pooled qPCR data can also represent the overall level of the targeted ARGs in one farm as precisely as pooled metagenomic data. Therefore, qPCR with sufficient aggregated samples can be an alternative and more cost-effective approach for the quantitative analysis of specific ARG targets in future population studies.

Based on the comparison of individual and pooled sampling procedures, we found a very stable estimate of average AMR per farm when using both on-farm pooled qPCR data and metagenomic data, but pooled samples cannot give an insight into the between-farm variation of AMR relative to the within-farm variation. Individual qPCR data allows us to assess within-farm variation and derive estimates of country, between and within farm variance components. Insight into these variance components may help us explain some of our observations, and may also help optimize future studies.

Table 2. Geographical (between countries) and between-farm and within-farm variation of faeces AMR in pigs, broilers, veal calves and turkeys.

Animal species	Sample sizes*	Variables	Degrees of freedom				Variance components				Variance component (%)				Standard deviation			
			aph(3')-III	*ermB*	*sul2*	*tetW*	*aph(3')-III*	*ermB*	*sul2*	*tetW*	*aph(3')-III*	*ermB*	*sul2*	*tetW*	*aph(3')-III*	*ermB*	*sul2*	*tetW*
Pigs	**7*20*9**	**Between-country**	7.50	6.96	7.36	5.00	0.20	0.12	0.28	0.01	**53.83**	36.33	**42.40**	10.63	**0.44**	**0.34**	**0.53**	0.09
		Between-farm	144.43	159.63	121.98	138.12	0.12	0.16	0.20	0.04	32.00	**50.94**	30.00	**50.59**	0.34	0.40	0.44	**0.20**
		Within-farm	879.33	1044.03	835.90	1047.14	0.05	0.04	0.18	0.03	14.17	12.73	27.6	38.78	0.23	0.20	0.43	0.17
Broilers	**5*20*9**	**Between-country**	4.99	7.17	4.35	5.83	0.04	0.19	0.05	0.04	6.93	38.00	6.78	11.83	0.20	0.43	0.23	0.19
		Between-farm	52.47	134.50	89.46	80.07	0.12	0.19	0.28	0.08	20.82	**38.00**	35.14	27.96	0.34	**0.43**	0.53	0.29
		Within-farm	646.00	697.03	638.74	693.20	0.41	0.12	0.47	0.18	**72.25**	24.00	**58.07**	**60.22**	**0.64**	0.34	**0.68**	**0.42**
Veal calves	**7*20*3**	**Between-country**	0.55	NA	1.51	1.30	0.00	0.00	0.02	0.01	3.01	0.00	11.53	7.91	0.07	0.00	0.14	0.09
		Between-farm	36.48	49.34	27.32	3.16	0.06	0.09	0.04	0.03	43.15	**62.31**	24.40	28.43	0.25	0.31	0.20	0.17
		Within-farm	270.46	340.03	312.31	345.15	0.08	0.06	0.10	0.07	**53.84**	37.69	**64.07**	**63.66**	0.28	0.24	0.32	0.26
Turkeys	**5*20*3**	**Between-country**	1.59	1.65	1.56	1.74	0.15	0.10	0.14	0.08	16.94	27.42	15.43	27.50	0.39	0.32	0.38	0.28
		Between-farm	24.17	48.08	23.82	35.37	0.24	0.19	0.25	0.09	27.24	**50.63**	27.03	32.10	0.49	0.44	0.50	0.30
		Within-farm	191.06	225.07	194.01	227.23	0.49	0.08	0.54	0.12	**55.83**	21.95	**57.54**	**40.39**	0.70	0.29	0.73	0.34

* n samples * n farms * n countries. All values are rounded at two digits behind the decimal point.

Variance component analysis

In a separate paper from the EFFORT group that used MIC data from multiple animal species,[12] no association was found between AMU (average treatment incidence at farm level stratified per country) and AMR (aggregated at country level) in all animal species, including turkeys. However, using the same MIC data for turkeys in this thesis (chapter 6), we found a significant positive association between farm-level ampicillin use and ciprofloxacin resistance. The first study only considered country effects, ignoring between-farm variation within a country. The study in Chapter 6 used a mixed model to account for both country and farm differences in AMR. This difference in the outcome of these two analyses, using basically the same data, shows that different results are obtained by making use of the between-farm variation in AMR or not. Analyzing potential variation sources (country (geographical variation), between- and within-farm variation) may help explain such observations. Therefore, a variance component analysis (VCA) was performed to evaluate AMR variations in individual qPCR data of pigs, broilers, veal calves and turkeys. First, we evaluated the results of the VCA null model (AMR ~ country + farm). Subsequently, we adjusted farm characteristics (AMU, herd characteristics and biosecurity measures) into the VCA null model to explore which variation components (country or farm or both country and farm) changed when including the determinants of AMR.

After adjusting AMU and other farm characteristics in the VCA null model, we found an obvious change in variation components, especially both the geographical (between country) and between-farm variation. Their contributions to AMR variation decreased highly, which mainly shifted to AMU and biosecurity measures (Chapter 2, Chapter 4). For example in the model of *ermB* in turkeys (Chapter 2), the between-country variance changed from 0.12 to 0.08 (33.33%) and the between-farm variance changed from 0.18 to 0.13 (27.8%) after the inclusion of AMU and biosecurity measures in the models. This indicates that both sources of variance contributed to the relation with AMU and biosecurity measures. Similar changes were also observed in the VCA results of three other farm animal species (pigs, broilers and veal calves). Again, this indicates that both geographical and between-farm variation in AMR contributed to associations between AMR with AMU and biosecurity measures. Although the small sample size of veal calves and turkeys may have reduced the statistical power of the analysis, the VCA results provide evidence that both sources of variation (country and farm) contributed to associations with AMU and farm biosecurity.

Among four animal species, within-farm variation generally showed the lowest contribution to the total AMR variation in pigs, but the highest in broilers, veal calves and turkeys (Table 4). This is in line with the correlation results, in which we found a high correlation ($\rho>0.7$, $p<0.01$) between individual qPCR data and pooled

metagenomic data for two ARG targets (*aph(3')-III* and *ermB*) only in pigs. This may also be in line with the comparison of our risk factor analysis results in qPCR data and metagenomic data, where we found much fewer AMU-AMR associations using individual/average-individual qPCR data than using metagenomic data in broilers, but not in pigs. Combining these results, we conclude that 7 individual samples per farm should be sufficient to estimate the farm-level AMR for pigs but not for veal calves, 5 individual samplers may not be sufficient for broilers and turkeys.

After fitting farm characteristics such as AMU and biosecurity, the main source of AMR variation remains unexplained geographical, between-farm and within-farm variation. For geographical and between-farm variation, we speculate that some unmeasured factors may also have contributed to the AMR variation, e.g., historical AMU levels in the farm, different farming practices, management factors, etc. Studies have shown that the impact of historical on-farm AMU is long-lasting, sometimes for several years.[13-16] For within-farm variation, this can only be explained when within-farm determinants for AMR at the stable level are available, which was not the case in our study. For example, due to the difficulty of AMU quantification, we have no individual animal AMU data. This misclassification of outcomes and exposure may have caused attenuation biases in our analysis. Therefore, we hope that future studies can include more and better-measured farm factors, so as to explain more AMR variation.

One Health approach

In chapter 2 of this thesis, we found that AMR abundance in the faeces of livestock workers appeared to be related to their working environment and was higher than the AMR abundance of control subjects (with no livestock exposure). We speculate that the spread of resistant bacteria or ARGs between animals and humans may contribute to this difference.[17, 18] For this mode of transmission, it is necessary to reduce workers' exposure to livestock pollutants and bacteria. Using appropriate hygiene measures and personal protective equipment (PPE) could be a possible preventive measure for livestock workers, although reduction of AMR in farm animals is a priority.

Regarding PPE, it reminds me of the COVID-19 epidemic that is still spreading around the world. The outbreak of COVID-19 was first reported in Wuhan, China in early December 2019.[19, 20] Subsequently, an investigation

on mink farms infected with the SARS-CoV-2 virus in 2020 in the Netherlands showed that new positive cases were diagnosed in the relevant farmworkers before and after the outbreak in minks.[21]

The outbreak and spread of the COVID-19 virus between animals and humans are similar to the AMR transmission in the entire ecosystem that we discussed in this thesis. This emphasizes the imperative of a One Health approach, through which the emergence and spread of ARGs and pathogens in humans, animals and the environment can be monitored, and the crucial information can be shared globally promptly. Only in this way can the government and relevant international organizations take measures in the initial stages.

Prospects and epilogue

It's well known that antimicrobials protect humans and animals from infectious pathogens. However, the emergence and development of AMR have put antimicrobials in a situation of whether to use them or not. As epidemiologists, we cannot condone or stand by and watch the uncontrolled development of AMR. It is our responsibility to identify potential risk factors of AMR, find effective and feasible measures to control AMR, and thereby help restore the effectiveness of antimicrobials in clinical treatment.

This thesis establishes a positive association between AMU and AMR in farm animals for different animal species. The result that AMU contributed the most to AMR variation of all farm characteristics in pigs and broilers further emphasizes the importance of controlling livestock AMU. The veterinary AMU reductions in Europe will certainly make an excellent contribution to the control of AMR, and I hope this will attract more and more countries and regions to gradually formulate policies and develop monitoring systems for the restriction of AMU and AMR in animals.

In addition to AMU, we also found certain herd characteristics and biosecurity measures have an impact on AMR levels, especially in veal calves and turkeys. This means that optimizing these aspects may represent additional measures for livestock workers to control AMR. For example, weaning piglets at an appropriate age and choosing appropriate disinfection products for stable cleaning. However, the various results obtained in different farm animals indicate that caution is needed when drawing conclusions about the impact and causality of the farm biosecurity measures. More in-depth, longitudinal and specific intervention experiments related to farm biosecurity and AMR should be carried out in the future.

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Appendix

Appendix Table 1. Main results of the association between AMR and herd characteristics in pigs, broilers, veal calves and turkeys.

Animal species	AMR vs herd characteristics			
	Individual qPCR* (p<0.05)	**Average-individual qPCR** (p<0.05)**	**Pooled qPCR*** (p<0.05)**	**Pooled Metagenome*** (p<0.05)**
Pigs	Negative between weaning age and *aph(3')-III*, (chapter 4) Positive between current number of fatteners and *aph(3')-III*, (chapter 4)	Negative between weaning age and *aph(3')-III* Negative between current age of fatteners and *aph(3')-III/ermB* Positive between average number of fatteners per year and *aph(3')-III* Negative between growing-finishing duration and *aph(3')-III*	Negative between presence of other animals and *ermB*, (chapter 2) Negative between growing-finishing duration and *ermB*, (chapter 2)	Negative between average number of fatteners per year and sulfonamides resistance, (chapter 5) Negative between current number of fatteners and sulfonamides resistance, (chapter 5)
Broilers	Positive between number of farmworkers and *ermB*, (chapter 4) Positive between weight of broilers at set-up and *ermB*, (chapter 4) **Positive between average number of rounds per year and *sul2*, (chapter 4)**	Positive between number of farmworkers and *ermB/tetW* Positive between weight of broilers at set-up and *ermB/tetW* **Positive between average number of rounds per year and *sul2***	**Positive between average number of rounds per year and *sul2*, (chapter 2)**	**Positive between average number of rounds per year and sulfonamides resistance, (chapter 5)**
Veal calves	Negative between arrival weight of calves and *aph(3')-III/ermB*, (chapter 3) Negative between presence of other animals and *aph(3')-II*I, (chapter 3)	Negative between arrival weight of calves and *aph(3')-III/ermB*	n.a. n.a.	NS NS
Turkeys	Negative between sampling age of turkeys and *ermB*, (chapter 6) Positive between other livestock present and *sul2*, (chapter 6) Negative between other livestock present and *tetW*, (chapter 6)		n.a. n.a. n.a.	NS NS NS

AMR is characterized by using individual qPCR results, the average of the 5-7 qPCR results, the qPCR results of the pooled samples and the metagenomic results from the pooled sample. Only the significant (p<0.05) associations for four ARG targets/AMR classes are shown (n.a.: Data was not available, NS: No significant association was observed). In bold: Associations found by both qPCR and metagenomic analysis. * Mixed model for qPCR data including all individual samples per farm. ** Meta-analysis including the average level of individual samples per farm. *** Meta-analysis including one pooled qPCR/metagenomic data per farm.

Appendix Table 2. Main results of the association between AMR and biosecurity measures in pigs, broilers, veal calves and turkeys.

Animal species	AMR vs biosecurity			
	Individual qPCR* (p<0.05)	Average-individual qPCR** (p<0.05)	Pooled qPCR*** (p<0.05)	Pooled Metagenome*** (p<0.05)
Pigs	**IB, positive between cleaning & disinfection and *ermB*, (chapter 4)** IB, positive between farrowing & suckling period and *aph(3')-III*, (chapter 4)	**IB, positive between cleaning & disinfection and *ermB*** EB, negative between environment region and *aph(3')-III*	**IB, positive between cleaning & disinfection and *ermB*, (chapter 2)** IB, positive between farrowing & suckling period and *aph(3')-III*, (chapter 2) **IB: positive between compartment measures & equipment use and *ermB*, (chapter 2)**	**IB, positive between cleaning & disinfection and MLS resistance, (chapter 5)** **IB: positive between compartment measures & equipment use and MLS resistance, (chapter 5)**
Broilers	**IB, negative between disease management and *tetW*, (chapter 4)** IB, negative between cleaning & disinfection and *tetW*, (chapter 4) EB, positive between removal of manure and *aph(3')-III/ermB/tetW*, (chapter 4)	**IB, negative between disease management and *tetW*** IB, negative between cleaning & disinfection and *aph(3')-III* EB, positive between removal of manure and *aph(3')-III/ermB/tetW* **IB: negative between compartment measures & equipment use and *tetW*** EB, positive between visitors and *aph(3')-III*	**IB, negative between disease management and *tetW*, (chapter 2)** **IB: negative between compartment measures & equipment use and *tetW*, (chapter 2)**	**IB, negative between disease management and tetW, (chapter 5)** EB, negative between visiting and sulfonamides/tetracyclines resistance, (chapter 5) **IB: negative between compartment measures & equipment use and tetracyclines resistance, (chapter 5)**
Veal calves	IB, positive between cleaning & disinfection and *ermB/tetW*, (chapter 3)		n.a.	NS
Turkeys	**EB, negative between visiting outside and *ermB/tetW*, (chapter 6)** EB, positive between bird/vermin proof placed and *aph(3')-III*, (chapter 6) EB, negative between staff keeps turkeys/birds at home and *aph(3')-III*, (chapter 6)	**EB, negative between visiting outside and *ermB/tetW*** 	n.a. n.a. n.a.	**EB, negative between visiting outside and MLS/tetracyclines resistance, (chapter 6)** EB, positive between presence of other farms in 500m and aminoglycosides resistance, (chapter 6) EB, negative between clean/dirty area division and aminoglycosides resistance, (chapter 6)

AMR is characterized by using individual qPCR results, the average of the 5-7 qPCR results, the qPCR results of the pooled samples and the metagenomic results from the pooled sample. Only the significant (p<0.05) associations for four ARG targets/AMR classes are shown (n.a.: Data was not available, NS: No significant association was observed). In bold: Associations found by both qPCR and metagenomic analysis. * Mixed model for qPCR data including all individual samples per farm. ** Meta-analysis including the average level of individual samples per farm. *** Meta-analysis including one pooled qPCR/metagenomic data per farm. IB: Internal biosecurity. EB: External biosecurity. MLS: Macrolide + lincosamide + spectinomycin.

Appendices

English Summary

Approximately 75 years ago benzylpenicillin was produced, purified and for the first time clinically used against infection. Antimicrobials, which enabled the prevention and treatment of bacterial infections, are one of the greatest inventions of the 20 century. With the mass production and sale of various classes of antimicrobials, a growing number of antimicrobial classes have been used for the clinical treatment of humans, companion animals and livestock. Moreover, as a result of the reported growth-promoting effects, large quantities of antimicrobials were administered to farm animals for commercial benefit—a practice that has been banned in the European Union since 2006.

There is an old Chinese saying, '全则必缺，极则必反', which means that when going to extremes, things will inevitably develop in the opposite direction at a certain time point. As a result of massive misuse and unrestricted use of antimicrobials in humans, animals and the environment, antimicrobials can become less effective or even ineffective in clinical treatment, based on a phenomenon known as antimicrobial resistance (AMR), which has caused the death of thousands of people as a result of treatment failure.

As a source of food production, farm animals may directly (e.g. food intake, contact) or indirectly (e.g. through the environment) transmit antimicrobial resistance genes (ARGs) to humans by clonal spread of bacteria or horizontal gene transfer (HGT) of ARGs. This means that AMR in farm animals is a challenging public health issue. Recent studies have shown that risk factors of farm animal AMR abundance are not only antimicrobial use (AMU) but also herd characteristics (e.g. age, weight, farm size) and biosecurity measures (e.g. cleaning and disinfection, access by outsiders). In order to better understand the ecology of AMR in humans, animals and the environment in Europe, and to explore potentially related risk factors of AMR, the European Union (EU) funded the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project in 2014 (http://www.effort-against-amr.eu/). In the EFFORT project, AMR abundances of various sources, animals species and humans from nine European countries were quantified by using a variety of quantification approaches (next-generation sequencing (NGS), real-time PCR (qPCR) and minimum inhibitory concentration (MIC)). Farm-related characteristics, such as AMU and farm biosecurity were collected through standard questionnaires. With such a large number of high-quality samples, substantial research results have been reported and published, including studies in this thesis.

The first chapter of this thesis introduces the definition and background of AMU and AMR in humans and animals in recent years. In addition, this chapter presents the phenotypic and genetic methods of AMR

quantification, as well as the determinants that may affect AMR, such as AMU, biosecurity, and other farm characteristics. The potential relationship of AMR between humans, animals, and the environment is briefly introduced. The monitoring and control of AMU and AMR through the One Health approach is mentioned.

In Chapter 2, a large number of samples from farm animals, livestock environments, companion animals, and humans collected from nine European countries are quantified by qPCR assays. ARG abundance varies highly across animal species and sample sources. In pigs and broilers, we observe a decreasing trend of ARG abundance along the production chain, and a positive association between AMR and corresponding AMU is observed. ARG targets quantified by qPCR and shotgun metagenomic sequencing in pooled faecal samples are highly correlated. In veal calves and turkeys, the geographical and between-farm variation can be partially explained by AMU (1.57%-8.68%) and farm biosecurity (6.23%-21.08%). Occupational livestock AMR exposure shows a relation to ARG abundance in faeces of livestock workers.

Chapter 3 and Chapter 4 describe the identification of potentially relevant AMR determinants in veal calves, pigs and broilers using epidemiological approaches. Chapter 3 focuses on veal calf farms from three European countries (France, Germany and the Netherlands), in which a significant positive association is found between *sul2* abundance in calf faeces and trimethoprim/sulfonamide use. In addition, veal calf farms that only used water for stable cleaning show lower relative *ermB* and *tetW* abundance than the other veal calf farms. Chapter 4 focuses on pig and broiler farms from nine European countries. A positive association is found between corresponding ARG abundance and AMU in both pigs and broilers. The biosecurity measure 'cleaning and disinfection' shows a positive association with relative *sul2* abundance in pigs, while on the contrary, it is negatively associated with relative *tetW* abundance in broilers. In both pigs and broilers, ARGs quantified by qPCR (using the median of individual samples) and pooled metagenomic data show a moderate correlation. In addition to the not completely reproduced risk factor analysis results of these two datasets, we speculate that a sample size of 5-7 individual faecal samples per farm may not cover sufficient AMR variation compared to pooled samples (25 mixed faecal samples). The geographical and between-farm variation of AMR in pigs and broilers can be partly (0.45%-14.23%) explained by farm-level AMU.

In Chapter 5, an updated AMR reference database and alternative bioinformatics/computational approaches are used for AMR assessment. In pigs and broilers, the updated metagenomic data highly correlated with previous metagenomic data from the same pooled faecal samples. Previously reported statistically significant associations between AMR (using the previous metagenomic data) and farm characteristics (e.g. AMU, biosecurity) could be

confirmed but not completely reproduced using the updated metagenomic data, which is mainly due to the updated reference databases and metagenomic alignment methods. Using the updated bioinformatics workflow without Bayesian multiplicative zero replacement is recommended to use.

Chapter 6 describes three AMR quantification methods (qPCR, NGS and MIC) in turkey faecal samples from three European countries (France, Germany and Spain). We find that beta-lactam and colistin resistance (metagenomic data) and ampicillin and ciprofloxacin resistance (MIC data) are associated with corresponding AMU, while no robust associations between AMU and AMR quantified by qPCR were observed. Interestingly, turkey farms with poor biosecurity measures in turn show lower AMR abundance than other farms. In the future, more specific and in-depth research on AMR and farm biosecurity in turkeys is necessary.

Finally, chapter 7 summarizes all the findings from previous chapters and compares them with related published research. qPCR with pooled data can be considered as an alternative to NGS for AMR quantification in large population studies. There are more AMU-AMR associations found in pooled data of 25 samples per farm than individual data of 5/7 samples per farm, which may emphasize the need to pay attention to the appropriate sample size in future epidemiological studies. In addition, after comparing the risk factor analysis and VCA results of individual qPCR data and metagenomic data among four animal species, we conclude that 7 individual samples per farm should be sufficient to estimate the farm-level AMR for pigs but not for veal calves, 5 individual samples may not be sufficient for broilers and turkeys. Furthermore, although AMU and biosecurity measures are identified as the main determinant among multiple farm characteristics in our study, they can only explain a limited part of the AMR variations. This indicates that there are still unidentified/unstudied determinants that need to be considered in future research.

The findings of this thesis shed light on AMR variations and potential links of AMR between humans, animals and livestock environments. It is recommended to limit AMU levels and implement appropriate biosecurity measures to subsequently restrict farm animal AMR. In the future, more in-depth longitudinal studies of AMR, AMU, and specific biosecurity measures will be needed, in which attention should be paid to the appropriate sample size. With the joint efforts of experts and researchers in various fields around the world, controlling AMR is expected to be achieved through the One Health approach. Ultimately, AMR levels in the natural ecosystem should be reduced to a level that safeguards the future use of antimicrobials in humans and animals.

Nederlandse Samenvatting

Ongeveer 75 jaar geleden werd benzylpenicilline gezuiverd en voor het eerst klinisch gebruikt tegen infecties. Antimicrobiële middelen, die preventie en behandeling van bacteriële infecties mogelijk maakten, zijn één van de grootste uitvindingen van de 20e eeuw. Met de massaproductie en verkoop van verschillende klassen antimicrobiële middelen, is een groeiend aantal antimicrobiële middelen beschikbaar gekomen voor de klinische behandeling van mensen, gezelschapsdieren en vee. Bovendien werden, als gevolg van de gerapporteerde groeibevorderende effecten, grote hoeveelheden antimicrobiële middelen toegediend aan landbouwhuisdieren vanwege commerciële doeleinden - een praktijk die sinds 2006 in de Europese Unie is verboden.

Er is een oud Chinees gezegde, '全则必缺，极则必反', wat betekent dat als we tot het uiterste gaan, dingen zich op een bepaald moment onvermijdelijk in de tegenovergestelde richting zullen ontwikkelen. Het massale misbruik en het onbeperkte gebruik van antimicrobiële stoffen hebben geleid tot een toename van antimicrobiële resistentie (AMR) -niveaus bij bacteriën van mens, dier en milieu. Als gevolg hiervan kunnen antimicrobiële middelen minder effectief of zelfs ineffectief worden in klinische behandelingen. Dit heeft als consequentie de dood van duizenden mensen als gevolg van het falen van de behandeling.

Landbouwhuisdieren kunnen direct (b.v. door consumptie van besmette dierlijke producten of fysiek contact met dieren of dierlijke producten) of indirect (b.v. via het milieu) resistente bacteriën of antimicrobiële resistentiegenen (ARG's) op mensen overbrengen door middel van klonale verspreiding van bacteriën of horizontale genoverdracht (HGT) van ARG's. Dit betekent dat AMR bij landbouwhuisdieren een potentieel volksgezondheidsprobleem is. Recente studies hebben aangetoond dat risicofactoren voor de mate van voorkomen van AMR bij landbouwhuisdieren niet alleen antimicrobieel gebruik (AMU) is, maar ook veehouderijkenmerken (b.v. leeftijd van de dieren, of hun gewicht, of bedrijfsgrootte) en hygiëne en infectiecontrolemaatregelen (b.v. reiniging en desinfectie, toegang door buitenstaanders). Om de ecologie van AMR bij mensen, dieren en het milieu in Europa beter te begrijpen en om potentieel gerelateerde risicofactoren van AMR te onderzoeken, is het Europese onderzoeksproject “Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT)” in 2014 gestart (http://www.effort-against-amr.eu/). In het EFFORT-project werden AMR-niveaus in faecesmonsters van verschillende diersoorten en mensen uit negen Europese landen gekwantificeerd met behulp van verschillende microbiologische methodes (next-generation sequencing (NGS), real-time PCR (qPCR) en minimale remmende concentratie (MIC)). Bedrijfsgerelateerde kenmerken, zoals AMU en hygiënestatus van de boerderij, werden verzameld via vragenlijsten. Met ’dit grote aantal

monsters van hoge kwaliteit zijn substantiële onderzoeksresultaten gerapporteerd en gepubliceerd, inclusief studies in dit proefschrift.

In het eerste hoofdstuk van dit proefschrift wordt de definitie en achtergrond van AMU en AMR onderzoek bij mens en dier in de afgelopen jaren ingeleid. Daarnaast worden in dit hoofdstuk de fenotypische en genetische methoden van AMR-kwantificering beschreven, evenals de determinanten die AMR kunnen beïnvloeden, zoals AMU, hygiënestatus en andere kenmerken van het bedrijf. De mogelijke verspreiding van AMR tussen bacteriën van mens, dier en milieu wordt kort geïntroduceerd. De monitoring en controle van AMU en AMR via de One Health-benadering wordt genoemd.

In Hoofdstuk 2 wordt een groot aantal monsters van landbouwhuisdieren, de veehouderij omgeving, gezelschapsdieren en mensen, verzameld uit negen Europese landen, gekwantificeerd met qPCR-assays. De mate van voorkomen van ARGs varieert sterk tussen diersoorten en soorten monsters. Bij varkens en vleeskuikens zien we een afnemende trend van ARG-concentratie langs de productieketen en wordt een positieve associatie tussen ARG en overeenkomstige AMU waargenomen. Specifieke ARG's gekwantificeerd met qPCR en shotgun-metagenome sequencing in gepoolde fecale monsters zijn sterk gecorreleerd. Bij vleeskalveren en kalkoenen kan de geografische variatie en variatie tussen boerderijen deels worden verklaard door AMU (1,57%-8,68%) en hygiënestatus op de boerderij (6,23%-21,08%). Beroepsmatige blootstelling aan AMR is geassocieerd met ARG-concentratie in ontlasting van veehouders.

Hoofdstuk 3 en Hoofdstuk 4 beschrijven de identificatie van potentieel relevante AMR-determinanten bij vleeskalveren, varkens en vleeskuikens met behulp van epidemiologische analyses. Hoofdstuk 3 richt zich op vleeskalverhouderijen uit drie Europese landen (Frankrijk, Duitsland en Nederland), waar een significant positief verband wordt gevonden tussen de *sul2*-concentratie in kalvermest en het gebruik van trimethoprim/sulfonamide. Daarnaast werd op kalverhouderijen die alleen water gebruiken voor stalreiniging een lagere relatieve *ermB*- en *tetW*-concentratie gemeten dan op andere kalverhouderijen. Hoofdstuk 4 richt zich op varkens- en vleeskuikenbedrijven uit negen Europese landen. Er is een positieve associatie gevonden tussen de ARG-concentratie en het corresponderende AMU bij zowel varkens als vleeskuikens. 'Reiniging en desinfectie' laat een positieve associatie zien met relatieve *sul2*-concentratie bij varkens, terwijl deze juist negatief is geassocieerd met relatieve *tetW*-concentratie bij vleeskuikens. Bij zowel varkens als vleeskuikens vertonen ARG's gekwantificeerd met qPCR en gepoolde metagenome gegevens een matige correlatie.

We denken dat met een steekproefomvang van 5-7 individuele fecale monsters per bedrijf (qPCR) de voorkomende variatie in AMR tussen dieren van een bedrijf niet voldoende tot uiting komt in vergelijking met gepoolde monsters van 25 gemengde fecale monsters (metagenome analyse). De geografische variatie en variatie tussen boerderijen van AMR bij varkens en vleeskuikens kon gedeeltelijk (0,45%-14,23%) worden verklaard door AMU op bedrijfsniveau.

In Hoofdstuk 5 worden een bijgewerkte AMR-referentiedatabase en alternatieve bio-informatica/rekenkundige benaderingen gebruikt voor de metagenome AMR-analyse. Bij varkens en slachtkuikens correleerden de bijgewerkte metagenome gegevens sterk met eerdere gegevens van dezelfde gepoolde fecale monsters. Eerder gerapporteerde statistisch significante associaties tussen AMR (met behulp van de eerdere metagenome gegevens) en boerderijkenmerken (bijv. AMU, hygiëne) konden worden bevestigd, maar niet volledig worden gereproduceerd met behulp van de bijgewerkte metagenome gegevens. Dit was voornamelijk te wijten aan de bijgewerkte referentiedatabases en het gebruik daarvan om ARGs te identificeren.

Hoofdstuk 6 beschrijft drie AMR-kwantificatiemethoden (qPCR, NGS en MIC) in fecale monsters van kalkoenen uit drie Europese landen (Frankrijk, Duitsland en Spanje). We vonden dat bètalactam- en colistine-resistentie (metagenome gegevens) en ampicilline- en ciprofloxacine-resistentie (MIC-gegevens) geassocieerd zijn met overeenkomstig AMU, terwijl er geen robuuste associaties werden waargenomen tussen AMU en AMR, gekwantificeerd door qPCR. Interessant is dat kalkoenbedrijven met slechte hygiënemaatregelen op hun beurt een lagere AMR-concentratie lieten zien dan andere boerderijen. In de toekomst is meer specifiek en diepgaand onderzoek naar AMR en hygiënemaatregelen op kalkoenenhouderijen nodig.

Ten slotte vat hoofdstuk 7 alle bevindingen uit voorgaande hoofdstukken samen en vergelijkt deze met gerelateerd gepubliceerd onderzoek. qPCR met gepoolde gegevens kan worden beschouwd als een alternatief voor NGS voor AMR-kwantificering in grote populatiestudies. Er zijn meer AMU-AMR-associaties gevonden in gepoolde gegevens van 25 monsters per bedrijf dan individuele gegevens van 5 à 7 monsters per bedrijf, wat de noodzaak kan benadrukken om in toekomstige epidemiologische studies aandacht te besteden aan de juiste steekproefomvang. Bovendien concluderen we, na risicofactoranalyse van individuele qPCR-gegevens en metagenome gegevens van DNA van faecesmonsters van vier diersoorten, dat 7 individuele monsters per bedrijf voldoende moeten zijn om de AMR op bedrijfsniveau voor varkens maar niet voor vleeskalveren te schatten, 5 individuele monsters zijn mogelijk niet voldoende voor vleeskuikens en kalkoenen. Bovendien, hoewel AMU en hygiënemaatregelen worden geïdentificeerd als de belangrijkste determinant van meerdere bedrijfskenmerken in

onze studie, kunnen ze slechts een beperkt deel van de AMR-variaties verklaren. Dit geeft aan dat er nog steeds niet-geïdentificeerde/bestudeerde determinanten zijn waarmee in toekomstig onderzoek rekening moet worden gehouden.

De bevindingen van dit proefschrift werpen licht op AMR-variaties en mogelijke verbanden van AMR tussen mens, dier en vee. Het wordt aanbevolen om AMU-gebruik te beperken en passende hygiënemaatregelen te nemen om AMR van landbouwhuisdieren te beperken. In de toekomst zullen meer diepgaande longitudinale studies van AMR, AMU en specifieke hygiënemaatregelen nodig zijn, waarbij aandacht moet worden besteed aan de juiste steekproefomvang. Met de gezamenlijke inspanningen van experts en onderzoekers op verschillende gebieden over de hele wereld, wordt verwacht dat het beheersen van AMR wordt bereikt via de One Health-benadering. Uiteindelijk moeten de AMR-niveaus in het natuurlijke ecosysteem worden teruggebracht tot een niveau dat het toekomstige gebruik van antimicrobiële stoffen bij mens en dier veilig stelt.

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Curriculum Vitae

Dongsheng Yang was born on December 19, 1992, in Xuchang, Henan province, China. After graduating from Heilongjiang Bayi Agricultural University with a bachelor's degree in veterinary medicine in 2014, he went to Jilin University to study for a master's degree in veterinary medicine. From 2016 to 2017, Dongsheng worked as an intern at Agro-product Safety Research Center, Chinese Academy of Inspection and Quarantine, mainly engaged in the detection of animal-derived ingredients in common edible animal products. In October 2017, Dongsheng started pursuing a doctorate degree at the Institute for Risk Assessment Sciences (IRAS) at Utrecht University and producing the work presented in this thesis. During this period, he obtained his second master's degree in Epidemiology at Utrecht University. Dongsheng has been engaged in the Ecology from Farm to Fork of Microbial Drug Resistance and Transmission (EFFORT) project, which aims to investigate the epidemiology of antimicrobial use and resistance in the meat and poultry production chain in Europe.

List of Publications

- **Yang D**, Van Gompel L, Luiken RE, et al. Association of antimicrobial usage with faecal abundance of aph (3')-III, ermB, sul2 and tetW resistance genes in veal calves in three European countries. *International Journal of Antimicrobial Agents*. 2020;56(4):106131.
- Horie M, **Yang D**, Joosten P, et al. Risk Factors for Antimicrobial Resistance in Turkey Farms: A Cross-Sectional Study in Three European Countries. *Antibiotics*. 2021;10(7):820.
- **Yang D,** Heederik DJJ, Mevius DJ, et al. Risk factors for the abundance of antimicrobial resistance genes aph (3′)-III, erm (B), sul2 and tet (W) in pig and broiler faeces in nine European countries. *Journal of Antimicrobial Chemotherapy*. 2022.
- **Yang D**, Heederik DJJ, Scherpenisse P, et al. Antimicrobial resistance genes aph(3')-III, ermB, sul2 and tetW abundance in animal faeces, products, production environments and human faeces in Europe. *Under revision*.
- **Yang D**, Munk P, Van Gompel L, et al. Risk Factors for the antimicrobial resistome in European livestock: An Updated Analysis of Metagenomic Data. *Manuscript in preparation*.
- Munk P, **Yang D**, Röder T, et al. The European Livestock Resistome. *Manuscript in preparation*.