Epidemiological analysis of antimicrobial resistance in the food chain in the metagenomics era

From antimicrobial resistant pathogens to the faecal resistome of humans and livestock

Liese Van Gompel

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Epidemiological analysis of antimicrobial resistance in the food chain in the metagenomics era

From antimicrobial resistant pathogens to the faecal resistome of humans and livestock

Epidemiologische analyse van antimicrobiële resistentie in de voedselketen in het metagenomics tijdperk: van antimicrobieel resistente pathogenen tot aan het humane en dierlijke fecale resistoom

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General introduction

1.1 Antimicrobial resistance: a brief history and its characteristics

Antimicrobial resistance: a natural phenomenon

'Antimicrobial resistance is ancient', D'Costa *et al.* declare in 2011¹ after discovering antimicrobial resistance genes (ARGs) in 30,000 year old Beringian permafrost sediments, and thus proving the existence of bacterial antimicrobial resistanceⁱ (AMR) long before modern medicine could have exerted its selective pressure. Generally speaking, AMR is considered an evolutionary response of microorganisms to the strong selective pressure caused by exposure to antimicrobial compounds.² This Darwinian selection process was initially thought to be the main origin of AMR in 'natural' non-clinical environments.³ Under this scenario, naturally produced antimicrobials (e.g. streptomycin produced by *Streptomyces griseus* or chloromycetin produced by *S. venezuelae*⁴) provide their microbial producers, and those microbes who can withstand the destructive powers of these antimicrobials in their environment, with a competitive advantage. However, research from the past two decades has suggested that some antimicrobials found in natural ecosystems are present at concentrations too low to inhibit bacterial growth, a condition needed for evolutionary selection. In addition, primary functions other than evading antimicrobials properties, such as metabolic activity or signal trafficking, have been described for certain ARGs.⁵ Low concentrations of antimicrobials have been shown to alter microbial transcription patterns, but not necessarily to affect antibiosis.⁶ The primary function of some of the antimicrobials found in these natural ecosystems would then be to serve an intercellular signalling role instead.⁵

i According to the definition from the World Health Organization, *antibiotic* resistance develops when bacteria adapt and grow in the presence of antibiotics.¹⁰⁹ Although also used to indicate resistance to other microorganisms, here, the general term *antimicrobial* resistance (AMR) is used to refer to *bacterial* resistance to antibiotics. For reasons of consistency, the term *antimicrobials* is used to refer to antibiotics.

The emergence of antimicrobial resistance

Although AMR has been present in the environment for aeons, its recent increase in the environment⁷ and especially its emergence in clinically relevant bacteria, is generally considered a man-made situation superimposed on nature.⁸ The use, misuse and overuse of antimicrobials in human medicine and animal husbandry has been acknowledged to be the key driver of AMR in the past 70 years.⁹ It all started with the discovery of the first antimicrobials at the end of the 19th century (Emmerich & Löw: *Pyocyanase*) and the beginning of the 20th century (e.g. Ehrlich, Bertheim & Hata: *Salvarsan*; Fleming: *Penicillin*), followed by the massive production and application of antimicrobials starting in the 1950sⁱⁱ.¹⁰ Not only did antimicrobials bring mankind medical success, they also introduced the problem of acquired antimicrobial resistance.^{8,10} Antimicrobials have been used for various purposes within human and animal health care (curative, prophylactic and metaphylactic usage), in non-clinical settings as animal growth promotor, and for household cleaning purposes. However, growing awareness about AMR is gradually leading to more prudent animal antimicrobial use (AMU), effectuated by banning the use of antimicrobial growth promoters (AGPs) in animals in Europe (2006¹¹), the US (2017¹²) and China (ban of colistin as AGP¹³). In addition, several focused efforts have been conducted towards reducing veterinary AMU and enhancing antimicrobial stewardship, such as those in Denmark¹⁴ and the Netherlands¹⁵.

Acquired antimicrobial resistance and the mobilome

Bacteria can express two types of phenotypic resistance: intrinsic resistance,ⁱⁱⁱ and acquired resistance.¹⁶ Mechanisms underlying AMR are diverse, are encoded by a myriad of different ARGs, and include enzymatic inactivation of antimicrobials, a reduced bacterial intracellular drug accumulation (by increasing efflux or decreasing influx) and the bacterial modification of cellular antimicrobial target sites.¹⁷ Next to the clonal spread of resistant bacteria, two other mechanisms are responsible for the spread of resistance and for (pathogenic) bacteria to acquire ARG determinants: mutation¹⁸ and horizontal gene transfer (HGT).^{19,20} ARGs are

ii The period between the 1950s and the 1970s is considered the so-called golden era of antimicrobial discovery after which only few new antimicrobials are discovered.¹¹⁰

iii Intrinsic resistance can be defined as a trait universally found within the genome of bacterial species, independent of antimicrobial selective pressure, which is not accredited to horizontal gene transfer.¹¹¹

often associated with mobile genetic elements, facilitating HGT (the mobilome, e.g. transposons, integrons, plasmids). HGT is the process in which a bacterial cell acquires ARGs through *conjugation* (cell to cell contact via cell surface pili or adhesins through which plasmids are transferred), *transformation* (the uptake and integration of naked fragments of extracellular DNA), *transduction* (DNA transfer through bacteriophages by a process in which the host cell is 'infected' with ARGs), or through *gene transfer agents* such as bacteriophage-like particles.²⁰ Prior studies have found several ARGs, involved in the process of AMR in environmental soil bacteria, to be homologous to those found in human clinical pathogens.²¹ The existence of HGT highlights the importance of the environmental resistome,^{22,23} or other potential reservoirs of ARGs through which HGT events could be initiated.

Antimicrobial resistance in the food chain

Smillie *et al.* (2011)²⁴ investigated potential HGT events by studying distantly related bacterial genomes isolated from human, environmental and animal samples. The authors reported on the transfer of ARGs between humans and livestock, known to provide resistance to human and veterinary antimicrobials. They concluded that livestock-associated bacteria can contribute to clinical resistance without directly infecting humans. This is not surprising when we consider farms or other settings involving livestock as examples of environments in which different ecosystems intersect, providing ample opportunity for transfer of resistant bacteria and possibly the occurrence of HGT events. In such settings, based on the presence of livestock, unwanted wildlife and pets, intensive (in) direct human-animal contact is to be expected. Similar types of contact can be found within a meat processing environment (e.g. through livestock or meat handling). Numerous studies have investigated the epidemiology of AMR, and point towards AMU as the principal driver for carriage of AMR bacteria in humans and animals,^{25,26} with livestock-associated Methicillin-resistant *Staphylococcus aureus* (LA-MRSA) and extended-spectrum β -lactamase (ESBL) producing bacteria as well-known examples. Prior studies have investigated determinants for human and livestock AMR carriage within farm and meat processing environments. Here, risk factors identified for livestock AMR carriage include specific farm or livestock batch characteristics.^{27–29} For human AMR carriage, specific determinants have been demonstrated such as duration and intensity of livestock contact.^{30–36} Determinants for AMR carriage in livestock and occupationally exposed farmers and slaughterhouse workers are the central subject of this thesis and will be further discussed in **chapters two to six** and the **general discussion**.

1.2 Methods to determine antimicrobial resistance

In the past decades, technological innovations have entirely transformed the field of AMR research, partly shifting the field from phenotypic AMR susceptibility testing (AST) towards the genomic characterization of AMR. At present, genotypic methods are primarily used to complement phenotypic methods. Drivers and barriers of genome sequencing to routine clinical use have recently been investigated,³⁷ as well as requirements for its use within surveillance activities.³⁸ In research, first applications of full metagenomic characterization of ARGs are emerging. The focus of this thesis will be on the benefits and practical use of genotypic methods within AMR epidemiology (**chapters two, three, five and six**), nonetheless, some culture-based techniques were employed for the realization of **chapter four**. This section consequently introduces some of the most commonly applied AMR detection techniques.

Phenotypic methods

Traditionally, phenotypic methods have been used to determine AMR patterns in target pathogens. When Alexander Fleming tested the properties of his 'penicillin' (a *Penicillium* broth filtrate) in 1929, he used an agar disk diffusion test – a phenotypic method – to show the inhibitory action of his filtrate on e.g. staphylococci.³⁹ Using this method, after culturing and isolating the target bacteria, antimicrobial susceptibility or resistance can be measured either qualitatively [e.g. agar disk diffusion (ADD)] or quantitatively [e.g. agar or broth dilution, antimicrobial gradient method (E-test®)]. The ADD method uses zone inhibition diameters to qualitatively classify an isolate as resistant, intermediate resistant or susceptible.⁴⁰ In contrast, quantitative methods report on the minimal inhibitory concentration (MIC, in mg/litre), which represents the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism. MICs are considered the gold standard of AST and are used to set clinical and epidemiological breakpoints.⁴¹ Clinical breakpoints inform clinicians on the likelihood of patient treatment failure, while epidemiological breakpoints/cut-off values (ECOFFs) can distinguish wild-type populations of bacteria from those with acquired or selected resistance mechanisms, enabling the guidance of AMR monitoring efforts.^{42,43} Breakpoints are officially established by the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) and the US-based Clinical and Laboratory Standards Institute (CLSI).⁴²

Genotypic methods

Although recent advances have been made towards culturing previously ‘unculturable’ human intestinal bacteria,⁴⁴ many – potentially resistant – bacteria remain unculturable or unknown.^{45–47} Those potential resistance reservoirs accordingly require AMR detection through other than solely phenotypic culture-based methods. Genotypic methods are particularly useful to detect AMR determinants in slow-growing or nonculturable microorganisms. In addition, these methods also enable us to further characterize types and mechanisms of resistance, to identify certain point mutations or genotypes, or to target the full microbial community for AMR.⁴⁸ The following methods will be briefly discussed: (q)PCR, the hybridization-based technique of microarrays, and whole-genome and metagenomic sequencing for the detection of AMR. Many more genomic AST methods are, however, available. Furthermore, a detailed comparison of phenotypic versus genotypic methods is presented in Table 1.1.

(q)PCR and DNA microarrays

The first description of the process called polymerase chain reaction (PCR) stems from the 1980s. Mullis *et al.* (1987)⁴⁹ report on a method which “*consists of repetitive cycles of denaturation, hybridization, and polymerase extension and seems not a little boring until the realization occurs that this procedure is catalyzing a doubling with each cycle in the amount of the [DNA] fragment defined [...], that this fragment is therefore increasing in concentration exponentially, and that the process can be continued for many cycles, and is inherently very specific*”. PCR can be used to target and amplify known ARGs in a sample or isolate, followed by ARG visualization through e.g. gel electrophoresis. Although having a wide scope of application, PCR largely depends on the sensitivity and specificity of the forward and reverse primers used,⁵⁰ and can only generate a qualitative result (ARG presence or absence). Since its discovery, the field of PCR research has experienced a vast number of improvements, leading among others towards the application of sequence-specific probes to increase assay specificity and towards the development of quantitative PCR (qPCR). qPCR is faster than conventional PCR, detects ARG amplification in real-time (generating a fluorescence signal) and quantifies the number of ARGs per sample through the use of a calibration curve.⁵¹ Other enhancements are the development of multiplex⁵² or high-capacity (q)PCR⁵³ assays, in which multiple ARGs are simultaneously targeted, consequently increasing efficiency while giving up the ability to optimize the assay per gene target. Since its discovery, PCR has found numerous applications in AMR research.⁴⁸

Table 1.1 General overview of AMR detection methods, their use and main (dis)advantages

Method	Phenotypic methods	Genotypic methods
<i>Examples of available techniques mentioned in this chapter</i>	<ul style="list-style-type: none">■ Selective plating■ Agar Disk Diffusion■ Agar or Broth Dilution■ E-test®■ Micro-fluidic AST method*	<ul style="list-style-type: none">■ (q)PCR methods■ Microarray■ Metagenomic sequencing (functional metagenomics, sequence-based metagenomics)■ Whole-genome sequencing■ Fluorescence in-situ hybridization*
<i>*(dis)advantages and impact not further included in this table</i>		
<i>What is tested?</i>	<ul style="list-style-type: none">■ Expression of antimicrobial resistance or antimicrobial susceptibility in known, viable and (relatively fast-growing) culturable bacteria.■ <i>In vitro</i> susceptibility.	<ul style="list-style-type: none">■ The presence (or prediction) of known or novel antimicrobial resistance gene determinants and gene mutations associated with specific resistance mechanisms.■ Functional expression of resistance (e.g. functional metagenomics).
<i>What is its practical use?</i>	<ul style="list-style-type: none">■ Determination of clinical breakpoints to guide patient treatment decisions.■ Determination of epidemiological cut-offs for monitoring and surveillance purposes.	<ul style="list-style-type: none">■ Detection of resistance determinants in any bacterium (including slow-growing or unculturable bacteria) against routinely and not routinely tested antimicrobial compounds.■ Extensive characterization of pathogens to differentiate between strains e.g. when clinically or epidemiologically relevant to make this distinction.■ Generating knowledge on the full spectrum of AMR, the biological pathways (e.g. also relevant for the development of new antimicrobials) and epidemiology and emergence of resistance. Guiding surveillance and monitoring efforts.

Table 1.1 Continued

Method	Phenotypic methods	Genotypic methods
<i>Advantages (not exhaustive):</i>	<ul style="list-style-type: none">■ Identification of (new) (multi)drug resistance and resistance patterns since resistance phenotypes result from gene interactions.■ Phenotypic methods can guide treatment decisions.■ Most techniques are very easy and practical to apply and do not require extensive training.■ Traditional non-automated phenotypic methods (e.g. plating, microdilution) are relatively inexpensive compared to genotypic methods (and more automated phenotypic systems) and are therefore suited to be applied in low-resource environments.	<ul style="list-style-type: none">■ The identification of antimicrobial resistance determinants is possible for difficult to culture or unculturable bacteria.■ In case of standardized assays and pipelines, rapid identification or exclusion of certain types of resistance can be performed which allows for early disease intervention.■ More insight can be gained into biological pathways and mechanisms of resistance.■ Detection of novel types of resistances and resistance mechanisms is possible (e.g. functional metagenomics, WGS or metagenomics combined with genome assembly).■ Metagenomic techniques are able to target the full bacterial community for antimicrobial resistance determinants simultaneously. In addition, existing metagenomic datasets can be employed for <i>in silico</i> retrospective detection of newly emerging ARGs.
<i>Disadvantages (not exhaustive):</i>	<ul style="list-style-type: none">■ Only insight into resistance phenotypes is obtained.■ Only known, relatively fast culturable bacteria can be investigated (culture bias).■ Susceptibility data alone can be insufficient in case of AMR risk analysis or monitoring and surveillance efforts (e.g. further characterization, comparison of strains and strain identification might be necessary for surveillance purposes).■ Low-level resistance might be difficult to detect by phenotypic methods alone.■ Lack of method harmonization can impede interlaboratory comparison, as well as shifting of interpretative standards (e.g. accepted breakpoints may vary in time and between countries).■ Phenotypic methods (e.g. plating and dilution) are limited to the number of agents which can be tested simultaneously and are rather laborious. However, automated assays exist for phenotypic antimicrobial susceptibility testing (e.g. Vitek 2 system, bioMérieux)	<p><u>Overall:</u></p> <ul style="list-style-type: none">■ Clinical decision making in microbial therapy is based on the detection of susceptibility, while genomic methods (with the exception of functional metagenomics) are mainly based on the screening of resistance determinants without regard of functional expression of resistance in the original host or the viability and the degree of intactness of the host bacterium. Susceptibility not necessarily correlates well with the presence or absence of genetic markers and is often a combination of the expression of multiple genes. Functional metagenomics can predict expression, but is bound to expression in its surrogate host (which might differ from expression in the wild-type host).■ Most genomic methods can only screen for antimicrobial determinants which are known and novel types of resistances are therefore missed. Examples of methods which might be able to detect novel AMR determinants are functional metagenomics, sequence-based metagenomic methods utilizing metagenomic assembly or WGS in case of high homology with known genes / linked to phenotypic testing.■ Standardized screening methods and protocols are limited (e.g. regarding assay optimization, applied limit of detection, sequencing depth and normalization strategies). In addition, standardized protocols for assembly, read and contig classification are lacking, while differences in sensitivity and specificity of algorithms are common. There is a need for standardized genomic reference databases since currently database curation strategies and database scopes diverge.

Table 1.1 Continued

Method	Phenotypic methods	Genotypic methods
<i>Disadvantages (continued)</i>		
		<ul style="list-style-type: none">■ Compared to traditional phenotypic methods, genomic methods require a more complex set-up of equipment (e.g. PCR), infrastructure and expertise (e.g. sequencing, metagenomics), and are therefore relatively more expensive and require extensive training of personnel. An exception to the complexity of equipment set-up is e.g. the pocket size nanopore sequencing device (Oxford Nanopore Technologies).■ In case of highly specific or expensive genomic characterization of strains, specialized reference laboratories might be required for testing, which impedes testing and characterization in low-resource environments. <p><u>Specifically for (q)PCR and microarray:</u></p> <ul style="list-style-type: none">■ Traditional assays can only target a limited number of distinct ARGs per run. Examples of high-throughput assays able to target a multitude of genes in parallel exist. These, however, come with the inability to optimize individual assays, which cause all assays to undergo the same cycling conditions.■ The presence of silent ARGs and pseudogenes, mutations and sample contaminants might inhibit PCR-based assays and cloud results (e.g. false-negative and false-positive results). <p><u>Specifically for sequencing methods:</u></p> <ul style="list-style-type: none">■ The accuracy, sensitivity and specificity of sequencing methods heavily rely on the depth of sequencing, the chosen read cleaning and trimming strategy, the assembly and read/contig classification algorithm and tool, and the curation strategy and scope (including bias towards human pathogens) of genomic reference databases.■ Although sequencing costs per raw megabase of DNA sequence have decreased in the recent two decades, there are still high costs involved in the design and maintenance of a bioinformatics and data analysis pipeline, including costs for training staff or costs in terms of time (e.g. computational time) and data storage depending on the techniques and algorithms used.

An alternative method for the assessment of multiple known ARGs in parallel is the application of DNA microarrays (or DNA chips). This method, originally designed for gene expression profiling in 1995,⁵⁴ has since then widely been applied in AMR research.^{55,56} It utilizes gene-specific probes deposited onto a solid surface in a lattice pattern, while target DNA is extracted (e.g. fluorescently) labelled, hybridized to the array and examined with e.g. an epifluorescence microscope.⁵⁷ While the principle of PCR is based on geometric/exponential product accumulation, microarray labelling product accumulation is arithmetic.⁵⁶ Microarray analysis is considered more expensive than PCR analysis, but to also have an outstanding value on a per gene basis,⁵⁵ especially when employing high-density microarray chips.⁵⁸

The era of sequencing and metagenomics

A. First and next-generation sequencing techniques

What initially started with an improvement of the rapid and simple ‘plus and minus’ method to define the genome of bacteriophage ϕ X174, later became known as ‘Sanger sequencing’. This first-generation sequencing technique consists of incorporating deoxynucleotide triphosphate analogues onto a growing oligonucleotide chain – so-called ‘sequencing-by-synthesis’ – triggering a chain-termination event.⁵⁹ Currently, Sanger sequencing has built a legacy of successors, referred to as next-generation sequencing (NGS) techniques, allowing for the mass parallelisation of sequencing reactions leading to an increased amount of DNA sequenced in one run. Multiple NGS methods and devices exist today.^{60,61} NGS methods have revolutionized the field of AMR research, and their popularity has readily increased, partly due to the plummeted costs of sequencing in the last decade.⁶²

B. Metagenomics and antimicrobial resistance

Increasingly affordable NGS technologies made it possible to bypass the need for culturing and target the whole microbial community randomly and *en masse*, a technique currently known as ‘shotgun metagenomics’. Roughly two types of approaches can be distinguished in the field of AMR: functional metagenomics and sequence-based metagenomics.⁶³ Functional metagenomics starts with the construction of a metagenomic library, by cloning and heterologous expression of (total) DNA in *E. coli* as the usual surrogate host.⁴⁶ Subsequently, the metagenomic library is screened for AMR activity using antimicrobial selection media and sequencing.⁴⁶ Previously unknown ARGs have been uncovered by applying functional metagenomics.^{21,64–66} In contrast to functional metagenomics, sequence-based metagenomics employs shotgun sequencing of total DNA

directly extracted from environmental samples (e.g. faecal samples) without the need for cloning into an expression vector and subsequent selective media screening. The resulting sequence reads are subsequently processed by e.g. assembly or read classification as further described in *paragraph 1.3*.

C. Whole-genome sequencing

With the rise of sequencing technologies, whole-genome sequencing (WGS) became feasible leading up to the sequencing of the first genome of a living organism in 1995: the bacterium *Haemophilus influenzae* Rd.⁶⁷ Through WGS an isolate or single cell is sequenced, followed by genome assembly. WGS eventually rose to acclaim within the field of AMR research as a tool for AMR characterization providing insights into the emergence and spread of antimicrobial resistance.⁶⁸ In recent years, concordances up to as high as 99% were observed between phenotypic AST results and WGS-predicted antimicrobial susceptibility in e.g. *Salmonella*, *E.coli* and *Campylobacter* isolates originating from pigs and retail meat.^{69–71} Nevertheless, for the investigated bacteria, the accumulated evidence for comparing WGS and phenotypic approaches is considered limited or poor, and consequently the use of WGS for *in silico* detection of antimicrobial susceptibility – thereby replacing phenotypic methods – is not yet considered appropriate in a clinical setting.³⁷ Conversely, its value for AMR epidemiological and surveillance purposes has often been underlined.^{68,72} Eventually, new data analysis techniques may turn the tide for the use of WGS as a clinical diagnostic tool: the combination of machine learning techniques and WGS has already been found to generate accurate MIC prediction models.⁷³

A selection of other AST techniques

As discussed before, a multitude of other phenotypic, biochemical or molecular AST methods are available for the detection of AMR. Other methods not described further herein include matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) to detect AMR biomarkers (biochemical method),⁷⁴ microfluidic AST methods (phenotypic method),⁷⁵ or fluorescence in-situ hybridization (genotypic method).^{76,77}

1.3 Analysing metagenomic data for epidemiological research

Within this thesis, next to phenotypic and biochemical methods (selective plating combined with MALDI-TOF MS, **chapter four**), mainly genotypic methods were used for the detection of AMR in animal and human samples (e.g. micro-array and sequencing, **chapter four**; qPCR, **chapter five**; metagenomics, **chapters two, three and six**).

Metagenomics, in theory, enables screening of the full bacterial resistome present in a sample. Although an innovative addition to the field of AMR, metagenomics – to the (un)experienced –, might sometimes feel more of a curse than a blessing, considering the number of technical, computational and ‘choice overload’ hurdles that have to be faced during preparation, processing and epidemiological analysis of metagenomic data. After DNA extraction, library construction and sequencing, a metagenomic workflow consists of three basic steps: computational pre-processing (e.g. base calling, quality trimming and adapter removal), the actual sequence analysis (e.g. read assembly and/or alignment), and post-processing (epidemiological analysis).^{78,79} In this paragraph some basic analysis options are provided with regard to the latter two steps (sequence analysis and analysis of metagenomic *abundance* data) to outline the context in which this thesis has been carried out.

Processing metagenomic data: assembly and read classification

Genomic assembly can be referred to as the stitching together of individual DNA sequences (reads) into genes or organisms,⁸⁰ and is considered beneficial to gaining insight into the genetic context of ARGs and the taxonomy of their carriers.^{80,81} In addition, assembly-based strategies are able to uncover e.g. novel bacteria⁷⁹ or plasmids⁸². Genomic assembly, and subsequent binning and annotation, requires high sequence coverage and computational power. It can potentially fail in case of complex samples when multiple genomes need to be constructed (e.g. metagenomic assembly),⁷⁹ while the principal ambition might only be to quantify and compare ARG abundances across samples (this thesis). Therefore, depending on the resources at hand and the research question, read classification strategies, which are relatively less computationally demanding, can be more appropriate.

A variety of strategies are available for metagenomic read classification, all employing different tools and algorithms. Reads (or assembled contigs) can be classified based on: (i) Sequence similarity (applied within this thesis), by aligning reads to known sequences through similarity searches (e.g. MGmapper⁸³); (ii)

Sequence composition, by calculating compositional nucleotide statistics (e.g. tetranucleotide or codon usage) prior to classifying reads (e.g. PhyloPythiaS), (iii) Hybrid methods incorporating components of both previous strategies (e.g. PhymmBL), and (iv) Marker sequences, in which classification is based on specific marker sequences (e.g. MetaPhlAn and ShortBRED⁸⁴).⁸⁵ All these tools and algorithms come with a trade-off between accuracy, sensitivity, specificity and run times.⁸⁶ Additionally, classification methods make use of existing reference databases and hence heavily rely on their quality (e.g. curation strategy) and substantive scope (e.g. specific versus general ARG databases, bias against human-associated organisms).^{72,87} Reliance on reference databases makes these methods also less suited for the detection of novel ARGs.

Epidemiological analysis of metagenomic abundance data

After ARG abundances have been generated, the analysis of metagenomic data is challenging. First, metagenomic data are often plagued by systematic variability between samples (e.g. caused by differences in average microbial genome sizes⁸⁸ or sequencing depth⁸⁹), necessitating the use of a normalization method to remove this variability. After normalization, analysis approaches additionally have to deal with the high dimensionality of metagenomic datasets, and an often limited number of observations ('large p, small n' property),⁹⁰ especially if combined with a large number of predictor variables. Furthermore, metagenomic datasets are count-based, may contain excess zeros and are usually not normally distributed.⁹¹ Finally, similar to all epidemiological analyses, a variety of metadata (covariates, confounders), need to be included to facilitate determinant analyses.

Different types of analysis approaches can be found in the literature, for instance: (i) those based on the situation in which the *abundance of each individual ARG* is compared between samples and related to one or more determinants and (ii) those methods which look at the *abundance profile as a whole* by comparing samples' dissimilarity, often called ordination and dimension reduction techniques.

Broadly speaking, the first approach incorporates statistical tests applied once for each ARG. Within (meta)genomic research, a variety of programming packages have been designed and applied to identify differentially abundant genes, while addressing the issue of a low number of observations and a high number ARGs. These methods all assume different underlying data distributions: *metagenomeSeq*⁹² models data following a normal distribution, therefore, data are first log-transformed before fitting a zero-inflated Gaussian model. Examples of approaches assuming a count distribution are e.g. *ShotgunFunctionalizeR*,⁹³

which uses a Poisson generalized linear model; or *Differential Gene Expression Analysis* (DESeq2, applied in **chapters two and six**)⁹⁴ and *Empirical Analysis of Digital Gene Expression* (edgeR)⁹⁵, which both apply negative binomial models initially developed for RNA sequence data; and a more recently proposed *zero-inflated overdispersed Poisson model*⁹⁶. The overall performance of these methods varies heavily and is affected by sample size, effect size and overall gene abundance.⁹⁷ Methods which pursue multiple ARG inferences in parallel, need to control for a high probability of type I errors (e.g. False Discovery Rate). This ability to control for false positives varies among approaches.⁹⁷ Although modelling data on a per gene basis offers relative flexibility, it must be noted that these methods cannot take into account the influence of a determinant on multiple genes and that most programming packages target only a small set of predictor variables.⁹⁰

The second commonly applied approach comprises ordination and dimension reduction methods, which aim to uncover relationships by searching for latent gradients in a multidimensional space. These methods can also easily accommodate 'large p, small n situations', for instance through summarizing a large predictor or response matrix.^{90,98} Most of these methods originate from the field of ecology and include *unconstrained/unsupervised/indirect gradient analysis methods* [e.g. principal component analysis (PCA),⁹⁹ correspondence analysis (CA), principal coordinates analysis (PCoA¹⁰⁰, **chapter two**), non-metric multidimensional scaling (NMDS¹⁰¹, **chapter six**) and cluster analysis] and *constrained/supervised/direct gradient analysis methods* [e.g. (distance-based) redundancy analysis (db-)RDA¹⁰⁰ and canonical correspondence analysis (CCA)].^{90,102} Simply put, unsupervised methods only use the dataset of response variables to explain the overall variance in the data. Conversely, supervised methods try to find the variability in the response variables that can be explained by a set of constraining explanatory variables.¹⁰² Of these methods, PCoA, NMDS, or distance-based RDA can be used within metagenomic research in a flexible manner based on their ability to use any type of distance matrix – calculated from a count based metagenomic dataset – as input for the analysis, while PCA and RDA or CA and CCA respectively assume linear (employing Euclidian distances) or unimodal (employing X^2 distances) relationships. However, dimension reduction techniques have a limited ability to estimate or interpret the influence of multiple predictors, random effects, as well as interaction, making these methods less suited for elaborate epidemiological analysis. Nevertheless, while not ideal, so-called 'partial ordination techniques' allow for factoring out the influence of covariates for the benefit of estimating a certain variable of interest.^{98,102} Furthermore, when hypothesis testing is required, ordination techniques need to

be combined with e.g. permutation tests [e.g. PERMANOVA (applied in **chapter six**), ANOSIM¹⁰³ or Monte Carlo permutation¹⁰²]. However, it must be noted that the above discussed dimension reduction techniques intrinsically cannot identify determinants for individual ARGs, while determinants might only be relevant for a subset of ARGs. This further complicates the interpretation and discovery of specific determinant-response relationships. In addition, distance-based multivariate analyses are prone to confound location and dispersion effects, which might lead to erroneous interpretation of results.¹⁰⁴

Next to the above methods, a plethora of other multivariate analyses (e.g. Procrustes analysis, **chapters two and six**) and tools are currently being applied within – multi-layer – omics research, ranging from dimension reduction (Partial Least Squares based methods) to variable selection techniques (lasso and ridge regression, elastic net and Bayesian variable selection).⁹⁰ Moreover, an increasing number of data mining and machine learning techniques are being used within microbiome research to detect genetic diversity and to support predictive modelling of AMR gene-phenotype relationships (e.g. combinations of k-means and hierarchical clustering, support vector machine and topic modelling¹⁰⁵, neural networks¹⁰⁶, random forests¹⁰⁶, variant mapping and prediction of AMR (VAMPr)¹⁰⁷ and pairwise comparative modelling¹⁰⁸).

1.4 Main objectives of this thesis

The epidemiology of AMR has a complex nature, considering the potential AMR reservoirs within the environment, the wide possibilities for AMR bacterial and gene transfer events, and the number of uncertainties with regard to the existence of AMR baseline levels despite antimicrobial pressure. Phenotypic studies have previously highlighted the occurrence of AMR in livestock, including associations between AMR in livestock and AMR in humans. However, comparatively little is known about determinants for the livestock resistome, and the resistome of humans occupationally exposed to AMR in livestock. Therefore, this thesis focuses on AMR in the food chain by mainly applying genomic methods for ARG detection.

The presented work has the following goals:

- To describe the pig and broiler faecal resistome in herds from nine European countries;
- To determine the role of AMU, farm management and biosecurity measures in shaping the livestock resistome;

- To assess faecal carriage of ESBL-producing bacteria in pig slaughterhouse workers and to determine the association between faecal carriage of ESBL-producing bacteria and occupational exposure;
- To determine environmental exposure to tetracycline (*tetW*) and macrolide (*ermB*) resistance genes in the pig slaughterhouse, to assess faecal carriage of these ARGs in pig slaughterhouse workers and to investigate the association between occupational environmental exposure and faecal ARG carriage;
- To describe the faecal resistome of humans occupationally exposed to AMR in livestock (pig and broiler farmers, pig slaughterhouse workers) as compared to a control population;
- To determine the role of occupational exposure to AMR in livestock in shaping the human resistome;
- To discuss the suitability of sequence-based metagenomics for application in AMR surveillance networks.

This thesis consists of the following parts:

Part I assesses the determinants for the faecal resistome in livestock. **Chapter two** starts off with a detailed metagenomic description of the pooled resistome of more than 9000 individual pigs and broilers sampled at 359 herds in nine European countries, and continues with a brief investigation of the relation between AMU and the faecal resistome. **Chapter three** elaborates on these associations by including detailed on-farm metadata gathered at the investigated pig farms.

Part II focuses on the determinants for faecal AMR carriage in pig abattoir staff working along the slaughter line. **Chapter four** investigates the determinants for carriage of ESBL producing *E. coli* in 334 pig slaughterhouse employees. **Chapter five** takes an in-depth approach by examining a variety of possible routes of environmental occupational exposure to tetracycline and macrolide resistance genes (e.g. pig carcasses and meat, hand contamination, ambient air), while relating this exposure to faecal tetracycline and macrolide resistance gene carriage among pig slaughterhouse workers.

Part III, i.e. **chapter six**, investigates the faecal resistome of farmers and slaughterhouse staff employed within the pig and poultry food production chain. Here, the resistome of occupationally exposed workers is described and determinants for and dissimilarities between their resistome components are explored as compared to a human control population.

Finally, **chapter seven** discusses the results presented in all chapters by integrating previous knowledge with research from this thesis, ending with a discussion on the benefits and limitations of metagenomic sequencing for surveillance purposes.

Research within this thesis is part of the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project (www.effort-against-amr.eu) which officially ended on 30 November 2018 and of which the main findings were presented to the European Commission in February 2019. One of the key goals within EFFORT was to investigate the epidemiology of AMR in the food chain employing a variety of molecular and bioinformatic technologies.

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Part I

Determinants for the faecal resistome of livestock

Chapter 2

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

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Supplementary data, excluding Excel tables, can be found at the end of this chapter
Supplementary Excel tables can be retrieved through Nature Microbiology

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Abstract

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

2.1 Introduction

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

Multiple studies have shown that the use of antimicrobials in livestock will lead to an increased occurrence of AMR and that the reduction of usage will eventually lead to reduced resistance.^{3–8} Several national surveillance programmes have been implemented to monitor the occurrence of AMR in different reservoirs and follow trends over time.^{1,9–11} There are major differences in antimicrobial consumption patterns between different countries globally and also within Europe.¹² Major differences in the occurrence of AMR have also been observed among indicator organisms (for example *E. coli*) isolated from different European countries.^{3,13} Current monitoring efforts are mainly based on culturing indicator bacteria followed by phenotypic AMR determination.^{13,14} This procedure only targets a limited number of species present in the gut microbiota and, therefore, probably represents only a fraction of its resistome (the collective pool of AMR genes). Metagenomic approaches have been used in several recent studies and have shown that metagenomic read mapping describes AMR abundance in bacterial communities more accurately than commonly used technologies on selected indicator organisms.^{15–17} A recent study focused on sampling a diverse group of individual pigs from 11 farms in 3 countries and showed that genetics, age, diet and geography all probably influence the pig microbiota, but little information is available for the poultry microbiota.¹⁶

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

2.2 Results

Acquired resistome characterization

The total AMR load varied significantly across samples, depending on both the host animal and the country of origin. In general, pigs had a higher AMR level than poultry (Figure 2.1a). The highest AMR levels were found in Italian pigs, where the top four resistance-scoring samples originated, all in excess of 10,000 fragments per kilobase reference per million bacterial fragments (FPKM) AMR. At the lower end of the spectrum were Danish poultry samples that occupied the 11 samples with the least AMR, all below 500 FPKM.

We summed the relative abundance of AMR to the corresponding drug class level for each sample to look for major trends across host species and countries (Figure 2.1b). When considering the proportion of the total resistome by AMR phenotype, the pig samples were relatively homogenous: tetracycline AMR was by far the most common, followed by macrolide AMR. β -lactam and aminoglycoside AMR genes followed with other kinds of AMR being rare. Italian pigs had a notably larger proportion of phenicol AMR compared with other countries and it seemed to be consistent across Italian farms. A subset of Bulgarian pig farms had a similar proportion of phenicol AMR.

Among the poultry farms, there was less consistency. Both within and between countries, the relative proportions of AMR per drug were more varied. As in pig samples, tetracycline, macrolide, beta-lactam and aminoglycoside AMR made up the majority, but the two latter classes had very minimal contributions in a subset of herds. Sulfonamide and trimethoprim AMR was more abundant in poultry samples than in pig samples across all countries. In many Polish poultry herds, quinolone AMR made up a sizeable fraction of the combined resistome. This was also true for a few non-Polish herds, notably in Bulgaria. For non-proportional graphical representations of the AMR load stratified by sample and drug class, see the Supplementary data for an unscaled, stacked bar chart (Supplementary Figure 2.1) and a heatmap (Supplementary Figure 2.2). Class level AMR relative abundances can be found in Supplementary Table 2.1.

To characterize the individual components of the resistome, we summed the relative abundance to the gene level, as was done for the phenotypic level. We found evidence for 407 different genes across all pig and poultry samples (Supplementary Table 2.2).

We calculated the dissimilarities between the gene-level resistomes of all samples and visualized it in a dendrogram (Figure 2.2). There was a perfect host separation, with all pig samples clustering separately from all poultry, suggesting

that pig and poultry resistomes are very distinct. In the pig cluster, the country separation was more pronounced than in the poultry cluster. An exception was Danish poultry, where 18 out of 20 farm resistomes clustered.

To assess the reproducibility of our protocol, from sampling through to sequencing, we evaluated the similarities between the resistomes of two triple-sampled swine herds. The Dutch triple-sampled herd had the highest similarities

Figure 2.1 Overview of AMR abundance and composition

From reads mapping to the ResFinder database, AMR abundance was calculated for each reference gene in each sample. (a) Boxplots showing the total AMR level per sample, stratified by host species and country. Each herd is also represented by a dot with sideways jitter to minimize overplotting. Horizontal box lines represent Q1, median and Q3. Whiskers denote the range of points within $Q1-1.5 \times IQR$ and $Q3+1.5 \times IQR$. $N=359$ metagenomes from independent herds. 20 metagenomes per livestock species per country was used, except for BG pig (21), BG poultry (19) and DE poultry (19). (b) Stacked bar chart of AMR abundance per type (colours) per sample (x-axis), proportional to total AMR within each sample.

Figure 2.2 Resistome clustering is influenced by both host animal and country

Dendrogram showing complete linkage clustering of BC dissimilarities between all pig and poultry resistomes. Triple-sampled pig herds are highlighted in separate colours. N=363 metagenomes from 359 independent herds.

between composite samples, ranging from 93.6% to 93.7% Bray-Curtis similarity. The Belgian triple-sampled herd pools had values ranging from 91.5% to 93.3% similarity. No replicated sample pool had a higher similarity to other herds than to its own replicates, and the two sets of three samples can therefore be seen clustering separately, indicating reproducibility in both sampling and sequencing (Figure 2.2). A farm resistome similarity heatmap is included in the Supplementary data (Supplementary Figure 2.3).

We ordinated the gene-level resistomes for all samples (Supplementary Figure 2.4) and pig and poultry samples separately (Supplementary Figure 2.5a,b). As with hierarchical clustering, there was a clear separation of pig and poultry samples, along the first principal coordinate, which explained 48% of the variation across all resistomes.

When analysing the two species separately, we observed clustering according to the country of origin in pigs (Supplementary Figure 2.5a), whereas

clustering was more diffuse for poultry (Supplementary Figure 2.5b). We tested for the country effect and found it to be significant in both pigs (adonis2, $p < 0.001$) and poultry (adonis2, $p < 0.001$). However, in poultry, the country effect only explained 24% of the variation, whereas the country effect explained 41% of the variation in pigs. In the pig resistome ordination, the Danish and Dutch samples clustered closely together. The same could be seen for the French and Belgian resistomes and to a lesser degree, the Italian and Spanish samples. Bulgaria, Germany and Poland showed larger dispersions than the other countries. Beta-dispersion levels varied significantly between countries in both pigs (beta-dispersion $p < 0.001$; Supplementary Figure 2.5c) and poultry (beta-dispersion $p < 0.001$; Supplementary Figure 2.5d).

We visualized the AMR gene abundances in a heatmap to look at the overall structure and composition of the resistomes and the co-occurrence of AMR genes (Supplementary Figure 2.6). Some AMR genes were more abundant in one species, whereas others, including *tet(W)* and *erm(B)*, were ubiquitous in all samples for both species. Among the pig samples, the Italian samples stood out: several chloramphenicol AMR genes, including *cat(pC194)*, *catP* and *cat_2*, were much more abundant in Italy than in the other countries, consistent with our inspection of AMR at the class level (Figure 2.1). Several AMR genes known to be co-located indeed co-occurred across samples. The genes in the vancomycin AMR *VanA* cassette were co-located in several poultry samples. This was also true for the *VanB* cassette members, clustering together but separately from *VanA*, showing an ability to distinguish variants of homologous genes. As indicated earlier, the poultry samples showed less country-based clustering than pigs. An exception was the Danish poultry samples; these had a noticeably lower abundance of many AMR genes that were widespread in other countries.

Core resistome

To determine whether specific genes were unique to each of the host animals, we examined the set of AMR genes that was consistently observed within each animal species (evidence for it in 95% of samples). We identified 33 core AMR genes in pigs and 49 core AMR genes in poultry, with 24 being shared between the two hosts (Supplementary Figure 2.7). Hence, only nine AMR genes were pig-core genes without also being poultry-core genes. These included the genes making up the Van-G vancomycin cassette, *tet(C)*, *bla_{ACI}* and *cfxA*. Twenty-five AMR genes were poultry-core genes without also being pig-core genes and include the Enterobacteriaceae-associated *strAB*, *sul2*, *blaTEM* and *tet(A)* genes.

Differential abundance analysis

To test which specific genes differed in abundance between countries, we carried out a differential abundance analysis for ResFinder gene cluster read counts. Heavy overrepresentation of low unadjusted p-values indicated a large effect of country in both the pig and the poultry datasets (Supplementary Figure 2.8). Of special interest was the newly characterized *Enterococcus*-associated linezolid-resistance gene *optrA*, which had a significantly higher abundance in Bulgarian poultry farms than in poultry farms in all other countries (false discovery rate (FDR) < 0.05) (Figure 2.3). However, a single Spanish farm did have an even higher

Figure 2.3 AMR genes differ in abundance between countries

A handpicked subset of genes that differed significantly in abundance between at least two countries in either pig or poultry farms are shown. The regularized log abundance (rlog) is shown on the y-axis in boxplots and points. Points were sideways jittered to reduce overplotting. The numbers along the x-axis denote the number of countries with significantly different mean abundance (DESeq2 Wald test, two-sided, $\alpha = 0.05$). Testing was done for all ResFinder genes and p-values were adjusted for multiple testing (FDR) for all country comparisons. Horizontal box lines represent Q1, median and Q3. Whiskers denote the range of points within $Q1-1.5 \times IQR$ and $Q3+1.5 \times IQR$. N=359 metagenomes from independent herds. 20 metagenomes per livestock species per country was used, except for BG pig (21), BG poultry (19) and DE poultry (19).

optrA abundance than any other farm. Among the pig herds, the *optrA* gene was more abundant in Bulgarian and Italian herds than anywhere else (except for two farms in Spain) (FDR<0.05).

The newly identified colistin-resistance gene *mcr-1* was significantly more abundant in Bulgarian and Italian poultry farms than in most other countries (FDR<0.05). France, Poland and Spain had intermediate levels, whereas Denmark, the Netherlands and Germany had the lowest levels (Figure 2.3). The Bulgarian poultry farms enrolled in this study did not report any polymyxin usage, whereas Italian farmers reported the highest average treatment incidents.

As previously noted from visual inspection of heatmaps, multiple chloramphenicol AMR genes including *cat(pC194)* were much more abundant in Italian pigs than in other pigs. The extended-spectrum β -lactamase *bla*_{CTX-M} gene cluster one also showed country dependency, being significantly more abundant in poultry samples from Spain, Poland, Italy, France and Belgium than in poultry samples from Germany (FDR<0.05). Differential abundance analysis results can be found in Supplementary Tables 2.3 and 2.4 for pig and poultry, respectively.

Alpha diversity and richness

We calculated several alpha diversity indexes for each farm resistome (Figure 2.4 and Supplementary Table 2.5). The range of AMR diversity was generally much larger for poultry samples, having both lower and higher diversity, than for pig samples, which had a tighter spread of diversity. The poultry samples had a higher estimated number of different AMR genes (that is, a higher Chao1-estimated richness).

Interestingly, countries with higher estimates of unique AMR genes in pigs also tended to have a high AMR richness in poultry (Spearman's rho: 0.88, $p=0.02$; Supplementary Figure 2.9). Spain, Italy, Bulgaria and Poland had the highest estimated number of unique AMR genes in both pigs and poultry. There was no such association for Pielou's evenness or Simpson diversity ($p>0.05$). Rarefaction curves for pig and poultry resistomes can be found in Supplementary Figure 2.10.

Association between the bacteriome and the resistome

To test the degree to which the bacterial composition of the microbiota dictates the resistomes, Procrustes analyses were performed. We found that for both pigs and poultry, the resistome correlated significantly with the bacterial composition ($p=0.001$; Figure 2.5). Thus, samples with similar taxonomic compositions tended

Figure 2.4 Resistome alpha diversity and richness differ between animal host and countries

From the read count pair matrix, several indexes were calculated: Simpson diversity index, Chao1 richness estimate and Pielou's evenness. Horizontal box lines represent Q1, median and Q3. Whiskers denote the range of points within $Q1-1.5 \times IQR$ and $Q3+1.5 \times IQR$. $N=359$ metagenomes from independent herds. 20 metagenomes per livestock species per country was used, except for BG pig (21), BG poultry (19) and DE poultry (19).

to have similar resistome compositions. In addition, most of the between-country differences in resistomes seem to be explained by systematic between-country differences in bacteriomes.

The correlation between AMR and taxonomy was similar in pigs (correlation: 0.86) and poultry (correlation: 0.88). Interestingly, in the pig samples, we saw a country effect on the strength of association between the bacteriome and the resistome. In the Dutch and Spanish pig herds, ordinations based on bacterial

Figure 2.5 Taxonomic variation explains resistome variation

Bacterial and AMR abundance profiles were correlated with each other using Procrustes analyses for pig (a) and poultry (c) herds, thus comparing the two multivariate datasets. The lines show the Procrustes residuals; the change in ordination position when using the resistome (dotted ends), compared to the bacteriome (non-dotted ends). The correlation coefficients and significance was derived using the protest function in vegan. Residual line plot for pig (b) and poultry (d) farms enables easier residual size comparison, showing the difference in the bacteriome-resistome association between farms. N=359 metagenomes from independent herds. 20 metagenomes per livestock species per country was used, except for BG pig (21), BG poultry (19) and DE poultry (19).

genera and AMR genes gave similar results (Figure 2.5b). In German farms, in particular, the resistome and bacteriome ordinations yielded more dissimilar results. This was less evident for poultry, although a single Danish poultry herd had a very unusual resistome, considering its taxonomic composition (Figure 2.5d).

Figure 2.6 National veterinary AMU is associated with total metagenomic AMR

Scatter plots of average total veterinary AMU (ESVAC) and pooled sample total AMR. A slight sideways jitter was added to the points to minimize overplotting. (a) Association between average veterinary drug use and total AMR load in pig farms. N=181 independent herds. (b) Association between average veterinary drug use and total AMR load in poultry farms. N=178 independent herds. See main text for description of trend lines.

AMR and drug use association

We found that the total country-level veterinary AMU from the European Medicines Agency's European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) was positively associated with AMR in both pigs and poultry. The AMR abundance increased by 1,736–3,507 FPKM (95% CI, $\beta=2621$) in pigs when the AMU increased by 1 $\log_e$ unit (a 36.8% increase in AMU) (Figure 2.6a) and to a lesser degree in poultry, where the AMR abundance increased by 68–1,330 FPKM (95% CI, $\beta=700$) when the AMU increased one $\log_e$ unit (Figure 2.6b). For pigs, the variance between farms within-country was seven times larger than the variance between countries in general, whereas in poultry, the variance was four times larger within-country than between countries.

We repeated the regression with the treatment incidents data for the farms (Supplementary Figure 2.11). Interestingly, these data were less associated with the AMR load than the national veterinary drug use data. The association remained for pigs, whereas poultry was not significant ($p>0.05$). Bulgaria had

low reported usage in both livestock species, whereas AMR was high. The Danish farms reported a higher average AMU in poultry than Bulgaria and the Netherlands, but the total AMR was far lower.

To test whether the AMU pattern across multiple antimicrobial classes was associated with AMR gene profiles, we compared the AMR gene cluster abundances for pig and poultry against both the ESVAC and the farm treatment incidents data (Supplementary Tables 2.6–2.8 and Supplementary Figure 2.12). Using Procrustes analyses, all matrix-matrix correlations were significant ($p=0.001$), although with low symmetric correlation coefficients (correlation: 0.34–0.45). As for the regression analysis, there was a better fit between pig AMR and the ESVAC data than between pig AMR and the farm treatment incidents data.

Functional AMR genes

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

Using the FRD, we found both similar and different patterns than using ResFinder. There was still a perfect separation between pig and poultry samples, but the country separation in pigs was less distinct than when using ResFinder (Supplementary Figure 2.13). Although less variation could be explained by two axes, the principal coordinates analysis (PCoA) plot of pig samples now clustered German and Spanish samples, with the remaining countries being more similar. The resistome richness showed similar patterns to ResFinder: Spanish, Italian, Polish and Bulgarian samples had a higher estimated richness in both pig and poultry than the other countries. The Procrustes correlation between the resistome and drug usage was lower (0.40 for pig and 0.25 for poultry). This result was echoed by the lack of association between the total AMR and the total AMU, for both pig and poultry ($p>0.05$; Supplementary Figure 2.14).

Discussion

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

A recent study including Chinese, Danish and French pigs showed that the Chinese pig resistomes clustered separately, whereas the Danish and French

pig resistomes overlapped.¹⁶ Here, we demonstrate that even among European countries, the livestock resistomes differ in a country-specific manner that might be explained by differential AMU so that countries with similarly high and diverse AMU (Spain and Italy) have similar resistomes, the same way as countries with similarly low AMU (Denmark and Netherlands) also have similar pig resistomes.

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

We found the recently discovered plasmid-borne colistin resistance gene *mcr-1* in numerous poultry herds, especially in Bulgaria, Spain and Italy. Spain and Italy had the highest reported veterinary colistin usage among the surveyed countries, whereas Bulgaria has a low reported usage, which is uncharacteristic for the high *mcr-1* level found here.¹³ This gene was recently discovered in China and identified throughout the world and has been identified in pigs, poultry and human clinical infections alike.¹⁸

A newly characterized enterococcal linezolid-resistance gene, *optrA*, was detected in a subset of pig samples, with Bulgaria, Italy and Spain having the highest abundances. The *optrA* gene provides AMR to both oxazolidinone and amphenicols, including the veterinary-used florfenicol.^{13,19} The high abundance of this gene in these countries can probably be explained by the fact that they have the highest veterinary amphenicol usage among the nine countries investigated. This explanation fits well with the fact that Bulgaria, Italy and Spain also had the highest abundances of chloramphenicol AMR genes, such as *cat(pC194)*, in poultry.

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

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

Interestingly, we observed that the number of unique AMR genes predicted (Chao1) significantly correlated between pig and poultry farms across countries. In addition, countries with a high estimated number of unique AMR genes also have

high AMR abundance (Italy, Spain, Bulgaria and Poland). The fact that countries' AMR abundance and the predicted number of unique AMR genes in pig and poultry tend to follow each other, could be explained with policy: if a country has strict AMU regulations in one livestock species, the chances are that similar regulations are in place for other livestock species. Indeed, the treatment incidents data showed that countries with higher AMU in pigs, had higher AMU in poultry. It might also be speculated that an ecological country effect plays a role; for example, the total country AMU might influence AMR abundance in all reservoirs. This might explain why the ESVAC data correlate better with the observed AMR than the treatment incidents data. Better AMU data, at the herd and country-level and over time, are needed to further explore the specific AMU-AMR associations.

It has previously been reported that the composition of the bacterial community structures the resistome.^{20,21} We found the same to be true for pig and poultry resistomes; in addition, we showed that the taxa-AMR association strength differs between countries. Horizontal gene transfer could explain this phenomenon, if a larger proportion of certain countries' resistome is mobile and AMR genes are more frequently introduced and re-introduced to genera. Conversely, vertical AMR transmission can also play a role, if, for example, one country's livestock is more isolated from trade. As we found that a large part of the observed resistome is dictated by the taxonomic composition, we expect much of the country resistome differences to be explained by systematic differences in feed and management.

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

The AMU data used in this study are not optimal. There is variation in drug use within each country's farms that we did not account for by using the available country-wide averages per drug class. Moreover, the population

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

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

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

The primary concern with read-mapping techniques is the lack of genomic context, which can be solved using metagenomic assembly and binning approaches.^{16,26,27} In this way, AMR alleles in full length, their genomic context and their associated taxa have been identified in both pig, poultry and human faecal samples.²⁸ As shown previously, the association between AMR and AMU is similar for metagenomics and traditional phenotypic methods, but several

aspects make metagenomics an intriguing monitoring tool.¹⁷ The fact that both types of analyses (quantitative, sensitive read mapping and qualitative, context-giving binning) use the same raw data makes metagenomics an attractive tool. In addition, the digital nature of sequence data would also allow future re-use and form the basis of an invaluable historical archive, potentially usable for both AMR and pathogen-tracking worldwide.

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

2.3 Methods

Farm selection and sampling

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

Selection of pig and poultry farms

In each participating country, 20 conventional integrated pig farrow-to-finisher non-mixed farms were selected. The farms needed to have a minimum of 150 sows and 600 fatteners and use batch production to ensure that most of the

animals of the sampled group originated from the same birth cohort. All-in all-out production at compartment level was preferred, and all fatteners sampled were required to have been on the same site during their entire life. Selected farms should have no contact through livestock trade and should have a random regional distribution.

In each country, 20 conventional broiler farms (no breeders) were selected. The farms had all-in all-out production, with a thinning procedure from day 30 onwards allowed. All selected farms should have no intended slaughter age higher than 50 days, no slow-growing breeds (intended growth rate of less than 55 g per day) and no stocking density lower than 10 birds per m². Only one flock per house per holding should be sampled and each flock should be between 20,000 and 40,000 birds. If possible, the selected farms should have a random regional distribution.

Procedure for sampling

We sampled pig farms between May 2014 and December 2015 and tried to minimize seasonal influences. The sampled fatteners were as close to slaughter as possible (that is, within the last week). A total of 25 fresh, still warm and undisturbed faecal droppings were sampled from pen floors (a minimum of 10 g faeces per sample) randomly divided over all eligible compartments or stables of fatteners close to slaughter.

Broilers were sampled between May 2014 and June 2016 and we tried to minimize seasonal influences. On each farm, 25 undisturbed, fresh main bowel droppings were collected from the floor of the house (a minimum of 3 g faeces per sample). The flocks were sampled as close to slaughter as possible (the last week before the final depopulation).

All samples were collected aseptically in plastic containers and were stored at 4 °C and transported to the laboratory within 24 hours after sampling.

Pooling and handling of samples

Upon laboratory arrival, individual faecal samples were homogenized by stirring thoroughly with a sterile tongue depressor or spoon for a few minutes. From each pig sample, two 2-ml cryotubes were filled and frozen immediately at -80 °C (alternatively at -20 °C for maximum 4 days, before transferring to -80 °C). For broiler samples, two cryotubes were prepared with at least 0.5 g faeces each. Sample pooling was either done immediately or the frozen tubes were shipped to the Technical University of Denmark (DTU) on dry ice for pooling.

Individual samples from the same herd were defrosted and placed on ice briefly before weighing. Following weighing, they were pooled with 0.5 g faeces from each sample and stirred for a few minutes with a sterile device (for example, a disposable wooden tongue depressor). All samples were only thawed once shortly before DNA extraction.

After the removal of two mislabelled samples, composite samples from a total of 178 broiler flocks and 181 pig herds remained.

Sampling to estimate the effect of random sampling

To study the potential effect of sampling randomness and the reproducibility of our sampling protocol, a Belgian and a Dutch pig herd were chosen for triplicate sampling. These two herds were sampled three times on the same day (25 samples x 3 sampling rounds), resulting in six pooled samples (2 herds x 3 sampling rounds), from which the within-farm resistome variation was assessed. A table with all samples and associated data is included as Supplementary Table 2.9.

DNA extraction and sequencing

From each of the pooled, herd-level faecal samples, DNA was extracted using a modified QIAamp Fast DNA Stool Mini Kit protocol (51604, Qiagen), as previously described.²³ One major modification is the addition of a bead-beating step at the beginning of DNA extraction. The protocol can be found at figshare.com/articles/SOP_-_DNA_Isolation_QIAamp_Fast_DNA_Stool_Modified/3475406. DNA purification of all the pooled samples was processed centrally at the DTU, and the DNA was stored in duplicates at -20 °C until further use.

DNA was shipped on dry ice for library preparation and sequencing at the Oklahoma Medical Research Foundation (OMRF; Oklahoma City, OK, USA). At the OMRF, DNA from all samples was mechanically sheared using ultrasonication to a targeted fragment size of 300 bp (Covaris E220evolution). For pooled pig samples, library preparation was performed with the NEXTflex PCR-Free library preparation kit (Bioo Scientific). For poultry samples owing to a lower DNA availability, the minimal amplification-based KAPA Hyper kit (Kapa Biosystems) was used. For all samples, the Bioo NEXTflex-96 adapter set (Bioo Scientific) was used. In batches of roughly 60 samples, the libraries were multiplexed and sequenced on the HiSeq3000 platform (Illumina), using 2 x 150-bp paired-end sequencing per flow cell. A total of 17 Belgian, Danish and Dutch pig faecal samples were sequenced on the HiSeq2500 platform (Illumina), using 2 x 100-bp paired-end sequencing before it became unavailable at the OMRF (see Supplementary Table 2.9).

In total, DNA from 365 pooled samples was extracted and shotgun sequenced, resulting in >36 billion sequences (18 billion paired-end reads), comprising >5,000 Gb of DNA. The sequencing yielded an average of 50 million (s.d.: 18×10^6) paired-end reads per pooled sample. This was similar for pig and poultry samples, although the sampling depth was more varied in pig samples.

Bioinformatics processing

The DNA sequences (FASTQ reads) from each sample were analysed following the principles from the previously described MGmapper tool.¹⁵ To avoid PCR copies in the poultry data, identical read pairs were removed using 'MarkDuplicates' from the Picard software (v2.8.3; broadinstitute.github.io/picard). Adaptor sequences were removed using BBduk (BBMap software).²⁹ Sequences from phiX174, which is an internal sequencing control, were removed using the BWA-MEM algorithm.³⁰ Trimmed read pairs were aligned using the BWA-MEM algorithm (Burrows-Wheeler aligner) to the prokaryotic RefSeq genomes from the NCBI GenBank with 'reference' and 'representative' tags (downloaded on 18 November 2016). The BWA-MEM algorithm (v0.7.15) normally estimates the insert size individually per computer CPU core. We used a per-sample estimate to increase the robustness of the estimated insert sizes and, therefore, the acceptable mapping distances for read pairs.

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

Properly paired read pairs, with at least 50-bp alignment in each read were accepted. ResFinder mapping counts were adjusted for differences in both gene lengths and bacterial sequence abundances by computing FPKM values for each ResFinder reference sequence.³² Raw mapping count data and their associated FPKM values can be found in Supplementary Tables 2.2 and 2.10.

Genes with many alleles in ResFinder result in unspecific mapping and randomly assigned read pairs. To avoid sensitivity loss and wrong assignments, we kept ambiguous hits, but aggregated their abundances to higher levels, corresponding to 90% gene identity clusters. To determine these clusters, we used CD-HIT-EST (v4.6.6) at a 90% identity level and otherwise default settings.³³ The resulting gene clusters were manually inspected and named to reflect their gene members (Supplementary Table 2.11). In addition to this 'gene cluster' level, we summed the FPKMs to resistance phenotype levels, as annotated in the ResFinder database.

FRD

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

Briefly, in each of these studies, DNA was extracted from environmental and human faecal samples, fragmented and cloned into a plasmid vector and screened for AMR functionality in *E. coli* cultured with one of several antimicrobials. Cloned fragments in plasmids that were found to confer AMR were sequenced and the AMR genes were identified. The protocol for the database construction can be found at cge.cbs.dtu.dk/services/ResFinderFG. Genes were quantified using MGmapper as was done for ResFinder. Genes with >90% identity to ResFinder genes were removed post-mapping to obtain the set of FRD genes that was absent from ResFinder. The reference gene abundances were summed to 90% gene clusters, using CD-HIT-EST, as was done for ResFinder.³³ The most frequent gene clusters remaining were derived from genes selected using trimethoprim, chloramphenicol, co-trimoxazole, cycloserine, amoxicillin, gentamicin, penicillin and tetracycline.

PCoA and resistome clustering

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

AMU in livestock

Data for the national livestock AMU was obtained from the ESVAC report and were stratified by major drug family.¹² The mass of active compound sold for use in animals in 2014 was divided by the PCU in 10⁶ kg, approximating the biomass.

The PCU is a unit that allows inter-species integration by adjusting for import/export and differences in the average weight between species when they are most likely to receive antimicrobial treatment. The estimate was multiplied by 1,000 to obtain drug per mg per PCU livestock. The country-specific veterinary drug use can be found in Supplementary Table 2.6 and Supplementary Figure 2.15.

In addition to national veterinary AMU, we obtained data from collaborating researchers on the average treatment incidents in the sampled farms, stratified by antimicrobial class, country and livestock species. The treatment incidence was calculated as the antimicrobial dose per defined daily animal doses (DDDvet) per 1,000 animals at risk, adjusting for 200-day and 40-day production cycles for pigs and poultry, respectively.³⁸ These average AMU values for pigs and poultry,^{39,40} stratified by drug group, are presented in Supplementary Tables 2.7 and 2.8, respectively. Data are visualized in Supplementary Figures 2.16 and 2.17.

Procrustes analyses

To determine the effect of the underlying microbiota on the resistome, we used Procrustes analysis. The gene cluster FPKM ResFinder matrix and the genus-level FPKM taxonomy matrix were Hellinger transformed and Bray-Curtis dissimilarities were calculated. Each dissimilarity matrix was ordinated using PCoA. The symmetric Procrustes correlation coefficients between the bacteriome and the resistome ordinations, p-values and plots were obtained using the 'protest' function in *vegan*.⁴¹

To test the association between AMU patterns and the resistomes, we also used Procrustes analysis as follows. A PCoA was generated from Euclidean distances between the samples in the AMU data. The AMU PCoA configuration was tested against the AMR gene cluster PCoA configuration using the 'protest' function with the default 999 permutations. This was done separately for pig and poultry samples.

Alpha diversity

For all samples, we computed the within-herd resistome diversity using the Simpson's Index of Diversity (1-D), the Chao1 richness estimate and Pielou's evenness.⁴² The gene cluster count matrix was rarified to 10,000 hits per sample for alpha diversity estimation, leading to the exclusion of samples with fewer hits to the AMR database.

Visualization

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

The circular Bray-Curtis resistome dendrogram was constructed by exporting the dendrogram in Newick format using the *ape* package and further annotating it using the Interactive Tree of Life tool.^{43,44} Barplots, boxplots and scatter plots were produced using the *ggplot2* R library.⁴⁵ The R library *RcolorBrewer* was used to generate the colour palettes used for the figures. This library is based on work by C. A. Brewer (www.ColorBrewer.org).

Statistical analyses

All statistics were done in Microsoft R Open 3.3.2, using the libraries and the procedures detailed below. The exact package versions can be found here: mran.revolutionanalytics.com/snapshot/2016-11-01/bin/windows/contrib/3.3. For statistical tests, only samples from the first visit to the triple-sampled herds were included (see Supplementary Table 2.9), meaning $N_{\text{pig}}=181$ and $N_{\text{poultry}}=178$. Unless otherwise mentioned, all statistical analyses were performed on pigs and poultry separately.

Association between AMU and AMR

To test the effect of the total AMU on the total metagenomic AMR abundance (the sum of all genes), we used the lme4 1.1-12 package to make linear mixed-effects regression models, with the total livestock drug usage (the sum of the ESVAC PCUs) as the independent variable, the total AMR abundance (the sum of FPKM) as the dependent variable and country as a mixed effect intercept.⁴⁶ The total AMU was log-transformed, which resulted in lower Akaike's information criteria. Pig sample residuals and country residuals showed normality and so did the poultry country residuals. Poultry sample residuals had a longer right tail, but square-root transformation of the poultry AMR data gave more-normal residuals and a similar conclusion ($p<0.05$). The effect and significance of drug usage were assessed using likelihood-ratio tests, comparing the random-effects models with

and without the AMU effect. The ResFinder-treatment incidents and FRD-PCU tests were done in the same way as the ResFinder-PCU tests.

Differential abundance analysis

To identify AMR genes that differ in abundance between countries, we analysed the gene cluster count matrix using the DESeq2 package as previously recommended for metagenomic read count data.^{47,48} This was done on the full count matrix, based on recommendations that rarefying is not warranted in metagenomic studies.⁴⁸ The read-pair count matrices for pigs and poultry were analysed separately. The number of mapped bacterial pairs was divided by the minimum number of mapped bacterial pairs and was used as the size factor. For each gene, we used a two-sided Wald test to determine whether the fold change between countries differed from zero and extracted all the country-versus-country results. P-values were adjusted for the false discovery rate (FDR) using the Benjamini-Hochberg approach and we used a significance threshold of alpha: 0.05.⁴⁹

Core resistome

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

Data availability

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

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Competing interests

The authors declare no competing interests.

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Supplementary data

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S2.1 Supplementary figures

All below supplementary figures are also available online and can be retrieved through: www.nature.com/articles/s41564-018-0192-9.

Supplementary Figure 2.1 Total AMR per sampled herd

Stacked bar chart of total relative AMR abundance in FPKM, separated by colour (AMR phenotype), sample (x-axis) and host species (top/bottom). This is an unscaled version of Figure 2.1b, where the height of each bar corresponds to the FPKM ResFinder AMR in a sample, rather than being proportional to within-sample AMR.

Due to its size, Supplementary Figure 2.2 can be found at the end of this document or can be retrieved through the online publication in Nature Microbiology.

Supplementary Figure 2.2 AMR phenotype heatmap

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

Supplementary Figure 2.3 Resistome sample similarity heatmap

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

Supplementary Figure 2.4 Pig and poultry farms have distinct resistomes

(a) PCoA plot of pig and poultry resistomes based on ResFinder gene clusters. $N=359$ metagenomes from independent herds. 20 metagenomes per livestock species per country was used, except for BG pig (21), BG poultry (19) and DE poultry (19). (b) Distribution of sample-wise distances to their group (livestock species) centroid. $N_{\text{pig}}=181$, $N_{\text{poultry}}=178$. Horizontal box lines represent Q1, median and Q3. Whiskers denote the range of points within $Q1-1.5 \times IQR$.

Supplementary Figure 2.5 Resistomes cluster according to country

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

Due to its size, Supplementary Figure 2.6 can be found at the end of this document or can be retrieved through the online publication in Nature Microbiology.

Supplementary Figure 2.6 AMR gene heatmap

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

Due to its size, Supplementary Figure 2.7 can be found at the end of this document or can be retrieved through the online publication in Nature Microbiology.

Supplementary Figure 2.7 AMR core gene heatmap

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

Supplementary Figure 2.8 Strong country effect on differential abundance of AMR genes

DESeq2 Wald tests were performed on the AMR mapping paired reads, testing for non-zero $\log_2$ fold change for each gene between countries. All country-versus-country unadjusted p-values were extracted and visualized for (a) pig (N=181 independent herd) and (b) poultry (N=178 independent herd) metagenomes. 20 metagenomes per livestock species per country was used, except for BG pig (21), BG poultry (19) and DE poultry (19).

Supplementary Figure 2.9 Number of unique AMR genes in pig and poultry are associated

Country-wise average AMR Chao1 indexes are plotted and were used for correlation (Spearman rank, N=9).

Supplementary Figure 2.10 Resistome sampling effort

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

Supplementary Figure 2.11 Association between drug treatment incidence (TI) and total AMR in sampled farms

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

Supplementary Figure 2.12 Procrustes analyses comparing pig and poultry resistomes with country-specific drug usage

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

Supplementary Figure 2.13 Resistome clustering for the functional resistance database (FRD)

Supplementary Figure 2.13 Resistome clustering for the functional resistance database (FRD)

The AMR genes present in FRD, but absent from ResFinder, were subjected to the same ordination analysis performed on the ResFinder genes. N=359 independent herds. 20 metagenomes per livestock species per country was used, except for BG pig (21), BG poultry (19) and DE poultry (19). This figure is analogous to Supplementary Figures 2.4 and 2.5, which should be referenced for details. Horizontal box lines represent Q1, median and Q3. Whiskers denote the range of points within $Q1-1.5*IQR$ and $Q3+1.5*IQR$.

Supplementary Figure 2.14 National veterinary AMU is not associated with FRD AMR

Analogous to Figure 2.6 in the main text (ResFinder). No association was found between the FRD genes' total abundance and ESVAC national veterinary AMU when analysed with a two-tailed, linear mixed-effects regression model for pigs ($p=0.3785$, $N=181$ independent herds) or poultry ($p=0.9392$, $N=178$ independent herds).

Supplementary Figure 2.15 National veterinary drug use per country

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

Supplementary Figure 2.16 Average drug use in sampled pig herds

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

Supplementary Figure 2.17 Average drug use in sampled poultry herds

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

S2.2 Supplementary tables

All below supplementary tables are only available online (Excel sheets) and can be retrieved through: www.nature.com/articles/s41564-018-0192-9.

Supplementary Table 2.1 ResFinder class abundance

The relative abundance of ResFinder-annotated drug classes (rows) per sample (columns) in fragments per Kilobase reference per million bacterial fragments (FPKM).

Supplementary Table 2.2 ResFinder count data

The number of paired-end reads assigned to each of the ResFinder reference sequences (rows) in each sample (columns).

Supplementary Table 2.3 Differential abundance in pigs

Results from testing for difference in mean abundance of AMR genes between any two countries' pigs (N=181 independent herds). The Benjamini- Hochberg procedure was used to adjust p-values for multiple testing (p-value (FDR) column).

Supplementary Table 2.4 Differential abundance in poultry

Results from testing for difference in mean abundance of AMR genes between any two countries' poultry (N=178 independent herds). The Benjamini- Hochberg procedure was used to adjust p-values for multiple testing (p-value (FDR) column).

Supplementary Table 2.5 Antimicrobial resistance alpha diversity

Indices for diversity, evenness and richness of AMR genes in the metagenomic samples. Samples with less than 10,000 fragments were excluded and the remaining samples were rarified to this level for the calculations.

Supplementary Table 2.6 National veterinary drug use

The relative drug use in the veterinary field, stratified by drug class and country. Numbers are in mg per PCU and are derived from the 2014 ESVAC report.

Supplementary Table 2.7 Pig farm drug use

Averages treatment incidence (TI) among the sampled pig farms, stratified by drug class and country.

Supplementary Table 2.8 Poultry farm drug use

Averages treatment incidence (TI) among the sampled pig farms, stratified by drug class and country.

Supplementary Table 2.9 Sample data

Each line corresponds to a pooled, herd-level metagenomic sample. 'Sample' is a unique sample ID. 'Read Pairs' refers to the number of read pairs remaining after quality- and adapter-trimming. 'Bacterial Read Pairs' refers to the number of read pairs assigned to bacterial genera. 'Country' refers to the country of origin of a herd. 'Sample type' specifies whether the pooled sample is from pig or poultry. The 'Note' column denotes whether a sample was sequenced on an Illumina HiSeq2500 instrument and which samples are not independent (i.e. from the same farm).

Supplementary Table 2.10 ResFinder relative abundance

The relative abundance of each ResFinder reference sequence (rows) in each sample (columns) in fragments per Kilobase reference per million bacterial fragments (FPKM).

Supplementary Table 2.11 ResFinder gene clusters

Table used for summarizing ResFinder reference abundances to higher levels. The C-E columns were derived by clustering the ResFinder reference sequences to 90% identity using CD-HIT (See methods section) and manually re-naming the clusters to reflect their contents. The 'AMR class' column reflects the drug(s) a given gene cluster protects against.

S2.3 Herd selection and sampling

Here we describe the selection of pig and broiler farms and corresponding sampling procedures as they were performed. Comparisons to the original criteria described in the sampling protocol were made using data collected at the farms through a questionnaire. This overview is based on data from 178 broiler farms and 181 pig farms. We sampled animals as close as possible to slaughter to elucidate the potential consumer exposure to AMR associated with meat production in Europe.

Selection of pig farms

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

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

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

The farms needed to have at least 150 sows and 600 fatteners on average. Overall, the average number of sows per farm in all the farms sampled was 496 and had a country-wise minimum average of 322 (FR) and maximum of 822 (ES). The total number of fatteners at the moment of sampling was unfortunately not investigated in the visited farms; however, the overall median number of fatteners set-up per year was 5,100, with a country-wise minimum median of 3250 (BG) and maximum of 13,700 (IT).

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

Belgium: A random list of pig farms was obtained from the government. From this list, we contacted farmers for participation.

Bulgaria: The farms were selected from two geographic regions: Northern- and Southern-Bulgaria. We visited farms where the owners/managers agreed to participate.

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

France: Farms were all designated by veterinary practitioners we contacted. We managed to involve different production organizations located in the west part of France (conveniently located within maximum 2h30min to the laboratory, to respect requirements regarding samples' transportation conditions).

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

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

The Netherlands: We recruited farms in collaboration with nine veterinary practices and one pig farming organization spread across the Netherlands. If the practice agreed to participate, the practice was asked to select 1 to 3 farms (depending on the size of the veterinary practice) that fulfilled the selection criteria. When a farmer showed interest, we contacted the farmer and explained the research, after which the farmer decided to participate or not. We kept a record with reasons for not participating.

Poland: We contacted veterinary practitioners by phone from a list of specialists in pig diseases and asked them for participation in the project. Of those who

accepted the invitation and did work with pig farms, we asked a list of farms complying with the selection criteria. The practitioners nominated the farm(s) of interest.

Spain: First, we contacted veterinary practitioners distributed across the country who proposed eligible farms. Then we selected farms randomly from this list and coordinated the sampling with the veterinarians. The stratification was based on the answers of the practitioners and the number of farms available per area of work. Regional stratification was not possible because we did not obtain feedback from practitioners of all regions.

Sampling of pig farms

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

To avoid seasonal influences, we planned to divide sampling over the year, however, most farms were sampled in the fall (58) and summer (68), and the least in winter (25) and spring (29). BG did not collect any samples in spring, and ES and NL did not sample in winter.

Per farm, we collected 25 fresh, still warm and undisturbed faecal droppings from the floor of separate pens (a minimum of 10 g faeces per sample), divided over all present compartments/stables of fatteners close to slaughter. When there were less than 25 pens, we divided the 25 samples over all pens.

Selection of broiler farms

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

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

The selected farms needed to be conventional with no intended slaughter age higher than 50 days, no slow-growing breeds (intended growth rate less than 55 g per day), and no stocking density lower than 10 birds per m². All sampled farms were conventional. The average slaughter weight across all farms visited

was 2,372 kg, with minimum 1,300 kg and maximum 3,700 kg. One country (FR) had a particularly low average weight at slaughter compared with the others (1,743 kg). The country-specific stocking density in birds per m² was calculated using the stocking density in kg per m² and the country's broilers average weight at slaughter in kg. Among all countries, the stocking density varied from a minimum of 12.26 (ES) and a maximum of 24.66 (FR), with country averages varying between a minimum of 13.77 (PL) and a maximum of 22.71 (FR). The overall average stocking density of all farms was 16.29 birds per m². We included only one flock/house per holding, and the flock size criterion was a minimum of 20,000 and a maximum of 40,000 broilers per house. The average size of the flocks across the 181 farms was 28,044 birds at set-up. One country did not fulfil the criterion for maximum flock size (DK-53,300), whereas most countries did not fulfil the criterion for minimum flock size (BE-16,500; BG-8,000; DE-17,200; ES-14,000; IT-11,340; NL-17,550; PL-18,500).

We planned to randomly select farms to participate in the study, using regional stratification whenever possible. This protocol was not fulfilled in all cases. Below, we describe the selection procedure applied in each country:

Belgium: A random list of broiler farms was obtained from the government. From this list, farmers were contacted for participation.

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

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

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

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

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

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

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

Spain: First, we contacted veterinary practitioners distributed across the country who proposed eligible farms. Then we selected farms randomly from this list and coordinated the sampling with the veterinarians. The stratification was based on the answers of the practitioners and the number of farms available per area of work. Regional stratification was not possible because we did not obtain feedback from practitioners of all regions.

Sampling of broiler farms

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

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

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

Chapter 3

The antimicrobial resistome in relation to antimicrobial use and biosecurity in pig farming, a metagenome-wide association study in nine European countries

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Abstract

Objectives

Previous studies in food-producing animals have shown associations between antimicrobial use (AMU) and antimicrobial resistance (AMR) in specifically isolated bacterial species. Multi-country data are scarce and only describe between-country differences. Here we investigate associations between the pig faecal mobile resistome and characteristics at the farm-level across Europe.

Methods

A cross-sectional study was conducted among 176 conventional pig farms from nine European countries. Twenty-five faecal samples from fattening pigs were pooled per farm and acquired resistomes were determined using shotgun metagenomics and the Resfinder reference database, i.e. the full collection of horizontally acquired AMR genes (ARGs). Normalized fragments resistance genes per kilobase reference per million bacterial fragments (FPKM) were calculated. Specific farm-level data (AMU, biosecurity) were collected. Random-effects meta-analyses were performed by country, relating farm-level data to relative ARG abundances (FPKM).

Results

Total AMU during fattening was positively associated with total AMR (total FPKM). Positive associations were particularly observed between widely used macrolides and tetracyclines, and ARGs corresponding to the respective antimicrobial classes. Significant AMU-ARG associations were not found for β -lactams and only few colistin ARGs were found, despite high use of these antimicrobial classes in younger pigs. Increased internal biosecurity was directly related to higher abundances of ARGs mainly encoding macrolide resistance. These effects of biosecurity were independent of AMU in mutually adjusted models.

Conclusions

Using resistome data in association studies adds accuracy and new insights to previously observed AMU-AMR associations. Major components of the pig resistome are positively and independently associated with on-farm AMU and biosecurity conditions.

3.1 Introduction

In many European countries, antimicrobial resistance (AMR) rates in human and zoonotic bacteria are high and/or on the rise.^{1,2} Unfortunately, antimicrobial drug discovery is not keeping pace with the rise of AMR.³ In 2014, this lack of appropriate treatment options was estimated to result in ~700,000 attributable human deaths per annum worldwide.⁴ While the need for research on new drugs is self-evident, aetiological research investigating determinants of AMR is equally warranted.

It is generally accepted that veterinary antimicrobial use (AMU) promotes AMR in animals, and AMR can be transmitted from animals to humans via the food chain or through direct animal contact.⁵ To date, the majority of association studies in food-producing animals have focused on the relation between AMR and AMU or other farm practices within one particular resistant bacterial species and/or pathogen, e.g. studies on MRSA (pigs, veal)^{6–9} or *Escherichia coli* (pigs, poultry, veal calves, dairy cows).^{10–15} Few studies report on multi-country data, but mostly report on associations between AMU and AMR at the ecological level.^{5,16} Since regional differences in AMR are observed between European countries,¹ insight into the role of on-farm practices that contribute to the observed differences is paramount to reduce AMR in farm animals.

In 2006 the term ‘resistome’ was proposed to designate the full collection of resistance genes in microorganisms and microbial populations.^{17,18} In recent years, the decreasing costs of high throughput metagenomics approaches¹⁹ have enabled researchers to study the resistome in a multitude of different reservoirs, e.g. humans, animals, soil, toilet waste, sewage and permafrost.^{20–27} Acquired resistance genes from the resistome can be transmitted between bacterial species through horizontal gene transfer. Rather than focusing on specific (pathogenic) resistant bacteria, studying this ‘mobile resistome’ can provide a more complete understanding of the epidemiology of AMR in the complex human-animal interface.²⁵ Currently, metagenomic resistome data are scarcely used in aetiologic studies. In 2016, Xiao *et al.*²⁸ investigated the influence of overall AMU in pigs on the composition of the pig resistome. Despite the limited number of investigated farms, higher abundances of ARGs were shown in pigs continuously fed low doses of antimicrobials.

More recently, our research group demonstrated positive associations between country-level AMU in pigs and the pig resistome (relative ARG abundances).²⁹ Within the present study we further build upon the latter study by including detailed farm-level data; in particular, we used a shotgun metagenomics approach to investigate associations between the pig resistome and farm-level

risk factors (e.g. AMU classes, biosecurity, farm production figures). To the best of our knowledge, the inclusion of detailed farm-level data in epidemiological resistome models (a model including both farm-level AMU and the resistome) is not yet performed before.

3.2 Materials and methods

Study design

Between 4 June 2014 and 9 December 2015, we conducted a cross-sectional study among a convenience sample of 181 pig farms in 9 European countries (Belgium, Bulgaria, Denmark, France, Germany, Italy, the Netherlands, Poland and Spain). At each farm, 25 pig faecal samples were collected and pooled as described in the supporting materials. Figure 3.1 presents the distribution of sampled farms across Europe. Only conventional, non-mixed, farrow-to-finish farms (sporadic exceptions as mentioned before)²⁹ were selected and sampled across seasons. Farm recruiting occurred directly from a randomized database or through veterinarians and slaughterhouses. The selected herds had to have only one owner and no contact through trade. Additional selection criteria were required at the farm (location of the fatteners, minimum number of sows and fatteners present on the farm) and herd-level (all-in all-out batch production), yet not always met, as previously described in detail.²⁹

Data collection

For an elaborate description of the pig sampling procedure, handling of samples and storage, sample pooling, DNA-extraction, sequencing, metagenomic data clean-up and bioinformatics, the reader is referred to Munk *et al.*²⁹ The DNA sequences (reads) from the metagenomic samples are deposited in the European Nucleotide Archive (ENA) under project accession number PRJEB22062.

Summaries regarding sampling, pooling, laboratory work, metagenomics, and questionnaire data are provided below and in the Supplementary data of the current paper. Owing to lack of complete data, six farms [farm identifiers (IDs): 1001, 1009, 9001-9003 and 9006] were excluded from the analysis resulting in 176 farms. Analyses using biosecurity data were performed in all countries except one, due to lack of contrast and being out of range of expected values (country I).

Figure 3.1 Pig farm sampling across nine European countries

Yellow dots represent either single farms or a multitude of sampled pig farms for which GPS coordinates were available. Farm GPS coordinates used to prepare this map were anonymized and could, therefore, deviate up to 7 km from the true location. Sampling in France and Italy is respectively concentrated within Brittany and in Northern Italy, as most pig farming is concentrated there [Eurostat: [http://ec.europa.eu/eurostat/statistics-explained/index.php/File:Number_of_sows_by_region_\(2013\).png](http://ec.europa.eu/eurostat/statistics-explained/index.php/File:Number_of_sows_by_region_(2013).png), accessed 22nd February 2020].

Questionnaire data

Standardized farm questionnaires (including AMU, farm technical and biosecurity questions) were completed by the farmers together with the visiting researcher. To avoid social desirability bias, certain biosecurity questions were verified during farm inspections. Questionnaire data were entered and checked for quality in EpiData 3.1. Further data quality checks were performed using ActivePerl 5.24.1³⁰ and SAS 9.4.³¹ SAS was subsequently used for database management. To check

data entry quality, 10% of all questionnaires were entered twice and compared. AMU data were re-evaluated in-depth after conversion to Microsoft Excel, while other farm data was analysed descriptively in 'R' v.3.3.1.³² Inconsistencies were thoroughly re-evaluated by the researchers responsible for the data. Finally, a database was created that consists of all AMR data and metadata (farm characteristics and AMU). All farms were anonymized to ensure that results could not be traced back to individual farms. Countries were anonymized as required by the farming organization in one participating country.

Calculation of antimicrobial treatment incidences

The calculation of antimicrobial treatment incidences (TIs) is summarized in the Supplementary data. AMU group TIs based on the Defined Daily Doses Animal (DDDvet) were computed at farm-level. A group treatment was defined as any treatment applied simultaneously to all animals present in, at least, the smallest housing unit, and includes any parental or other curative group treatment next to metaphylactic or prophylactic group treatments. TI expresses the percentage of pigs receiving a dose of antimicrobials each day, or equivalently, during the percentage of time a pig is treated with antimicrobials in a certain production phase or its entire life. TIs were determined for three age categories (TI sucklers, weaners, fatteners) and a standardized 200-day lifespan³³ across age categories (TI 200, which corrects for possible differences in slaughter ages between herds and countries), per farm and by AMU class.

Since multiple farms did not use any antimicrobials, we also performed a separate analysis while dichotomizing certain AMU variables. Country AMU variables for which farms in at least seven countries expressed non-use were dichotomized at the median value. If non-use for a specific AMU variable was observed in all farms, except for one country, we excluded this country from this variable.

Furthermore, TIs based on antimicrobial purchases were calculated, which includes AMU purchase data from a standardized time period before the farm visit.³⁴ Unlike AMU purchase data, AMU group treatments directly relate to the sampled batch. Therefore, we only used AMU purchase data within a sensitivity analysis.

Biosecurity indexes calculation

The algorithm behind Biocheck.ugent®, a risk-based scoring tool to evaluate the quality of biosecurity within a pig herd, was used to calculate a biosecurity score for each farm based on 108 biosecurity questions.^{35,36} Each question was part of a biosecurity subcategory of external (measures to prevent disease introduction on

farms) or internal (measures to prevent disease spread within farms) biosecurity. Within these two subcategories, weights were assigned to each subcategory (Supplementary Table 3.1), and to each particular answer to a subquestion within each subcategory.³⁵ Total biosecurity was defined as the mean of external and internal biosecurity. One question from the original Biocheck.ugent® could not be included (“*How frequently is the fluid of footbaths changed*”) and the algorithm was therefore slightly modified. Overall, three main and 12 subscores were calculated per farm (Supplementary Table 3.1).

Statistical analysis

‘R’ software (version 3.3.2)³² was used for statistical analysis and creating figures. Farm technical variables were selected and checked for correlation. In case of high correlation (Spearman’s $\rho \geq 0.7$), we chose the most appropriate variable based on the risk-factor literature. To increase power and to evaluate known cross-resistance, all AMR variables were clustered by AMU class, and all AMU class variables were used as input for the models, together with several combinations of these AMR and AMU class variables (Supplementary Table 3.1). Since AMR was previously linked to local temperature, we also included sampling season as a potential determinant.³⁷

As described in the supporting materials, relative ARG abundances [fragments per kilobase reference per million bacterial fragments (FPKM)] were generated and aggregated [cluster levels: 90% identity level and AMR class]. Based on previous analyses²⁹ a large country effect was expected and therefore a random-effects meta-analysis (DerSimonian-Laird estimator, linear regression, by country) was chosen to relate, univariately, each ARG cluster to AMU, biosecurity and technical farm data (‘R’ packages *rmeta*, *metafor*). ARG abundances and AMU data were strongly skewed to the right requiring $\log_{10}$ transformation. Owing to excess zeros, a pseudocount of 1 was added before the $\log_{10}$ transformation.

Choosing a meta-analysis as statistical technique inevitably results in country-exclusion when no AMU/AMR is observed. To avoid spurious associations and overestimation of results, summary estimates were only preserved when estimates from at least four countries were incorporated.

The meta-analysis returned two p-values: one regarding the overall effect size [Benjamini-Hochberg false discovery rate (FDR) adjusted, $q < 0.1$ threshold], and the other was between-country heterogeneity ($p_h > 0.05$, absence of heterogeneity). Within a random-effects meta-analysis weights are assigned by country which takes the between and within-country variation into account. To prevent certain countries from influencing these weights excessively, ARG

clusters were standardized (mean=0, SD=1) by country. Models were first adjusted for potential confounders from the main list of univariate significant associations ($p<0.05$, $q<0.1$). To exclude confounding by AMU and biosecurity, we separately re-analysed the data adding both internal biosecurity and AMU to a model with its main associated AMR type. Internal biosecurity was chosen over other biosecurity variables (total biosecurity, internal biosecurity subcategories) based on high correlations (Spearman's $\rho\geq 0.7$) between biosecurity variables. For the most important associations defined in the univariate meta-analysis, the lower aggregation ARG clusters (90% identity level) were regressed on AMU and biosecurity.

Overall, the meta-analyses provide estimates of the association between the determinants and the relative ARG clusters (FPKM). These estimates should be considered as associations without direct straightforward and practical implementation. First, FPKM values are interpreted as the number of fragments of a certain clustered resistance gene within the full bacterial population present within a faecal sample, and do not allow direct absolute gene count comparison between samples. Since we added a pseudocount prior to $\log_{10}$ transformation (AMU and ARGs) 'calculating back' to easily interpretable estimates becomes challenging. Lastly, since we scaled the ARG abundances, the change in the determinants shall be interpreted as a change in standardized units of the ARGs.

3.3 Results

Farms

Questionnaires and resistome data were available from 176 farms. At least one farm per season was visited within each country, except for countries F and H (no winter), and country I (no spring and winter). Table 3.1 provides summary statistics of the sampled farms and batches. Certain variables indicate large within- and between-country differences, e.g. the number of fatteners sampled, fatteners set-up per year and producing sows.

Pig resistome and AMU group treatment data

Earlier reported resistome²⁹ and AMU data³⁴ for the 176 farms included in this study are summarized in the Supplementary data (Supplementary figures 3.1-3.5).

Table 3.1 Overview of sampled farms and batches of pigs

Characteristics	Country A	Country B	Country C	Country D	Country E	Country F	Country G	Country H	Country I	Overall
General farm information ^a										
No. of included farms	20	20	20	20	20	19	20	20	17	176 farms ^b
No. of farms which held other animals at farm for non-commercial purposes, <i>n</i> (%)**	14 (70.0)	4 (20.0)	16 (80.0)	5 (25.0)	11 (55.0)	17 (89.5)	3 (15.0)	9 (45.0)	5 (29.4)	84 (47.7)
No. of people working at the farm in total, <i>median</i> (10 th ,90 th percentiles)**	2.0 (1.0-3.1)	3.0 (2.0-6.2)	3.5 (2.0-7.1)	5.0 (2.9-10.0)	3.0 (2.0-5.0)	3.0 (1.8-8.0)	4.0 (2.0-17.8)	5.0 (2.0-12.2)	5.0 (2.0-23.2)	3.0 (2.0-10.0)
No. of farms with a certain type of batch management production system, <i>n</i> (%)***										
1-week (%)	3 (15.0)	7 (35.0)	11 (55.0)	10 (50.0)	3 (15.0)	10 (52.6)	11 (55.0)	11 (55.0)	6 (35.3)	72 (40.9)
2-week (%)	3 (15.0)	4 (20.0)	6 (30.0)	1 (5.0)	5 (25.0)	1 (5.3)	0	2 (10.0)	0	22 (12.5)
3-week (%)	4 (20.0)	5 (25.0)	1 (5.0)	8 (40.0)	9 (45.0)	2 (10.5)	7 (35.0)	4 (20.0)	0	40 (22.7)
4-week (%)	8 (40.0)	0	0	0	0	2 (10.5)	2 (10.0)	3 (15.0)	9 (52.9)	24 (13.6)
5-week (%)	2 (10.0)	3 (15.0)	0	1 (5.0)	3 (15.0)	3 (15.8)	0	0	2 (11.8)	14 (8.0)
other (%)	0	1 (5.0)	2 (10.0)	0	0	1 (5.3)	0	0	0	4 (2.27)
Characteristics of the sampled batch ^a										
No. of fatteners sampled, <i>median</i> (10 th ,90 th percentiles)**	307 (109-909)	160 (93-2623)	394 (154-777)	342 (226-713)	319 (120-495)	200 (95-568)	240 (129-433)	1100 (641-3000)	600 (122-1753)	333 (106-1339)

Table 3.1 Continued

Characteristics	Country A	Country B	Country C	Country D	Country E	Country F	Country G	Country H	Country I	Overall
Age of the fatteners at sampling in days, median (10 th -90 th percentiles) ^{a**}	182 (168-214)	192 (170-210)	150 ^c (123-172)	270 (232-287)	175 (161-189)	178 (165-191)	162 (149-172)	168 (150-182)	128 (110-164)	173 (135-246)
Farm technical data ^a										
No. of fatteners set-up/year, median (10 th -90 th percentiles) ^{a*}	4442 (2920-11306)	4429 (2870-10039)	4600 ^c (3292-7080)	13700 (4602-23070)	6371 (4009-13692)	6473 (3016-17417)	5075 (3000-12818)	4225 (1980-7380)	2000 (720-54800)	5100 (2052-17248)
No. of producing sows present at the farm/year, median (10 th -90 th percentiles) ^{a**}	266 (152-527)	341 (148-1353)	253 (149-629)	629 (276-1123)	276 (164-489)	290 (189-882)	250 (155-565)	550 (200-2056)	100 (44-750)	297 (149-1091)
No. of producing boars present at the farm/year, median (10 th -90 th percentiles) ^{a**}	2.0 (1.0-5.1)	2.0 (1.0-5.8)	4.0 (2.0-8.0)	4.5 (1.9-13.1)	2.0 (1.0-5.1)	2.0 (1.0-5.0)	2.0 (1.0-6.2)	3.0 (2.0-8.1)	5.0 (2.6-10.0)	2.0 (1.0-7.7)
Age of transfer of the weaners to growing-finishing department in days, median (10 th -90 th percentiles) ^{a**}	70 (63.9-84.6)	70 (67.8-85.5)	78 ^c (69.0-84.0)	90 (81.8-111.0)	70 (62.9-81.3)	70 (58.4-81.6)	68.5 (56-77.7)	60 (60.0-75.0)	72 (68-75)	71 (60-90)
Weaning age in days, median (10 th -90 th percentiles) ^{a**}	23.5 (20.0-25.2)	27.0 (21.0-28.0)	28.0 (25.0-32.1)	28.0 (24.6-28.0)	24.0 (21.0-28.0)	27.0 (22.6-28.0)	28.0 (24.6-28.0)	24.0 (21.0-28.0)	28.0 (28.0-35.0)	28.0 (21.0-29.0)
No. of weaned piglets/sow/year, median (10 th -90 th percentiles) ^{a**}	30.1 (23.5-32.1)	29.0 (22.3-31.0)	28.1 (26.3-31.1)	24.5 (22.4-27.1)	29.5 (27.9-31.6)	29.6 (27.7-31.3)	25.5 (22.9-30.1)	26.0 (22.9-27.6)	23 (18-26)	27.2 (22.5-30.9)

Letters A-I represent the nine countries. Percentages and percentiles are rounded to one or no digits behind the comma.

^a Significant difference between countries based on the Kruskal-Wallis test or continuity corrected X² test: <0.01*, <0.001**, not possible to test***.

^b In total, 33.0% (N: 58) of all farms were visited in fall, 16.5% (N: 29) in spring, 38.6% (N: 68) in summer and 11.9% (N: 21) in winter.

^c One observation was missing. Letters A – I represent the nine countries.

Figure 3.2 Overview of the three main biosecurity scores (total, external and internal) computed per farm presented by country (176 farms, boxplots)

Letters A – I represent the nine countries. Scores can vary between 0% (low) to 100% (high). A box represents the 25th (Q1) to 75th (Q3) percentile, the centerline depicts the median (Q2). Dots represent outliers (values smaller than $Q1 - 1.5 \times IQR$ or larger than $Q3 + 1.5 \times IQR$). An asterisk (*) presents the mean by country.

Biosecurity scores

Figure 3.2 displays the main calculated biosecurity scores from the 176 farms, showing large within- and between-country variation. The higher the score, the higher the respective type of farm biosecurity. Overall, farm external biosecurity (median=73.9) was higher than internal biosecurity (median=65.0). Furthermore, within these two biosecurity categories, the medians differed significantly between countries (Kruskal-Wallis, $p < 0.001$).

Random-effects meta-analysis

In total, we regressed 16 main ARG classes (clustering of ARGs based on AMR class) on 14 farm meta-variables (including season), 15 main AMU classes and 15 biosecurity scores in a random-effects meta-analysis by country (excluding AMU/AMR combinations; Supplementary Table 3.1). The univariate meta-analysis showed a positive association between total AMU during the fattening phase and total ARG (AMR class clustering) in pigs ($\beta = 0.76$, $p < 0.01$; Figure 3.3), but not

Figure 3.3 Univariate meta-analysis by country: total AMU during fattening phase versus total relative ARG abundance (total FPKM), $p < 0.01$

Letters A – I represent the nine countries. The heterogeneity test (p_h) is significant only when including Country F (very low AMU). Without country F: summary estimate $\beta = 0.64$, $p = 0.014$ and $p_h > 0.05$. β [CI], estimate of the linear regression and [upper and lower CI]. p_h , p-value heterogeneity test. Weight, the assigned weight per country.

for AMU during other life phases. In total, 122 significant associations between ARG classes and determinants ($p < 0.05$) were identified including potential confounders. After excluding confounders and correcting for multiple testing, 18 associations remained ($q < 0.1$, $p_h > 0.05$; Table 3.2).

Among the main determinants for AMR, we found positive corresponding AMU-ARG associations for macrolides and tetracyclines. Interestingly, no similar associations existed between the most commonly used antimicrobials colistin and aminopenicillins and their corresponding ARG classes. When aminopenicillin use was high in sucklers or weaners, and low in fatteners, no association was found with the corresponding ARG class (β -lactam resistance was relatively low). Similarly, colistin use was high in sucklers, lower in fatteners (only 9% of the farms use colistin in fatteners, compared with 18% that use macrolides) and low colistin resistance was observed.

We also found positive associations between non-corresponding AMU-ARG classes such as β -lactam use (penicillins, aminopenicillins and cephalosporins) and amphenicol resistance. Next to AMU, we identified higher internal biosecurity

as a determinant for (mainly) macrolide resistance. These latter associations remained after adjustment for AMU (Table 3.2).

Many farms did not use critically important antimicrobials such as (fluoro)quinolones or aminoglycosides, and dichotomous AMU was deemed more appropriate in these cases. This partly dichotomized analysis confirmed the results from the main analysis (except the association between penicillin/aminopenicillin use and amphenicol resistance), although after correcting for the FDR, these associations were no longer statistically significant. Furthermore, in comparison with the main analysis, amphenicol use (TI 200) was positively associated with amphenicol resistance ($q < 0.001$). Another sensitivity analysis using AMU purchase data instead of AMU group treatments only confirmed the positive associations involving macrolide resistance (results not shown).

Table 3.3 displays an overview of the top 10 ARG clusters (90% identity level) within the main ARG classes identified in the meta-analysis. Large between-country differences (20%-40%) were observed when examining the contribution of the most abundant gene cluster within each antimicrobial class, e.g. for macrolide, *mefA_3_AF227521*; tetracycline, *tetQ*; and amphenicol, *cat2*. The univariate meta-analysis was repeated by including these ARG clusters, for which the most important associations ($q < 0.1$, $p_h > 0.05$) are presented in Table 3.4.

3.4 Discussion

The present study evaluates associations between farm characteristics and faecal (largely) mobile resistomes of conventionally held fattening pigs across nine European countries. The amount of macrolides and tetracyclines used was associated with the abundance of macrolide and tetracycline resistance genes. Furthermore, internal biosecurity was independently associated with (mainly) increased macrolide resistance.

Our results show robust evidence of direct ARG selection by these two widely used antimicrobial classes (macrolides, tetracyclines). These effects were observed when evaluating both levels of ARG clustering (ARG class and 90% identity level). Macrolide and tetracycline use in pigs have previously been linked to, respectively, macrolide and tetracycline resistance in specific bacterial species.^{10,38–41} We also showed evidence for cross-ARG selection (a combination of macrolide, lincosamide and lincomycin/spectinomycin use versus macrolide resistance) and co-selection of ARGs (a combination of penicillin, aminopenicillin and cephalosporin use versus amphenicol resistance). The observed cross-resistance to macrolide and lincosamide use is expected based

on the mechanism behind macrolide resistance.^{42,43} However, to the best of our knowledge, the association between β -lactam use and amphenicol ARGs has not been described before in pigs. Nevertheless, co-selection in pigs has been suggested in metagenomic studies. It was, for instance, previously observed that in-feed ASP250 (chlortetracycline, sulfamethazine and penicillin) treatment in experimentally held pigs, increased ARG abundance beyond the administered ASP250, including amphenicol resistance.⁴⁴

Sensitivity analyses using antimicrobial purchase and dichotomized AMU data did not fundamentally change our main findings involving macrolide AMU-AMR associations. Nonetheless, dichotomization of AMU only confirmed our results prior to FDR correction. We consider this a negligible discrepancy since FDR correction could be considered conservative⁴⁵ and dichotomizing continuous variables likely results in loss of information and statistical power.⁴⁶

In our study, the observed AMU-ARG associations might be characterized in different ways. Most antimicrobial group treatments were administered during early production stages (suckling and weaning), but we mainly found AMU-ARG associations corresponding to treatments administered during the fattening period (with the least AMU recorded).³⁴ Interestingly, when some antimicrobial classes were highly and specifically used in early life (e.g. aminopenicillins and colistin), no AMU-ARG relationships and only few colistin ARGs were found. Considering the assessment of the resistome in the final fattening phase, these findings suggest short-term selective pressure posed by early age AMU. Declining or lower AMR in the (final) fattening phase compared with AMR during earlier ages has been described before in longitudinal studies regarding ESBLs⁴⁷ and MRSA⁴⁸ in pigs. Although read-mapping was chosen over assembly-based strategies for greater sensitivity, this lack of associations might also be assigned to low(er) ARG cluster levels (e.g. *mcr* or *bla*_{CTX-M}) detected within our metagenomic survey [e.g. in comparison to phenotypic assays (*bla*_{CTX-M})⁴⁹ and since we only mapped against ResFinder⁵⁰]. Another way to characterize these findings is by hypothesizing that the lack of AMU-ARG associations related to high colistin and β -lactam use potentially reflects natural history. It could be imagined that sustained past high use of certain antimicrobials, together with potentially differential bacterial fitness costs related to resistance,^{51,52} developed and established the currently circulating pool of resistance genes.

Biocheck.ugent® scores for internal and total biosecurity were shown to be positively associated with macrolide resistance. The separate country estimates are consistently positive in most countries. Since the estimates hardly changed when adding both internal biosecurity and AMU to the model for macrolide resistance, we conclude that internal biosecurity is an independent determinant

Table 3.2 Results random-effects meta-analysis: AMR class clusters ($q < 0.1$, $p_h > 0.05$)

AMU group treatments and biosecurity scores	AMR class	β	β^*	β^{**}	[LCI, UCI]	[LCI, UCI]*	[LCI, UCI]**	q	p*	p**	Countries***
Lincosamide & macrolide use (fatteners)*	macrolide, oxazolidinone†	1.076	1.047	1.018	[0.602,1.550]	[0.530,1.563]	[0.567,1.469]	0.006	0.0001	<0.0001	8
Lincosamide & macrolide use (fatteners)*	macrolide	1.076	1.046	1.018	[0.602,1.550]	[0.530,1.563]	[0.567,1.469]	0.006	0.0001	<0.0001	8
Internal biosecurity	macrolide	0.027	0.022	0.029	[0.014,0.039]	[0.007,0.038]	[0.015,0.042]	0.013	0.0049	<0.0001	8
Internal biosecurity	macrolide, oxazolidinone†	0.027	0.022	0.029	[0.014,0.039]	[0.007,0.038]	[0.015,0.042]	0.013	0.0049	<0.0001	8
Macrolide use (fatteners)	macrolide	1.061	1.118	1.258	[0.537,1.585]	[0.506,1.730]	[0.795,1.721]	0.015	0.0003	<0.0001	7
Macrolide use (fatteners)	macrolide, oxazolidinone†	1.061	1.118	1.258	[0.537,1.585]	[0.506,1.730]	[0.795,1.721]	0.015	0.0003	<0.0001	7
Lincosamide & macrolide use (200 days)*	macrolide, oxazolidinone†	0.825	0.925	0.750	[0.410,1.239]	[0.458,1.393]	[0.191,1.310]	0.016	0.0001	0.0085	8
Lincosamide & macrolide use (200 days)*	macrolide	0.825	0.926	0.750	[0.410,1.240]	[0.459,1.393]	[0.191,1.309]	0.016	0.0001	0.0085	8
IB: Cleaning & disinfection	macrolide	0.015	0.011		[0.007,0.023]	[0.002,0.020]		0.024	0.0126		8
IB: Cleaning & disinfection	macrolide, oxazolidinone†	0.015	0.011		[0.007,0.023]	[0.002,0.020]		0.024	0.0126		8
IB: Measures comp. & equipment use	vancomycin	0.016	0.016		[0.008,0.025]	[0.006,0.025]		0.025	0.0017		8
β -lactam use (fatteners)#	phenicol (amphenicol)	1.212	1.332		[0.569,1.855]	[0.619,2.044]		0.025	0.0002		7
Tetracycline use (200 days)**	tetracycline	0.754	0.558		[0.346,1.163]	[0.089,1.027]		0.031	0.0197		9

Table 3.2 Continued

AMU group treatments and biosecurity scores	AMR class	β	β^*	β^{**}	[LCI, UCI]	[LCI, UCI]*	[LCI, UCI]**	q	p*	p**	Countries***
IB: Measures comp. & equipment use	macrolide	0.016	0.016		[0.007,0.024]	[0.007,0.025]		0.032	0.0006		8
IB: Measures comp. & equipment use	macrolide, oxazolidinone†	0.016	0.016		[0.007,0.024]	[0.007,0.025]		0.032	0.0006		8
Total biosecurity	macrolide	0.032	$p_h < 0.05$		[0.014,0.051]	$p_h < 0.05$		0.042	$p_h < 0.05$		8
Total biosecurity	macrolide, oxazolidinone†	0.032	$p_h < 0.05$		[0.014,0.051]	$p_h < 0.05$		0.042	$p_h < 0.05$		8
β -lactam use (fatteners)##	phenicol (amphenicol)	1.163	1.275		[0.500,1.825]	[0.561,1.989]		0.044	0.0005		7

All figures are rounded. Presented q-value is only applicable to β , [LCI, UCI], lower and upper CI. * β [LCI, UCI] and associated p-value adjusted for three confounders: 'number of fatteners set-up/year', 'number of producing sows present/year' and 'sampling age'. ** β [LCI, UCI] and associated p-value adjusted for internal biosecurity (AMU) and adjusted for AMU (internal biosecurity). An adjustment was not performed for the internal biosecurity subcategories and total biosecurity due to high overall correlation (Spearman's $\rho > 0.7$) with internal biosecurity. Internal biosecurity is adjusted for 'TI fatteners lincosamide and macrolide'. When internal biosecurity is adjusted for 'TI 200 lincosamide and macrolide' or 'TI fatteners macrolide', the internal biosecurity estimates and CIs become respectively 0.029 [0.014, 0.045] and 0.035 [0.015, 0.054]. ***Countries included in each association: countries were excluded if no AMU was recorded on all farms from the respective country. All biosecurity analyses included eight countries.

Variable explanations: lincosamide and macrolide use (fatteners)*, macrolide + lincosamide + lincomycin/spectinomycin use in fatteners. Lincosamide and macrolide use (200 days)*, macrolide + lincosamide + Lincomycin/spectinomycin use corrected for a lifespan of 200 days. β -lactam use (fatteners)†, β -lactam use (penicillin + aminopenicillin + cephalosporin) in fatteners. β -lactam use (fatteners)##, β -lactam use (penicillin + aminopenicillin) in fatteners. Tetracycline use (200 days)**, tetracycline use corrected for a lifespan of 200 days. Macrolide, oxazolidinone†, macrolide + macrolide, oxazolidinone and phenicol resistance. IB: Cleaning & disinfection, internal biosecurity subcategory 'Cleaning & disinfection'. IB: Measures comp. & equipment use, internal biosecurity subcategory 'Measures between compartments & use of equipment'.

Table 3.3 Prevalence of ARG clusters per AMR gene class cluster identified in the meta-analysis

Macrolide	Overall %	A %	B %	C %	D %	E %	F %	G %	H %	I %
mef(A)_3_AF227521	32.13	31.90	29.07	34.11	24.70	33.52	48.25	51.61	22.24	33.67
Inu(C)	27.88	38.48	25.77	36.72	26.02	29.30	31.30	15.04	27.73	23.11
erm(F)_clust	18.74	20.36	9.78	3.94	28.73	23.27	8.12	13.12	22.28	13.29
erm(B)_clust	10.96	5.93	17.98	5.54	10.14	8.49	5.48	10.61	16.42	14.83
erm(G)_clust	3.58	0.98	4.34	10.83	2.38	2.72	2.69	3.97	4.17	3.67
Inu(A)	1.77	0.22	1.80	0.22	3.87	0.21	0.04	0.13	2.81	4.42
mef(A)_10_AF376746	1.11	0.83	1.89	1.48	1.11	1.07	1.25	1.33	0.82	0.86
msr(D)	1.01	0.23	2.31	5.89	0.38	0.31	1.68	0.91	0.26	0.86
Inu(B)	0.88	0.51	3.62	0.69	0.57	0.22	0.37	0.60	0.92	1.39
erm(Q)	0.52	0.24	1.75	0.30	0.51	0.34	0.37	0.38	0.54	0.62
Tetracycline	Overall %	A %	B %	C %	D %	E %	F %	G %	H %	I %
tet(Q)	38.57	42.05	27.54	44.25	39.34	35.28	46.63	51.54	27.48	41.93
tet(W)	30.78	33.51	34.42	33.22	29.01	31.88	28.81	24.76	33.16	26.63
tet(40)	11.55	7.51	8.71	4.46	15.38	15.69	9.50	5.94	18.79	10.97
tet(O)	7.36	7.22	9.07	9.05	6.35	8.13	7.54	5.74	6.91	6.83
tet(44)	5.14	5.23	8.51	6.05	3.96	4.23	4.78	4.24	4.96	4.98
tet(X)_clust	1.89	2.10	0.85	1.03	2.98	2.62	0.75	1.81	2.32	1.23
tet(L)_clust1	1.79	0.80	5.73	0.25	0.60	0.33	0.36	1.44	3.69	2.17
tetA(P)	0.73	0.45	2.15	0.71	0.52	0.46	0.57	0.55	0.58	0.70
tet(32)	0.73	0.74	1.50	0.55	0.38	0.75	0.59	0.44	0.89	0.62
tet(M)	0.59	0.16	0.47	0.13	0.90	0.35	0.13	1.24	0.60	1.24

Table 3.3 Continued

Amphenicol	Overall %	A %	B %	C %	D %	E %	F %	G %	H %	I %
cat_2	60.05	48.95	25.59	27.41	63.40	30.32	42.28	16.14	41.06	54.47
catP	14.36	8.93	7.43	7.16	16.77	2.67	7.17	7.19	4.37	1.77
cat(pC194)	10.49	10.48	12.33	1.72	10.40	45.50	15.74	2.45	6.02	10.22
fexA	4.50	1.69	0.40	0.00	4.06	0.20	2.10	7.90	10.97	6.61
floR_clust	3.32	1.85	19.29	5.02	1.21	0.92	3.63	51.60	4.89	9.70
catQ	3.08	3.75	3.08	7.78	2.90	0.00	4.37	3.33	2.69	4.65
cmx	1.23	4.62	12.10	2.79	0.27	6.29	2.99	2.96	18.52	2.09
catS	0.69	6.32	5.53	23.30	0.43	3.17	13.75	2.44	1.67	0.23
cml_clust	0.48	2.02	3.45	0.00	0.05	0.30	0.00	0.75	5.25	2.30
cat_clust	0.48	0.00	5.19	0.89	0.07	0.92	0.00	0.56	0.86	3.20
Vancomycin	Overall %	A %	B %	C %	D %	E %	F %	G %	H %	I %
VanT-G	16.77	19.64	15.44	19.30	9.42	17.75	18.45	18.81	15.71	14.92
VanXY-G	16.15	16.47	12.68	18.95	8.77	18.99	15.77	17.38	17.15	20.17
VanW-G	13.32	13.55	11.36	14.31	9.08	15.01	13.73	13.73	13.62	17.13
VanG	11.50	10.55	8.96	12.71	7.77	11.35	14.49	13.35	12.17	12.34
VanX-B	5.70	7.41	6.21	5.20	10.82	2.58	5.39	4.86	4.53	4.13
VanH-B	4.96	4.20	6.70	3.42	9.24	4.86	4.96	4.23	4.41	1.51
VanA-B_1_AF192329	4.80	5.22	5.56	4.91	8.72	3.59	4.49	3.80	3.70	1.96
VanR-G	4.78	4.45	4.26	3.36	3.88	7.12	4.64	5.54	6.69	2.07
VanS-B	4.33	3.71	6.51	4.54	7.05	2.60	3.27	4.42	3.09	2.76
VanY-B	4.04	3.96	6.27	3.02	7.63	2.50	3.82	3.02	3.57	1.52
Oxazolidinone & Macrolides & Amphenicols	Overall %	A %	B %	C %	D %	E %	F %	G %	H %	I %
cfr_clust	100.00	0.00	100.00	0.00	100.00	100.00	0.00	100.00	100.00	100.00

Letters A – I represent the nine countries. Country percentages: The contribution (%) of an ARG cluster to an AMR class cluster detected in a particular country. Only the top ten (highest abundance) ARG clusters per AMR gene class cluster are presented. The blue scale indicates high (dark blue) to lower (light blue) contributions.

Table 3.4 Results random-effects meta-analysis: main ARG clusters ($q < 0.1$, $p_h > 0.05$)

AMU and biosecurity	AMR class clustering	90% identity level clustering	β	[LCI, UCI]	q	Countries*
Lincosamide & macrolide use (fatteners)*	macrolide	<i>erm</i> (T)_4_AJ488494	1.274	[0.823,1.726]	<0.001	8
		<i>erm</i> (B)_clust	1.225	[0.769,1.680]	<0.001	8
		<i>erm</i> (G)_clust	0.991	[0.305,1.678]	0.047	8
		<i>erm</i> (F)_clust	0.702	[0.188,1.217]	0.068	8
Macrolide use (fatteners)	macrolide	<i>erm</i> (B)_clust	1.392	[0.918,1.867]	<0.001	7
		<i>erm</i> (T)_4_AJ488494	1.323	[0.834,1.812]	<0.001	7
		<i>mef</i> (A)_3_AF227521	0.980	[0.453,1.507]	0.011	7
		<i>erm</i> (A)_3_EU348758	0.757	[0.250,1.265]	0.038	7
		<i>erm</i> (Q)	0.745	[0.175,1.316]	0.088	7
Lincosamide & macrolide use (200 days)*	macrolide	<i>erm</i> (T)_clust	0.864	[0.365,1.362]	0.017	8
		<i>erm</i> (F)_clust	0.724	[0.300,1.148]	0.018	8
		<i>mef</i> (A)_3_AF227521	0.684	[0.262,1.106]	0.023	8
		<i>mef</i> (A)_10_AF376746	0.626	[0.206,1.047]	0.038	8
		<i>msr</i> (D)	0.687	[0.210,1.165]	0.047	8
		<i>erm</i> (B)_clust	0.700	[0.162,1.238]	0.089	8

Table 3.4 Continued

AMU and biosecurity	AMR class clustering	90% identity level clustering	β	[LCI, UCI]	q	Countries*
Internal biosecurity	macrolide	<i>erm</i> (F)_clust	0.027	[0.014,0.040]	0.002	8
		<i>erm</i> (B)_clust	0.023	[0.010,0.036]	0.013	8
		<i>erm</i> (G)_clust	0.022	[0.009,0.035]	0.020	8
		<i>mef</i> (A)_3_AF227521	0.019	[0.007,0.032]	0.036	8
		<i>erm</i> (T)_4_AJ488494	0.018	[0.005,0.031]	0.070	8
Tetracycline use (200 days) [‡]	tetracycline	<i>mph</i> (B)	0.017	[0.004,0.030]	0.087	8
		<i>tet</i> (M)	0.947	[0.437,1.457]	0.011	9
		<i>tet</i> (W)	0.735	[0.318,1.152]	0.014	9
		<i>tet</i> (40)	0.605	[0.190,1.020]	0.046	9
		<i>tet</i> (P)	0.615	[0.190,1.040]	0.047	9
β -lactam use (fatteners) [#]	phenicol (amphenicol)	<i>tet</i> (L)_clust1	0.673	[0.146,1.199]	0.094	9
		<i>cat</i> _2	1.184	[0.268,2.099]	0.089	7
		<i>cat</i> _2	1.174	[0.268,2.080]	0.089	7

All figures are rounded. [LCI, UCI], lower and upper CIs. *Countries included in each association: countries were excluded if no AMU was recorded on all farms from the respective country. All biosecurity analyses included 8 countries. Variable explanations: Lincosamides & macrolides use (fatteners)^{*}, macrolide + lincosamide + lincomycin/spectinomycin use in fatteners. Lincosamides & macrolides use (200 days)^{*}, macrolide + lincosamide + lincomycin/spectinomycin use corrected for a lifespan of 200 days. β -lactam use (fatteners)[#], β -lactam use (penicillin + aminopenicillin + cephalosporin) in fatteners. β -lactam use (fatteners)^{##}, β -lactam use (penicillin + aminopenicillin) in fatteners. β -lactam use (200 days)[‡], tetracycline use corrected for a lifespan of 200 days.

of macrolide resistance. Within internal biosecurity, the subcategories ‘cleaning and disinfection’ (eight countries with positive slope) and ‘measurements between compartments and use of equipment’ (M&E) (seven countries with positive slope) are shown to be positively associated with macrolide and vancomycin resistance (only M&E). In recent decades, evidence has built pointing to cross-resistance to antimicrobials and biocides (e.g. quaternary ammonium compounds) mediated by the hyperexpression or acquisition of bacterial efflux pumps.⁵³ Moreover, co-resistance has been suggested by linkage/co-occurrence of ARGs and biocide and metal resistance genes in bacteria and on plasmids.^{53–56} Since both internal biosecurity ‘cleaning and disinfection’ (largely) and M&E (restrictively) are based on questions related to farm disinfection, a positive association between internal biosecurity and resistance might potentially be explained by high use of specific types of disinfectants.

Munk *et al.*, 2018²⁹ showed a strong correlation between the pig resistome and genus-level bacteriome by means of a Procrustes analysis. This analysis suggested that much of the between-country variation was explained by bacteriome differences. To the best of our knowledge, the present study is a first attempt at finding associations between the pig faecal resistome and farm-level data. To avoid spurious associations and to support the data complexity (~20 farms in nine countries), we took the strong between-country variation in resistomes and risk factors into account using a random-effects meta-analysis. Future larger studies might focus on implementing zero-inflated binomial or Hurdle models⁵⁷ to support further the excess zeros when analyzing more thoroughly the ARGs at lower aggregation levels.

Adjustments for confounding were made during analysis, but other bias potentially remains. Our study design made extensive effort to randomize farm selection, but did not always succeed. The farmers’ willingness to participate might correlate with an interest in AMU/AMR reduction and consequently low AMU/AMR levels. No AMU/AMR also leads to a decreased sample size and potentially spurious associations, as the influence of non-use/no AMR cannot be estimated in a country-wise linear regression meta-analysis. We consequently excluded associations including less than four countries. Furthermore, while good farm AMU administration records were required, social desirability and recall bias could nevertheless have resulted in AMU and biosecurity misreporting or AMU underreporting and hence misclassification of exposure. Likewise, although the majority of treatments in pig production consist of group treatments,^{58,59} the exclusion of individual treatments (due to an even higher risk of recall bias), might have led to misclassification of exposure. Despite strict protocols, bias could have resulted from countries employing their own field and lab workers.

Using resistome data provides a great level of detail and multiple outcome variables. This makes classical risk factor analysis less straightforward in comparison to studies involving specific AMR bacteria. First, ARG data are expressed as relative abundances (FPKM) and consequently less intuitive to interpret. Second, these ARGs could be localized in clinically irrelevant or dysfunctional/dead bacterial species, and no phenotypic resistance patterns (the combined expression of multiple ARGs) are studied. Third, less prevalent but still important resistant types (e.g. β -lactam resistance: *bla*_{CTX-M}) are more difficult to detect when using metagenomic sequencing data despite the high sequencing depth in our study. Finally, our study was limited by the ARGs present within the ResFinder database (largely acquired ARGs).

In conclusion, our study is (as far as we are aware) unique in combining a large resistome dataset with risk factors. We established associations between ARGs and farming practices, which provides more insight into the biological pathways of resistance. Our approach enabled the evaluation of a large set of antimicrobial classes in parallel while checking for cross- and co-resistances (~2000 associations within 9 countries). We, therefore, believe that our study provides an important first step in inferring the main drivers for AMR in pigs at the metagenomic level.

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Competing interests

None to declare.

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Supplementary data

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S3.1 Materials and methods

S3.1.1 Summary: Procedure for sampling and pooling

Per farm, a batch of pigs of more or less the same age category, which received the same group treatments during their entire life, were randomly sampled across pens and compartments. At each farm, 25 individual pigs were sampled in their last weeks before slaughter. Faecal samples were collected using sterile spoons directly after defaecation from faecal parts without floor contact. Animal faecal sampling was non-invasive and therefore no additional animal ethics approval was needed from the respective national authorities. In any case, sampling did not cause pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice (Directive 2010/63/EU). After sampling, the individual samples were homogenized and pooled by farm.

Note regarding farm selection: The current farm selection in each country is based on inclusion criteria as defined in the sampling protocol. Farms were selected based on these criteria in agreement with local farming organizations, partially based on convenience (e.g. distances to farms). Therefore, the resulting farm selection in each country cannot be considered representative of the livestock sector in the respective country.

S3.1.2 Summary: Laboratory analysis

DNA was centrally extracted and processed at DTU from the pooled samples (N=176) by means of a modified QIAamp Fast DNA stool mini kit protocol. Frozen DNA was consecutively shipped to Oklahoma Medical Research Foundation (OMRF) for library preparation (DNA fragmentation: 300bp, Covaris E220evolution, Bioo NEXTflex PCR-free library preparation kit, Bioo NEXTflex-96 adapter set) and sequencing (Illumina HiSeq3000, 2x150bp paired-end sequencing). In total, 17 Belgian, Danish and Dutch pig faecal samples were sequenced by means of HiSeq2500, using 2x100bp paired-end sequencing, before it became unavailable at OMRF.

S3.1.3 Summary: Metagenomics and bioinformatics results

Sequence data were analyzed according to the MGmapper tool principles.¹ First, read cleaning was performed: Adapters were removed using BBDuk (BBMap v39.92 - Bushnell B. -<https://sourceforge.net/projects/bbmap/>) and reads aligning to the internal spike-in control phi-X174 were filtered. BWA-MEM mapping (Burrows-Wheeler Alignment Tool Maximal Exact Matches algorithm),² slightly modified as previously described (Munk *et al.*, 2018),³ was used to align

trimmed read pairs to all bacterial and archaeal genomes from NCBI Genbank marked as reference or representative for a taxon, and to the (largely) acquired AMR genes in the ResFinder database of November 2016. Subsequently, technical read duplicate reads were considered as artefacts and removed.

For each ResFinder reference and NCBI microbial genome, the number of read pairs properly aligning with at least 50bp, from both forward and reverse, were counted. ResFinder gene counts were normalized by dividing the count for each Resfinder reference by its length in bp (thousands) and the total number of bacterial reads per sample (millions), and next multiplied by 10^9 for reasons of readability. This resulted in a fragment per kilobase reference per million bacterial fragments (FPKM) value.

To overcome unspecific mapping to highly homologous sequences within ResFinder, the relative antimicrobial resistance gene (ARG) abundances (in FPKM) were aggregated at the 90% identity level (CD-HIT software).⁴ Furthermore, a second high-level clustering was performed separately at the antimicrobial resistance class level (AMR class clustering).

S3.1.4 Calculation of antimicrobial treatment incidences

Antimicrobial use data (AMU) was reported before in Sarrazin *et al.*, 2018.⁵ Antimicrobial use (AMU) quantification was based on Defined Daily Doses animal (DDDvet) and long-acting (LA) factor (a value to represent the duration of activity of a long-acting product) in accordance with Timmerman *et al.*, 2006.⁶ DDDvet was used as provided by ESVAC. Whenever for a given combination of active substance and administration route a DDDvet value was missing, the values as described by Postma *et al.* 2015⁷ were used or the information was obtained from the summary of product characteristics. In addition, LA factors were used as described before.⁷ Treatment Incidence (TI) calculations were performed for each administered group treatment or purchased product per age category (sucklers, weaners and fatteners; Formula 3.1).

Formula 3.1

$$TI = \frac{\text{Total amount of active substance administered or purchased (mg)}}{\text{DDDvet (mg/kg/day)} \times \text{number of days at risk} \times \text{kg pig at risk} \times \text{LA factor} \times 100 \text{ pigs at risk}}$$

Next to TIs by age category, a TI for a standardised lifespan of 200 days was calculated in accordance with Sjölund *et al.*, 2016⁸ to correct for possible differences in slaughter age between herds and countries (Formula 3.2).

Formula 3.2

$$TI\ 200 = \frac{TI\ suckler \times suckling\ period + TI\ weaner \times nursery\ period + TI\ finisher \times finishing\ period}{lifespan\ in\ a\ farm} \times \frac{200\ days\ lifespan}{lifespan\ in\ a\ farm}$$

S3.2 Results

Summaries include data from 176 farms selected for analysis. Datasets including 176 farms are derived from Munk *et al.*, 2018³ (N=181 farms) and Sarrazin *et al.*, 2019.⁵

S3.2.1 Summary: Pig resistome

Previously defined resistomes yielded an overall average of 50 million (SD: 18×10^6) PE reads per pooled sample. Corresponding rarefaction curves can be found in Munk *et al.*, 2018.³ Large differences in total ARG loads (total FPKM) within and between countries were observed (Supplementary Figure 3.1). Mean relative ARG abundance varied highly between countries (e.g. country D and H versus C and F). In total, 312 clustered ARGs (clustering at 90% identity level) were identified. Interestingly, the majority of ARGs were attributed to a small set of ARG clusters (Supplementary Figure 3.2). Tetracycline resistance gene clusters *tetQ* and *tetW* were most abundant, representing respectively 22% and 18% of the total ARG abundance (total FPKM), followed by gene clusters encoding macrolide, lincosamide, aminoglycoside and β -lactam resistance. Clustering of ARGs by AMR class showed high ARG levels corresponding to widely used antimicrobials such as tetracyclines, macrolides, aminoglycosides and β -lactam antimicrobials (Supplementary Figure 3.3).

Supplementary Figure 3.1 Total ARG abundance in the pig faecal microbiome per farm (total FPKM, 176 farms) and summarized by country

Boxplots: Letters A – I represent the nine countries. A box represents the 25th (Q1) to 75th (Q3) percentile, the centerline depicts the median (Q2). Dots represent outliers (values smaller than $Q1 - 1.5 \times IQR$ or larger than $Q3 + 1.5 \times IQR$). An asterisk (*) presents the mean by country. Dataset including 176 farms is derived from Munk *et al.*, 2018³ (N=181 farms).

Supplementary Figure 3.3 AMR clustered by antimicrobial class presented per country (total FPKM)

Top plot: Most abundant AMR classes. Bottom plots: Less abundant AMR classes. A box represents the 25th (Q1) to 75th (Q3) percentile, the centerline depicts the median (Q2). Dots represent outliers (values smaller than $Q1 - 1.5 \times IQR$ or larger than $Q3 + 1.5 \times IQR$). An asterisk (*) presents the mean by country. Letters A – I represent the nine countries. Dataset including 176 farms is derived from Munk *et al.*, 2018³ (N=181 farms).

S3.2.2 Summary: AMU volumes and consumption patterns (group treatments)

Four Treatment Incidences (TIs) were calculated: TIs for sucklers, weaners, fatteners and a TI corrected for a lifespan of 200 days (TI 200). 20 farms reported that no antimicrobial group treatments were used in the sampled batch. Two of these farms also didn't report purchased antimicrobials. Overall, the highest use was observed during the weaning phase, the lowest use during the fattening phase (Supplementary Figure 3.4 and 3.5 bottom plot).

Supplementary Figure 3.4 Total AMU (group treatments) expressed as treatment incidence (TI) per pig age category and by country (176 farms)

TI expresses the percentage of pigs receiving a dose of antimicrobials each day. TI 200 = Total TI corrected for a lifespan of 200 days. TI sucklers/weaners/fatteners = Total TI for the respective age categories. All TIs are based on Defined Daily Doses Animal (DDDvet). A box represents the 25th (Q1) to 75th (Q3) percentile, the centerline depicts the median (Q2). Dots represent outliers (values smaller than $Q1 - 1.5 \times IQR$ or larger than $Q3 + 1.5 \times IQR$). An asterisk (*) presents the mean by country. Letters A- I represent the nine countries. Dataset including 176 farms is derived from Sarrazin *et al.*, 2018.⁵

Country H showed the highest overall AMU and I and F the lowest (Supplementary Figure 3.5). Overall, antimicrobials of the aminopenicillin class (β -lactam antimicrobials) were mostly used (TI 200: total=785, mean=4.46, data not shown), closely followed by polymyxin use (colistin) (TI 200: total=732, mean=4.16, data not shown). Tetracyclines and macrolides were also widely used but in a second

Supplementary Figure 3.5 AMU group treatments by country stratified by antimicrobial class (% of total AMU)

Top plot: Treatment Incidence corrected for a lifespan of 200 days. Bottom plot: Treatment Incidence fatteners. Total and mean treatment incidences are displayed at the right. Dataset including 176 farms is derived from Sarrazin *et al.*, 2018.⁵ Letters A – I represent the nine countries. AG = aminoglycoside, AMP = aminopenicillin, PHE = amphenicol, CEP = cephalosporin, LIN = lincosamide, L&S = lincomycin and spectinomycin, MAC = macrolide, PEN = penicillin, PLM = pleuromutilin, COL = polymyxin (colistin), SUL = sulphonamide, TET = tetracycline, TMPS = trimethoprim and sulphonamide, FLQ = fluoroquinolone, OTQ = other quinolone.

order (TI 200: respectively total=408, 399 and mean=2.31, 2.26, data not shown). Critically important antimicrobials like cephalosporins and fluoroquinolones were used less frequently (TI 200: total=95, 40 and mean=0.54, 0.23, data not shown). The overall distribution of certain antimicrobials within a country particularly differed for countries with low total AMU (countries I and F) and for country A (high cephalosporin use, due to two cephalosporin using farms, data not shown) when compared to higher use countries (H, D). Additionally, the relative within-country contribution of colistin use largely varied between countries (e.g. from relatively no use in country C to up to 30% of total use within countries H, E and I).

S3.3 Overview of all variables

Supplementary Table 3.1 Lists of all outcomes and determinants used in the meta-analysis.

Response variable	Predictors
AMR classes**3	AMU classes**45
<ul style="list-style-type: none">AminoglycosideAminoglycoside & quinoloneβ-lactamColistinFosfomycinMacrolideMacrolide & oxazolidinone & phenicol (amphenicol)NitroimidazolePhenicol (amphenicol)Oxazolidinone & phenicol (amphenicol)QuinoloneRifampicinSulfonamideTetracyclineTrimethoprimVancomycinTotal AMR	<ul style="list-style-type: none">AminoglycosideAminopenicillin (β-lactam)Penicillin (β-lactam)Cephalosporin (β-lactam)Polymyxin (colistin)MacrolideLincomycin-SpectinomycinLincosamideAmphenicolFluoroquinoloneOther quinolonesSulfonamideTetracyclineTrim & sulfaPleuromutilinTotal AMU
Created AMR class combinations	Created AMU class combinations
<ul style="list-style-type: none">Aminoglycoside + aminoglycoside & quinolonequinolone + aminoglycoside & quinoloneMacrolide + macrolide & oxazolidinone & phenicol (amphenicol)Phenicol (amphenicol) + oxazolidinone & phenicol (amphenicol)Macrolide & oxazolidinone & phenicol (amphenicol)Oxazolidinone & phenicol (amphenicol) + macrolide & oxazolidinone & phenicol (amphenicol)	<ul style="list-style-type: none">Fluoroquinolone + other quinolonesPenicillin + aminopenicillinPenicillin + aminopenicillin + cephalosporinMacrolide + lincosamide + lincomycin-spectinomycinLincosamide + lincomycin-spectinomycinTrim & sulfa + sulfonamide
Other farm and batch characteristics	
<ul style="list-style-type: none">Sampling seasonFarm technical dataMean no. of producing sows present/yearMean no. of producing boars present/yearNo. of weaned piglets per sow/yearMean weight at weaning (kg)Mean weight of fatteners at set-up (kg)Mean weight of fatteners at delivery at the slaughterhouse (kg)Mean no. of fatteners set-up/yearGeneral farm characteristicsOther animals present at the farm (yes/no)No. of people working at the farmType of batch production system (weekly system)Characteristics of the sampled batchWeaning age piglets (days)Age of weaners to growing-fattening barns (days)Sampling age fatteners (days)	
Biosecurity variables ⁹	<ul style="list-style-type: none">Total biosecurityExternal biosecurityInternal biosecurityExternal biosecurity subcategoriesPurchase of animals and semenTransport of animals, removal of manure & dead animalsFeed, water and equipment supplyPersonnel & visitorsVermin & bird controlEnvironment & regionInternal biosecurity subcategoriesDisease managementFarrowing and suckling periodNursery unit managementFattening unit managementMeasures between compartments, use of equipmentCleaning & disinfection

The top parts of the first two columns represent the available AMU or AMR classes, the bottom parts represent combinations of AMR and AMU classes used in the analysis. All single or combined AMR categories were univariately regressed against all single or combined AMU categories and all listed biosecurity and other farm and batch characteristics (by country). * Expressed as relative ARG abundances (FPKM). **Treatment Incidences were available for a lifespan of 200 days (T1 200) and for sucklers, weaners and fatteners (T1 sucklers, T1 weaners and T1 fatteners).

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Part II

Determinants for antimicrobial resistance and antimicrobial resistance genes in slaughterhouse workers

Chapter 4

ESBL carriage in pig slaughterhouse workers is associated with occupational exposure

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Abstract

We investigated the prevalence of extended-spectrum β -lactamase (ESBL) carriage in slaughterhouse workers and the association with occupational exposure to slaughter animals and products. Stool samples from 334 employees in a Dutch pig slaughterhouse were obtained. Presence of ESBL was determined by selective plating, microarray analysis, and gene sequencing. Questionnaires were used to collect personal and occupational information. The overall prevalence of ESBL carriage was 4.8% (16/334). All ESBL-producing isolates were *Escherichia coli*. The ESBL genes detected were *bla*_{CTX-M-1} (N=8), *bla*_{CTX-M-15} (N=3), *bla*_{CTX-M-27} (N=2), *bla*_{CTX-M-24} (N=1), *bla*_{CTX-M-55} (N=1), and *bla*_{SHV-12} (N=1). A higher prevalence of ESBL was seen in workers in jobs with as tasks 'removal of lungs, heart, liver, tongue' (33%) and 'removal of head and spinal cord' (25%). For further analysis, participants were divided into two groups based on potential exposure to ESBL as related to their job title: one group with an assumed higher exposure to ESBL (e.g. stable work, stabbing, dehairing, removal of organs) and another group with an assumed lower exposure to ESBL (e.g. refrigeration, packaging and expedition). In the 'higher exposure' group, ten out of 95 (10.5%) were carrying ESBL versus six out of 233 (2.6%) in the 'lower exposure' group. Human ESBL carriage was significantly associated with job exposure in the slaughterhouse (OR=4.5, 95% CI=1.6-12.6). Results suggest that ESBL carriage in slaughterhouse workers overall is comparable with the Dutch population. Within the slaughterhouse population a difference in carriage exists depending on their position along the slaughter line and tasks involved.

4.1 Introduction

In humans, infections with extended-spectrum β -lactamase (ESBL)-producing Enterobacteriaceae are associated with an increased burden of disease and costs.¹ Livestock can carry ESBL-producing Enterobacteriaceae with *bla*_{CTX-M-1} as the most prevalent ESBL gene found in Europe.² ESBL-producing Enterobacteriaceae can be transferred from animals to humans through food or direct contact.^{3,4} Direct contact with livestock mainly occurs in an occupational setting. In farmers, carriage of ESBL-producing Enterobacteriaceae is associated with the presence of ESBL-producing Enterobacteriaceae in animals.^{3,5,6} Slaughterhouse workers might also be occupationally exposed to ESBL-producing Enterobacteriaceae. Depending on the job task, slaughterhouse workers have frequent contact with live animals, animal carcasses or animal products. ESBL-producing Enterobacteriaceae have frequently been found in intestinal content of pigs at the slaughter level.⁷⁻⁹ Besides that, ESBL-producing Enterobacteriaceae can also be detected on pig carcasses at the slaughterhouse.¹⁰ A higher prevalence of antimicrobial resistant *Escherichia coli* in pig slaughterhouse workers has been reported.¹¹⁻¹³ However, carriage of ESBL-producing Enterobacteriaceae in slaughterhouse workers has not been established yet. In addition, *E. coli* contamination on carcasses seems to be reduced during the slaughter process.^{14,15} Therefore exposure to, and as a consequence human faecal carriage of, ESBL-producing Enterobacteriaceae might be dependent on the working area and job task within a slaughterhouse.

We investigated the prevalence of ESBL carriage in pig slaughterhouse workers and the association with occupational exposure.

4.2 Materials and Methods

Study design

A large pig slaughterhouse in the southern part of the Netherlands (Vion, Boxtel) was visited by the researchers during one week in June 2015. All slaughterhouse workers with a slaughter process-related job were asked to participate in the study by the use of flyers and information on screens. In total, 1781 eligible slaughterhouse workers were employed by the slaughterhouse at the time of the study. Participants were asked to provide a faecal specimen and to fill out a consent form and a questionnaire containing items on personal and occupational information, including job function in the slaughterhouse. Due to the heterogeneity in nationality of the slaughterhouse workers (14 different

nationalities), the flyer, questionnaire and consent form were provided in 11 different languages. Participants were motivated for participation with a voucher worth 25 euro. Faeces samples and documents were handed in to the researchers at the slaughterhouse within the same week. At the same day, to detect ESBL-producing Enterobacteriaceae, <1 g faeces was collected from the tube by the use of a swab and stored in the refrigerator immediately. All swabs were sent refrigerated in one batch to the laboratory. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The Medical Ethical Committee of the University Medical Centre Utrecht confirmed that the Medical Research Involving Human Subjects Act did not apply for this study and that therefore an official approval by the Medical Ethical Committee was not required (Protocol no. 14-346/C). All participants gave written informed consent.

Laboratory analysis

At the day of arrival in the laboratory, all faecal specimens were analysed for the presence of ESBL-producing Enterobacteriaceae by selective plating. Samples were suspended in 10 ml LB-medium with cefotaxime (1 µg/ml) and incubated overnight at 37°C. Approximately 10 µl of each suspension was streaked on MacConkey agar plates with cefotaxime (1 µg/ml) and incubated overnight at 37°C. Individual colonies with different morphology were selected for bacterial species identification performed by MALDI-TOF MS. All isolates suspected of producing ESBLs were selected for further molecular analysis to confirm the presence of ESBL genes. DNA was isolated using DNeasy 96 Blood & Tissue Kit (Qiagen, Hilden, Germany). A β-lactamase microarray assay (Check-MDR CT101, Checkpoints, Wageningen, the Netherlands) was used to detect genes encoding carbapenemases (KPC and NDM), ESBLs (CTX-M groups 1, 2, 8/25 and 9, TEM, SHV), and AmpCs (CMY-1/MOX, ACC, DHA, ACT/MIR, CMY-2, FOX). DNA from ESBL/AmpC microarray positive isolates was amplified and sequenced with group-specific primers to determine the exact gene type.¹⁶ DNA sequences were interpreted with Basic Local Alignment Search Tool (National Center for Biotechnology Information). Plasmids encoding the ESBL (or AmpC) genes were determined using a transformation-based approach. Purified plasmid DNA was electro-transformed into *E. coli* DH10B cells (Invitrogen, Van Allen Way, CA, USA) under the following conditions: 1.25 kV per cm, 200 Ω, 25 µFar.¹⁷ When strains recovered from workers were not giving transformants (workers 12 and 15), conjugation was performed using the plasmid-free rifampin-resistant *E. coli* E3110

as a recipient strain for liquid-mating assays in 1:1 ratio as previously described.¹⁷ Transformants were selected on lysogeny broth (LB) agar supplemented with cefotaxime (1 µg/ml), whereas transconjugants on LB agar with rifampin (100 µg/ml) and cefotaxime (1 µg/ml). PBRT (PCR-based replicon typing) on the transformants and multilocus sequence typing (MLST) on the parental strains were performed to analyse genetic similarities of isolates and plasmid content.¹⁷

Statistical analysis

Slaughterhouse workers were classified as ESBL positive if any ESBL gene was detected in their faeces sample. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The association between carriage of ESBL-producing Enterobacteriaceae in humans and job function was calculated with logistic regression analysis (proc GENMOD). For this analysis, participants were divided in two groups based on potential exposure within their specific job task. One group with an assumed higher exposure to ESBL-producing Enterobacteriaceae consisted of job tasks in the early stages of the slaughter process (e.g. stable work, stabbing, dehairing, removal of organs, i.e. direct contact with pigs and pig carcasses). The other group, assumed to have lower exposure to ESBL-producing Enterobacteriaceae, consisted of job tasks in the latter stages of the slaughter process (e.g. refrigeration, packaging, expedition, i.e. contact with meat or meat products) (see Table 4.1). When participants reported having more than one job function, they were assigned to the first job function at the slaughter line. Other potential risk factors such as nationality, consumption of raw meat, hospitalization, use of antimicrobials, living on a farm, travelling, and potential confounders (age, gender and smoking) were analysed univariately as well and selected for multivariate analysis when the p-value was below 0.2. Model selection was performed by a backward procedure. Model fit was checked with the QIC-statistic (quasi-likelihood under the independence model criterion). The final model retained variables significant at $p \leq 0.05$.

Table 4.1 Distribution of slaughterhouse workers and ESBL carriage over different job tasks.

	Job task	Frequency		
		Total	ESBL carriers	Non-ESBL carriers
'Higher exposure' group	Stable / lairage part, stunning part	12	0	12
	Stabbing and bleeding part	5	0	5
	Dehairing and hanging part	6	1	5
	Removal of penis, stab wound and loosing/ligation anus (anal duct)	6	0	6
	Evisceration	10	1	9
	Removal of lungs, heart, liver, tongue	12	4	8
	Inspection platform	8	0	8
	Dressing (first, second), removal abdominal fat, diaphragm	17	1	16
	Removal of head, spinal cord	8	2	6
	Organs (tongues, hearts, livers, kidneys)	11	1	10
'Lower exposure' group	Refrigeration and cooling area	26	0	26
	Cutting room	75	2	73
	Deboning area	63	1	62
	Packaging	30	2	28
	Expedition (moving and shipment)	8	0	8
	Other (e.g. technical services)	31	1	30

Results

Human stool samples from 334 employees were obtained. Most of the participants were male and did not originate from the Netherlands (Table 4.2). The overall prevalence of ESBL carriage was 4.8% (16/334), and all ESBL-producing isolates were *E. coli*. The ESBL genes detected were *bla*_{CTX-M-1} (N=8), *bla*_{CTX-M-15} (N=3), *bla*_{CTX-M-27} (N=2), *bla*_{CTX-M-24} (N=1), *bla*_{CTX-M-55} (N=1) and *bla*_{SHV-12} (N=1). In addition, three other slaughterhouse workers carried an AmpC gene [*bla*_{CMY-2} (N=2) and *bla*_{DHA-1} (N=1)]. Plasmids encoding ESBL genes were assigned to multi-F (N=6), I1α (N=4), N (N=3) and K (N=1) replicon types. For two isolates, transformation and conjugation experiments failed to give transformants and transconjugants suggesting the chromosomal location of these genes. MLST revealed that ESBL-

Table 4.2 Overview of participant characteristics and Odds Ratios from the univariate analysis for the probability of ESBL carriage

Characteristics of participants	Frequency			Odds Ratio
	Total	ESBL carriers	Non-ESBL carriers	OR (95% CI)
Exposure group in slaughter process (N=328)				
'Higher exposure' group	95	10	85	4.45 (1.57-12.62)
'Lower exposure' group	233	6	227	Ref.
Age (mean) (N=328)	39.8	38.1 (N=16)	39.9 (N=312)	0.99 (0.94-1.03)*
Gender (N=324)				
Male	276	14	262	2.51 (0.32-19.54)
Female	48	1	47	Ref.
Nationality (N=305)				
Non-Dutch†	245	12	233	0.98 (0.27-3.58)
Dutch	60	3	57	Ref.
Consumption of raw meat (N=294)				
Yes	106	4	102	0.70 (0.21-2.28)
No	188	10	178	Ref.
Hospitalization in the past 12 months (N=295)				
Yes	32	2	30	1.28 (0.28-5.96)
No	263	13	250	Ref.
Use of antimicrobials in the past 12 months (N=323)				
Yes	48	3	45	1.34 (0.37-4.90)
No	275	13	262	Ref.
Living on a farm (N=297)				
Yes	17	0	17	NE
No	280	13	267	
Travelling in the past 12 months (N=311)				
Yes	216	7	209	0.36 (0.13-1.04)
No	95	8	87	Ref.
Smoking (N=321)				
Yes	140	3	137	0.31 (0.09-1.12)
No	181	12	169	Ref.

Ref. = Reference category; NE = Not estimated due to empty cells. *Per one year increase. †Not from the Netherlands: Cape Verdean (N=1), Ghanese (N=1), Hungarian (N=46), Latvian (N=3), Polish (N=119), Portuguese (N=12), Romanian (N=41), Slovakian (N=20), Turkish (N=2).

producing *E. coli* belonged to 11 different sequence types (STs), with ST131 (N=4), ST88 (N=3) and ST93 (N=3) as the most frequent ones. All molecular aspects are listed in Table 4.3.

Table 4.3 Molecular aspects of ESBL/AmpC positive *E. coli* isolates from slaughterhouse workers

Slaughterhouse worker	ESBL/AmpC gene	Plasmid rep/ inc-type	Sequence types (ST)	Job task
1	<i>bla</i> _{CTX-M-1}	IncI1a	88	Evisceration
2	<i>bla</i> _{CTX-M-1}	IncI1a	88	Removal of lungs, heart, liver, tongue
3	<i>bla</i> _{CTX-M-1}	IncI1a	88	Removal of lungs, heart, liver, tongue
4	<i>bla</i> _{CTX-M-1}	IncI1a	10	Dressing (first, second), removal abdominal fat, diaphragm
5	<i>bla</i> _{CTX-M-1}	IncN	93	Removal of head, spinal cord
6	<i>bla</i> _{CTX-M-1}	IncN	93	Removal of head, spinal cord
7	<i>bla</i> _{CTX-M-1}	IncN	93	Organs (tongues, hearts, livers, kidneys)
8	<i>bla</i> _{CTX-M-1}	IncFIA-FII	131	Packaging
9	<i>bla</i> _{CTX-M-15}	IncK	156	Cutting room
10	<i>bla</i> _{CTX-M-15}	incFIB-FII	410	Deboning area
11	<i>bla</i> _{CTX-M-15}	IncFIA-FII	131	Dehairing and hanging part
12	<i>bla</i> _{CTX-M-24}	—*	354	Packaging
13	<i>bla</i> _{CTX-M-27}	IncFIA-FIB-FII	131	Other (e.g. technical services)
14	<i>bla</i> _{CTX-M-27}	IncFIB-FII	131	Cutting room
15	<i>bla</i> _{CTX-M-55}	—*	95	Removal of lungs, heart, liver, tongue
16	<i>bla</i> _{SHV-12}	IncFIB-FIC-FII	665	Removal of lungs, heart, liver, tongue
17	<i>bla</i> _{CMY-2}	IncI1a	752	Cutting room
18	<i>bla</i> _{CMY-2}	IncI1a	752	Cutting room
19	<i>bla</i> _{DHA-1}	IncFIA-FII	38	Stabbing and bleeding part

* No successful transformation or conjugation.

ESBL carriage was not equally distributed across different jobs in the slaughterhouse. A higher prevalence of ESBL seemed to appear in workers involved in 'removal of lungs, heart, liver, tongue' (33%) and 'removal of head and spinal cord' (25%) (See table 4.1). In the 'higher exposure' group, ten out of 95 (10.5%) were carrying ESBL versus six out of 233 (2.6%) in the 'lower exposure' group. Human ESBL carriage was significantly associated with job exposure in the slaughterhouse [odds ratio (OR)=4.45, 95% CI=1.57-12.62]. Smoking was univariately and negatively borderline significantly associated with ESBL carriage (OR=0.31, 95% CI=0.09-1.12) as well as travelling (OR=0.36, 95% CI=0.13-1.04). None of the other potential risk factors (nationality, consumption of raw meat, hospitalization, use of antimicrobials, living on a farm) or confounders (age, gender) were significantly associated with ESBL carriage. In the multivariate analysis, the effects of smoking and travelling appeared independent of job exposure (only a slight change in OR) and did not remain in the final model. Therefore the final model only contained job exposure as a risk factor for ESBL carriage.

When looking at ESBL carriage by nationality, Romanian workers had a higher prevalence [five out of 41 (12.2%)] compared to the other three well-represented nationalities: Hungarian [one out of 46 (2.2%)], Dutch [3 out of 60 (5.0%)] and Polish [five out of 119 (4.2%)]. However, 23 out of 41 (56.1%) of the Romanians worked in the 'higher exposure' group, which is a considerably higher percentage than within Hungarian, Dutch and Polish slaughterhouse workers [seven out of 46 (15.2%), nine out of 60 (15.0%), and 30 out of 119 (25.2%) respectively]. When in the final model the effect of job exposure was adjusted for Romanian nationality, the association between ESBL carriage and job exposure hardly changed (OR=4.85, 95% CI=1.54-15.24). No significant interaction between Romanian nationality and job exposure was observed for ESBL carriage.

Focusing on *bla*_{CTX-M-1} gene solely, the prevalence was 7.4% (7/95) in the 'higher exposure' group and 0.4% (1/233) in the other group. When the analysis was restricted for *bla*_{CTX-M-1} alone, the association between ESBL carriage and exposure group was confirmed (OR=18.46, 95% CI=2.24-152.16).

From the 74 participants who reported to have more than one job title, 11 participants mentioned job titles not within the same exposure group. This misclassification of exposure might have changed the association between exposure and ESBL carriage. When a sensitivity analysis was performed by switching these 11 participants from the 'higher exposure' to the 'lower exposure' group, the observed association between ESBL carriage and occupational exposure did not change significantly and had a similar point estimate (OR=5.36, 95% CI=1.89-15.25).

4.3 Discussion

The study revealed that slaughterhouse workers were more likely to carry ESBL when working in early slaughtering steps (before chilling of the pig carcasses) than slaughterhouse workers working from the cooling onwards. The overall prevalence of ESBL carriage was 4.8%, which is more or less comparable to numbers found in two recent Dutch studies in residents of Amsterdam (8.6%) and residents living in the vicinity of livestock farms (4.5%).^{18,19} However, the ESBL prevalence differed between the assumed higher and lower exposed groups (10.5% vs 2.6%) pointing at higher occupational exposure during specific tasks performed by individual slaughterhouse workers.

A previous study in the same slaughterhouse showed a decline in presence and counts of *Salmonella* on the skin surface and the exterior of carcasses from bleeding till chilling.²⁰ In addition, a decline in the presence of *E. coli* during the slaughter process, especially after chilling of the pig carcasses, has been described in the literature.^{14,15,21,22} It is likely that ESBL-producing *E. coli* shows the same decline as *E. coli* in general, although this remains an assumption since exposure to ESBL was not measured in this study. This could explain the higher carriage of ESBL among slaughterhouse workers when involved in the slaughter steps before chilling. For some slaughter steps only low numbers of participants could be included. As a consequence, it was not possible to estimate differences in ESBL carriage in more detail. However, when focusing on the descriptive figures, ESBL carriage is considerably higher when working in the slaughter steps 'removal of lungs, heart, liver, tongue' and 'removal of head and spinal cord'. Exposure to ESBL might be higher for workers in these slaughter steps, because both steps involve the handling of the throat area, including the pharyngeal tonsils which are colonized with high counts of several bacteria (potentially including ESBL-producing *E. coli*).^{23,24}

In this study, *bla*_{CTX-M-1} was the most predominantly detected ESBL gene in pig slaughterhouse workers. Out of the ESBL positive slaughterhouse workers, eight were carrying *bla*_{CTX-M-1} (50%), which is significantly higher than the proportions of *bla*_{CTX-M-1} reported in two Dutch studies. In ESBL positive residents of Amsterdam and ESBL-positive residents living in the vicinity of livestock farms 26 out of 145 (18%) and 13 out of 99 (13%) carried a *bla*_{CTX-M-1} gene, respectively.^{18,19} In addition, *bla*_{CTX-M-1} is also the most frequently found ESBL gene in slaughter pigs in the Netherlands.²⁵ Moreover, pig farmers were found to carry the same ESBL gene type as exclusively or predominantly detected in their pigs, of which *bla*_{CTX-M-1} was most frequently found in both of them.³ In contrast, *bla*_{CTX-M-15} has mostly been found in humans in a clinical setting and in the Dutch human population, but not that often in livestock.^{2,18,26} A great diversity was seen in STs, therefore clonal

transmission between slaughterhouse workers is probably not the dominant route of transmission. Besides, half of the STs (ST10, ST38, ST88, ST93, ST354) have previously been associated with *E. coli* from pig origin.^{8,27,28} In addition, IncN and IncI1 plasmids are known to play a role in the dissemination of *bla*_{CTX-M-1} in *E. coli* in livestock, including in pigs.^{29–31} Although no samples from carcasses or slaughter products were obtained, these results regarding ESBL gene types, plasmids and STs, suggest transmission from animals to humans.

Besides occupational exposure, none of the analysed determinants (nationality, consumption of raw meat, hospitalization, use of antimicrobials, living on a farm, travelling, age, gender and smoking) were found to be risk factors for ESBL carriage in slaughterhouse workers. ESBL carriage was higher in slaughterhouse workers with a Romanian nationality compared with other nationalities, although the association between ESBL carriage and occupational exposure remained when adjusting for Romanian nationality. Besides that, the effect of being in the 'higher exposure' group is similar for Romanian slaughterhouse workers as for the other nationalities, considering the non-significant interaction. Also, in three out of five Romanian ESBL carriers the ESBL gene type detected was *bla*_{CTX-M-1}. Smoking and travelling were protective factors for ESBL carriage univariately, although they were not retained in the final model. From the 173 non-Dutch slaughterhouse workers who reported their travelling destiny, 88% travelled to their country of origin. Although travelling frequencies were not assessed, travelling might be interpreted as long-distance commuting. To our knowledge, there is no explanation described in the literature for any effect of smoking on ESBL carriage.

Although ESBL carriage was detected in slaughterhouse workers, the risk of acquiring ESBL-producing *E. coli* when handling meat intensively seems to be limited. When this is extrapolated to consumers, exposure to ESBL in pork through handling might not be of a high public health concern. In combination with environmental exposure data, this information can be used in formal risk assessments. Although not addressed in this study design, duration and persistence of ESBL carriage can vary greatly.^{32,33} Duration of ESBL carriage is not only important for assessing the risk of infection, but for the risk of transmission of ESBLs from slaughterhouse workers into the general population as well.

The results suggest that the overall carriage in slaughterhouse workers is comparable with the Dutch population, but the proportion of the *bla*_{CTX-M-1} gene (commonly found in livestock) is higher. Differences exist between slaughterhouse workers depending on their job tasks. A higher prevalence of ESBL carriage was found in slaughterhouse workers working before the chilling process of the carcasses compared to workers in the cooling area, cutting and deboning departments.

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Competing interests

Vion Food Group was not involved in the statistical analysis, the interpretation of the data, nor the decision to publish.

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Chapter 5

Occupational exposure and carriage of antimicrobial resistance genes (*tetW*, *ermB*) in pig slaughterhouse workers

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Supplementary data can be found at the end of this chapter

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Abstract

Objectives

Slaughterhouse staff is occupationally exposed to antimicrobial resistant bacteria. Studies reported high antimicrobial resistance gene (ARG) abundances in slaughter pigs. This cross-sectional study investigated occupational exposure to tetracycline (*tetW*) and macrolide (*ermB*) resistance genes and assessed determinants for faecal *tetW* and *ermB* carriage among pig slaughterhouse workers.

Methods

During 2015–2016, 483 faecal samples and personal questionnaires were collected from workers in a Dutch pig abattoir, together with 60 pig faecal samples. Human dermal and respiratory exposure was assessed by examining 198 carcass, 326 gloves, and 33 air samples along the line, next to 198 packed pork chops to indicate potential consumer exposure. Samples were analyzed by qPCR (*tetW*, *ermB*). A job-exposure matrix was created by calculating the percentage of *tetW* and *ermB* positive carcasses or gloves for each job position. Multiple linear regression models were used to link exposure to *tetW* and *ermB* carriage.

Results

Workers are exposed to tetracycline and macrolide resistance genes along the slaughter line. *TetW* and *ermB* gradients were found for carcasses, gloves, and air filters. One packed pork chop contained *tetW*, *ermB* was non-detectable. Human faecal *tetW* and *ermB* concentrations were lower than in pig faeces. Associations were found between occupational *tetW* exposure and human faecal *tetW* carriage, yet, not after model adjustments. Sampling round, nationality, and smoking were determinants for ARG carriage.

Conclusion

We demonstrated clear environmental tetracycline and macrolide resistance gene exposure gradients along the slaughter line. No robust link was found between ARG exposure and human faecal ARG carriage.

5.1 Introduction

Emerging antimicrobial resistance (AMR) poses a threat to global human and animal health.¹ Antimicrobial use (AMU) is the major driver for AMR in humans and animals in general.^{2,3} Human AMR carriage has been associated with direct and indirect contact with livestock,^{4–6} while a high AMR prevalence has been documented in persons with regular occupational livestock contact including veterinarians, farmers and abattoir workers.^{7–13}

Contact with live animals was identified as the major risk factor for nasal carriage of (livestock-associated) methicillin-resistant *Staphylococcus aureus* [(LA-) MRSA] in pig and broiler slaughterhouse workers.^{10–12} Similarly, a higher risk for faecal carriage of extended-spectrum β -lactamase-producing Enterobacteriaceae (ESBL-PE) was found for pig slaughterhouse employees working at earlier positions in the slaughter line.¹³ Since AMR can also be indirectly transmitted to humans via the environment,^{5,6} it is necessary to quantify AMR in the air or on environmental reservoirs like carcasses or surfaces throughout the slaughter line.

Abovementioned studies focused their efforts on the detection of specific resistant bacteria (MRSA, ESBL-PE). Our recent resistome studies demonstrated that antimicrobial resistance genes (ARGs) conferring resistance to tetracyclines and macrolides were among the most abundant ARGs in the resistome of European fattening pigs close to slaughter and were respectively associated with tetracycline and lincosamide/macrolide use in pigs.^{14,15} These findings led us to adopt an in-depth study of the risk for ARG carriage in pig slaughterhouse workers by specifically studying faecal tetracycline (*tetW*) and macrolide (*ermB*) resistance genes. To assess occupational exposure to *tetW* and *ermB*, we simultaneously studied a variety of host (pig faeces) and environmental samples (pig carcasses, gloves worn by staff as a proxy for hand contamination, ambient air) at various positions along the pig slaughter line and assessed determinants for faecal *tetW* and *ermB* carriage among workers. In addition, we studied packed meat further down the chain as an indication for potential consumer exposure to ARGs.

5.2 Methods

Study design

Samples were collected within the largest Dutch pig slaughterhouse (Vion, Boxtel, the Netherlands), slaughtering daily ~18,000 Dutch fattening pigs. Sampling was performed in two one-week sampling rounds (second week June 2015, first week July 2016). The human population from 2015 was previously largely tested for

ESBL-PE.¹³ Pig slaughter was divided into two main phases, slaughter in the ‘black area’ [successively: lairage, stunning, bleeding, scalding/dehairing, and carcass scorching (singeing)] and the ‘clean area’ [successively: evisceration, pluck removal (removal liver, lungs, heart, oesophagus, and tongue), carcass splitting, meat inspection, dressing, and carcass cooling], followed by a third phase of cutting, deboning, and some further processing (Supplementary Figure 5.1).

Respectively in 2015 and 2016, we collected in total (convenience sampling): 325 and 158 faecal samples from abattoir and other staff; 96 and 102 pooled carcass [skin (cork borer) and meat] samples; 16 and 17 air samples, 156 and 170 gloves worn by slaughterhouse staff, and 30 and 30 pig faecal samples. Carcass and glove sampling was divided over different time points to avoid bias based on daily bacterial build-up. Furthermore, 198 separate meat end-products (packed pork chops) were collected at the meat slicing and packing centre (Vion, Groenlo, the Netherlands) from June to September 2016.

Sample collection

Human faecal samples (N=483) and gloves (N=326)

Slaughterhouse employees were recruited by means of flyers, posters, and information provided on screens. Written information was translated into 11 languages to accommodate the 14 nationalities present and was distributed within all canteens together with stool sampling packages and questionnaires. Participants collected a stool sample and completed a questionnaire regarding personal details and occupational exposure (i.e. job position, AMU, animal contact, hospitalization, travelling, and meat consumption). A sample collection booth was installed at the main entrance of the slaughterhouse and was occupied by someone from the research team around all main breaks (from around 5 am to 5 pm). In 2015 and 2016, respectively 1781 (all slaughterhouse production workers) and 354 (production workers from lairage to cooling, to increase samples from earlier and scarcer slaughter line positions) employees were initially targeted to participate. Participants were compensated (25 euro) and gave written consent. The Medical Ethical Committee of the University Medical Centre Utrecht confirmed that the Dutch ‘Medical Research Involving Human Subjects Act’ did not apply for this study (Protocols 14-346/C, 14-403/C).

To determine occupational exposure to ARGs through hand contact, we examined the surface of disposable gloves worn by abattoir staff. At two time points (morning, afternoon) of one single day (for locations refer to Supplementary Figure 5.1), we collected gloves (inside-out, chosen from the hand with most meat contact) in a stomacher bag (BagLight PolySilk®, Interscience, 400 ml, UK).

Pig carcass (N=198) and faecal samples (N=60), meat-end products (N=198)

Carcass samples were collected on two consecutive days. The first day, sampling was divided over four time points (early morning, late morning, early afternoon and late afternoon, Supplementary Figure 5.1). Each carcass sample consisted of four cork borer samples from four pigs closely succeeding in line, taken from the pigs' shoulder ($\varnothing$ 25 mm), subsequently pooled and collected in a stomacher bag (BagPage[®]+, with separation membrane, Interscience, 400 ml, UK). In parallel, at each time point, 14-16 pig faecal colon samples were collected at the veterinary inspection platform. The second day, we collected and similarly pooled meat samples in the morning (carcass cooling), and the morning and afternoon (cutting and deboning) from pigs slaughtered the previous day. Finally, packed meat end-products, intended for retail, were collected on six different mornings (pork chops, unconnected to previous sampling, N=198) after packing under a modified atmosphere ($\sim$ 30% CO₂, $\sim$ 70% O₂) at Vion Groenlo.

Air samples (N=33)

Personal inhalable air samples were collected over the course of one shift ($\sim$ 8 h) by abattoir staff along the slaughter line (Supplementary Figure 5.1) by means of a GilAir-5 pump [flow rate of 3.5 l/min, GSP conical sampler (JS holdings, UK)] containing a Teflon (PTFE) SKC filter (2.0 μ m, pore size, $\varnothing$ 37 mm, SKC Inc., USA). We only included successful 4 to 6 h measurements, excluding breaks, for measurement consistency.

Environmental samples laboratory preparation

Laboratory preparation steps including overall sample handling are described in detail in the Supplementary data. Briefly, Ringer's solution was applied to the outside of each glove (by filling the inside-out turned gloves within a stomacher bag) and poured into stomacher bags containing the carcass samples or meat-end products, followed by further processing using a stomacher and centrifuge. Pellets were subsequently obtained and stored at -80°C until DNA extraction. Air filters were sterilely transferred to Greiner tubes which were then filled with extraction fluid and centrifuged. The fluid was freeze-dried and the resulting lyophilizate was stored at -20°C until DNA extraction.

DNA extraction and qPCR analysis

Samples were thawed just prior to DNA extraction. DNA from human and pig faeces was extracted by the modified QIAmp Fast DNA stool mini kit (Qiagen, cat. no. 51604) as described before.¹⁶ Glove DNA was extracted by the NucleoSpin®96 Food kit (Macherey-Nagel, Germany), while meat and air DNA was respectively extracted by the modified Nucleospin® Food kit (Macherey-Nagel) and the modified NucleoSpin® 8 Plant II kit. Following DNA extraction, *tetW*¹⁷ and *ermB*¹⁸ genes were targeted by qPCR (Supplementary Table 5.1). Additionally, qPCR was performed to target 16S rRNA,¹⁹ a general molecular marker for microbial communities, used for normalization of *tetW* and *ermB* gene copies per total bacterial load. Refer to the Supplementary data for more details regarding DNA extraction and qPCR.

Data preparation and statistical analysis

Questionnaires and field forms were entered and checked for consistency in EpiData 3.1 before importing into 'R' v.3.5.1 for data cleaning and statistical analysis.²⁰ Potential determinants from the questionnaire were used in the analysis when at least 95% of the questions were answered. Due to potential difficulties in identifying antimicrobials by its users, antimicrobial usage was recorded after evaluating two questions from the questionnaire: (i) The question regarding the usage of any antimicrobials or medication in the past year and (ii) In case medication was used, a question had to be answered with regard to the characteristics of the drugs used (name, indication, duration of usage, administration method). In case of doubt with regard to the type of medication used, the answer was marked as 'potential antimicrobial usage'. Participants were asked to choose up to three of the presented slaughter line positions where they worked the most and to describe their role in their own words. If workers indicated more than one job position, the staff was assigned to the last, presumably cleaner, position in the slaughter line favouring specificity over sensitivity.²¹ If other positions were mentioned, they were assigned to the category 'other' unless the position clearly fitted in one of the slaughter line categories.

An ARG job-exposure matrix (ARG-JEM) for glove and carcass contamination was created by calculating the percentage of detects (*tetW*, *ermB*) in carcass and glove samples for seven job area groups for which both carcass and gloves samples were available (Table 5.1, model population N=327). These percentages were then allocated to workers in these positions for each combination of gene (*tetW/ermB*) and sample type (carcass/glove). Other environmental samples

Table 5.1 General characteristics of the human slaughterhouse population and the human population included in the models

Population characteristics	Full population ^a (N=482) N/total (%)	Population included in the models ^a (N=327) N/total (%)
Gender (<i>female</i>)	76/476 (16.0)	47/322 (14.6)
Smoking (<i>no</i>)	256/470 (54.5)	177/319 (55.5)
Antimicrobial use (<i>no</i>)	427/475 (89.9)	291/324 (89.8)
Mean age (10 th -90 th percentile)	39.0 (24.0-54.1) N=480 ^b	38.7 (25.0-54.0) N=325 ^b
Job position^c		
■ <i>Black area:</i>		
■ Lairage, bleeding	23/482 (4.8)	23/327 (7.0)
■ Scalding to singeing	26/482 (5.4)	26/327 (8.0)
■ <i>Clean area:</i>		
■ After singeing	8/482 (1.7)	8/327 (2.5)
■ Evisceration, pluck removal	52/482 (10.8)	52/327 (15.9)
■ Inspection, dressing	79/482 (16.4)	79/327 (24.2)
■ <i>Cutting and deboning:</i>		
■ Cutting room	60/482 (12.5)	60/327 (18.4)
■ Deboning room	79/482 (16.4)	79/327 (24.2)
■ <i>Other areas:</i>		
■ Cooling	18/482 (3.7)	n.a.
■ Organ area	42/482 (8.7)	n.a.
■ Other (e.g. office, facility, cleaning)	95/482 (19.7)	n.a.
Nationalities		
■ <i>Dutch</i>	77/482 (16.0)	46/327 (14.1)
■ <i>Polish</i>	186/482 (38.6)	124/327 (37.9)
■ <i>Romanian</i>	72/482 (15.0)	55/327 (16.8)
■ <i>Other^d</i>	124/482 (25.7)	39/327 (11.9)
■ <i>Unknown (missing)</i>	23/482 (4.8)	13/327 (4.0)

^a N of the full (N₂₀₁₅=325, N₂₀₁₆=157) and model population (N₂₀₁₅=211, N₂₀₁₆=116) is an approximation of the size of the datasets used for the *tetW* and *ermB* (log₁₀ copies) analyses before outlier removal. The *tetW* and *ermB* full and model datasets have 97.3% (full population) and 96.6% (model population) of all observations in common. Missings were excluded in the summary figures per variable.

^b N = part of the population for whom a date of birth was available.

^c In total, around 24% of the workers worked in more than one area. Due to the targeting of workers from lairage to cooling in 2016, only 4 workers reported working in the cutting and deboning area (last position in the slaughter line) in 2016. *n.a.* = category not included in the models (gloves and carcass samples were not both available).

^d E.g. Hungarian, Slovakian and Portuguese nationalities

were only used for descriptive analysis due to localized sampling (pig faeces, meat-end products) or a limited number of samples (air).

All eligible questionnaire and ARG-JEM variables were separately linearly regressed against workers' *tetW* or *ermB* carriage, expressed as $\log_{10}$ copies per gram faeces. Then, three separate multiple regression models were fitted per gene (*tetW*, *ermB*) including either the ARG-JEM derived carcass or gloves variable or the job position variable (with similar job positions included as in the JEM), next to covariates from the univariate analysis ($p < 0.1$) and potential confounders. Potential interactions and assumptions of the final models were checked (with and without potential outliers based on model diagnostics). Subsequently, geometric mean ratios (GMR) were computed by exponentiating regression coefficients and their 95% confidence intervals (CIs). Finally, a sensitivity analysis was performed including the 16S-normalized human faecal *tetW* and *ermB* $\log_{10}$ copies as the outcome variable.

5.3 Results

TetW and *ermB* were detectable in all human and pig faecal samples, whereas a smaller proportion of carcass (~20%), glove (~70%), and air (~30%) samples showed levels above the limit of detection (LOD, Supplementary Table 5.2).

Human and pig samples

The human population (mean age 39 years, range 18-64) mostly consisted of men (84%), with 54.5% non-smokers (Table 5.1). Most common nationalities were Polish (38.6%), Dutch (16%), and Romanian (15%). Dutch, Polish, and Romanian staff are roughly proportionally represented within the black area (respectively 26.5%, 24.5%, and 28.6% of all workers in the black area), while their majority is working at cutting and deboning (of all Dutch workers: 50.0%, of all Polish workers: 45.5%) or the clean area (of all Romanian workers: 57.4%). Most participants working at fixed positions along the slaughter line worked at inspection and dressing and within the deboning room (both 16.4%). Furthermore, the majority of staff (89.9%) reported not to have used any form of antimicrobials one year before sampling.

All human samples were found positive for *tetW* and *ermB*. *TetW* and *ermB* concentrations found in human faeces are lower compared to pig faeces. In both human and pig faeces, we observed higher concentrations of *tetW* than *ermB*. Human ARG concentrations varied across slaughter steps (Figure 5.1).

Figure 5.1 Prevalence of *tetW* and *ermB* log₁₀ copies per gram stool from slaughterhouse staff along the slaughter line

The slaughter line runs from left to right: categories ‘organs’ and ‘other’ are technically not part of the slaughter line. Asterisks (*) depict the mean *tetW* or *ermB* log₁₀ concentrations at a specific job location along the slaughter line. A box represents the 25th (Q1) to 75th (Q3) percentile, the centreline depicts the median (Q2). Dots represent values larger or smaller than Q1 – 1.5 or Q3 + 1.5 times the interquartile range.

x-axis: N (numbers displayed above the X-axis): The number of human faecal samples eligible for analysis after qPCR quality control per slaughter position for each gene target (*tetW*, *ermB*). The following job positions are included per slaughter step: Lairage: Stables, stunning area. Bleeding: Hanging and bleeding of stunned pigs. Scalding: Positions after carcass scalding and carcass dehairing, before carcass singeing. Singeing: Positions after carcass singeing, before evisceration. Intestines: Removal of intestines (evisceration). Pluck: Pluck removal (removal of liver, lungs, heart, oesophagus and tongue). Inspection: Veterinary inspection platform. Dressing: Dressing of carcasses, removal of fat and diaphragm. Removal: Removal of heads and spinal cords. Cooling: Cooling, includes carcass cooling but also several other cooling units. Cutting: Separate carcass cutting room (within the same building as the main abattoir). Deboning: Separate deboning area (within the same building as the main abattoir). Packing: Packing and preparing meat for transport. Organs: Multiple separate ‘warm’ and ‘cold organ units’, e.g. working with hearts, kidneys and/or livers in separate organ areas. Other: Includes office, facility and cleaning staff, and all other staff not working at a fixed position along the slaughter line.

Meat, glove, and air samples

Carcass samples were predominantly *tetW* and *ermB* positive within the first slaughter steps (respectively both 100% after bleeding, 89% and 54% after scalding, Figure 5.2a). Remarkably, a rise in *tetW* and *ermB* positive samples was seen within cutting and deboning, although nearly all samples had gene concentrations below the limit of quantification (LOQ) (Figure 5.2b). Additionally, we identified only one *tetW* (<LOQ, not originating from the sampled slaughterhouse) and no *ermB* positive packed pork chops. All pork chops originated from Dutch pigs, 21.3% came from the examined slaughterhouse.

In comparison to carcass samples, gloves more frequently tested positive for *tetW* and *ermB*, and overall demonstrated higher median concentrations (samples >LOQ) (Figures 5.3A-5.3B). Additionally, a slight decrease was found after singeing, and an increasing-decreasing trend before and after evisceration & pluck removal. After cooling another decrease was visible, while slightly higher median *tetW* and *ermB* concentrations were found within gloves collected in the organ units. In gloves, *tetW* and *ermB* concentrations were also frequently low (of all positive samples respectively 2-31% were <LOQ). After 16S-normalization, the same, but slightly more distinct trends were visible after 'hanging and bleeding' when examining *ermB* (Supplementary Figure 5.2).

All air samples tested positive for *tetW* and *ermB*, except for samples from the cutting (*tetW* 0/3 and *ermB* 0/2 positive) and the deboning rooms (*tetW* 2/4 and *ermB* 2/3 positive). A clear declining gradient along the slaughter line was visible in samples >LOQ within the first steps of the slaughter line (Figure 5.4). This overall trend was corroborated after 16S-normalization, apart from a seemingly lower median *tetW* and *ermB* concentration found at lairage and stunning (Supplementary Figure 5.3).

Determinants for *tetW* carriage in human stool

The percentage of *tetW* detected on carcasses and gloves was positively associated with the concentration of *tetW* in human stool (GMR for a 10% increase in detected *tetW*=1.05, $p=0.052$ for carcasses, and 1.07, $p=0.01$ for gloves, Table 5.2). Adjustments for gender, age, smoking, AMU and nationality mainly altered the carcass association (carcass: GMR=1.03, $p=0.26$; gloves: GMR=1.08, $p=0.004$). Additionally adjusting for sampling round yielded non-significant estimates for both carcasses and gloves (carcass: GMR=1.004, $p=0.88$, gloves: GMR=1.01, $p=0.70$; Table 5.2).

In the univariate analysis, higher *tetW* concentrations were found within personnel working in the black (GMR=1.91, $p=0.003$) and clean area (GMR=1.41, $p=0.03$) compared with staff working within the cutting and deboning rooms. When the black, clean, and cutting-deboning areas were subdivided, only employees working at lairage and bleeding showed significantly different *tetW* concentrations (GMR=1.94, $p=0.03$) from staff working at deboning. Smoking was associated with lower *tetW* abundance (current versus non-smokers, GMR=0.59, $p=0.0002$), while age was positively associated. Additionally, faecal *tetW* abundances were significantly higher in Dutch staff compared with staff having other nationalities. After adjusting the models including either carcasses, gloves, or job position (for gender, age, smoking, antimicrobial use, nationality, and sampling round), only smoking, nationality and sampling round remained as determinants for *tetW* carriage (Table 5.2). Finally, a sensitivity analysis including the 16S-normalized human *tetW* outcome data, did not change our main conclusions (data not shown).

Determinants for *ermB* carriage in human stool

No significant association was found between the percentage of *ermB* positive carcasses or gloves and *ermB* prevalence in workers' stool (Table 5.2). Compared with Dutch staff, Polish staff carried higher and Romanian staff lower *ermB* abundances in their stool. Smoking had a negative effect (GMR=0.59, $p=0.01$) and sampling round was positively associated with *ermB* concentrations in stool (GMR=2.24, $p=0.003$). Similar observations were found in a sensitivity analysis with the 16S-normalized data, with the following differences in the adjusted model: females carried significantly higher *ermB* concentrations in their stool than males, while the effect of smoking was not significant (data not shown).

5.4 Discussion

Our findings indicate occupational exposure of slaughterhouse employees to *tetW* and to a lesser extent to *ermB* through dermal and inhalation exposure. Carcass and gloves contamination were analysed as a proxy of dermal exposure, potentially resulting in oral uptake via hand-mouth contact. We also found univariate associations between *tetW* in the slaughterhouse environment and human faecal *tetW*, which were attenuated in adjusted models. No such associations were found for faecal *ermB* abundance.

**Figure 5.2 Barplot (a): Percentage of *tetW* and *ermB* detects on pig carcasses and meat collected along the slaughter line (N = number of samples eligible for analysis after qPCR quality control)
Boxplot (b): Total number of *tetW* and *ermB* log₁₀ copies on carcasses along the slaughter line (N = number of samples > LOQ)**

The slaughter line runs from left to right. A box represents the 25th (Q1) to 75th (Q3) percentile, the centreline depicts the median (Q2). Dots represent values larger or smaller than Q1 – 1.5 or Q3 + 1.5 times the interquartile range.

Figure 5.3 Barplot (a): Percentage of *tetW* and *ermB* detects on gloves worn by slaughterhouse staff along the slaughter line (N = number of samples eligible for analysis after qPCR quality control) Boxplot (b): Total number of *tetW* and *ermB* log₁₀ copies on gloves worn by slaughterhouse staff along the slaughter line (N = number of samples > LOQ)
The slaughter line runs from left to right. A box represents the 25th (Q1) to 75th (Q3) percentile, the centreline depicts the median (Q2). Dots represent values larger or smaller than Q1 – 1.5 or Q3 + 1.5 times the interquartile range. Blue dotted lines represent the location of the cooling areas (including the carcass cooling) in the slaughter line.

Figure 5.4 Total number of *tetW* and *ermB* log₁₀ copies on air filters collected along the slaughter line (N = number of samples > 100)

The slaughter line runs from left to right. A box represents the 25th (Q1) to 75th (Q3) percentile, the centreline depicts the median (Q2). Dots represent values larger or smaller than Q1 – 1.5 or Q3 + 1.5 times the interquartile range. Blue dotted lines represent the location of the cooling areas (including the carcass cooling) in the slaughter line.

Table 5.2 Determinants for *tetW* and *ermB* log₁₀ copies per gram human stool

Determinants	<i>tetW</i>		<i>ermB</i>	
	GMR [95% CI]	Adjusted GMR [95% CI]	GMR [95% CI]	Adjusted GMR [95% CI]
Environmental exposure variables				
Carcass (% detects per 10%)	1.05 [0.9997, 1.10]	1.004 [0.96, 1.05]	0.99 [0.91, 1.08]	1.01 [0.93, 1.09]
Gloves (% detects per 10%)	1.07 [1.02, 1.13] ^a	1.01 [0.95, 1.08]	1.005 [0.92, 1.09]	0.98 [0.89, 1.08]
Job position				
■ Black area	1.91 [1.25, 2.92] ^a	1.23 [0.76, 1.97]	0.93 [0.46, 1.88]	1.01 [0.48, 2.14]
■ Clean area	1.41 [1.04, 1.90] ^a	1.15 [0.81, 1.64]	1.03 [0.63, 1.69]	0.94 [0.54, 1.63]
■ Cutting and deboning	reference	reference	reference	reference
Potential covariates and confounders				
Gender (males vs ^b females)	1.23 [0.83, 1.82]	1.09 [0.75, 1.58]	0.60 [0.32, 1.14]	0.61 [0.34, 1.10]
Smoking (current vs ^b non-smoker)	0.59 [0.45, 0.78] ^a	0.61 [0.47, 0.80] ^a	0.71 [0.45, 1.12]	0.59 [0.38, 0.89] ^a
Age	1.02 [1.01, 1.03] ^a	1.004 [0.99, 1.02]	1.01 [0.99, 1.03]	1.00 [0.98, 1.02]
Antimicrobial use (use vs ^b non-use) ^c	1.12 [0.71, 1.76]	1.17 [0.76, 1.80]	0.87 [0.41, 1.86]	1.11 [0.56, 2.20]
Nationalities				
■ Dutch	reference	reference	reference	reference
■ Polish	0.44 [0.28, 0.67] ^a	0.51 [0.32, 0.81] ^a	2.23 [1.15, 4.32] ^a	2.31 [1.12, 4.73] ^a
■ Romanian	0.43 [0.26, 0.71] ^a	0.47 [0.27, 0.81] ^a	0.19 [0.09, 0.41] ^a	0.19 [0.08, 0.44] ^a
■ Other	0.33 [0.21, 0.52] ^a	0.41 [0.25, 0.66] ^a	0.85 [0.42, 1.71]	0.88 [0.41, 1.88]
■ Unknown	0.35 [0.15, 0.85] ^a	0.48 [0.20, 1.15]	3.41 [0.83, 13.96]	4.69 [1.14, 19.27] ^a
Sampling year (2016 vs ^b 2015)	1.81 [1.36, 2.41] ^a	1.86 [1.32, 2.63] ^a	1.87 [1.17, 3.01] ^a	2.24 [1.32, 3.80] ^a

Unadjusted and adjusted regression coefficients are expressed as geometric mean ratios (GMRs). Three models were fitted per gene (*tetW*, *ermB*) including either carcass (JEM), gloves (JEM), or job position. These models were then adjusted for all potential covariates or confounders (gender, smoking, age, antimicrobial use, nationality, sampling year). Adjusted estimates and CIs from the latter variables are shown from the model including gloves (JEM). No major differences were observed between these estimates and estimates from models including carcass (JEM) or job position.

^a p-values <0.05. All numbers are rounded at two figures or the first integer behind the comma unless rounding would result in the misinterpretation of a non-significant effect.

^b This category was taken as the reference category in the regression analysis.

^c Antimicrobial use was recorded as (potential) antimicrobial use in the past year before sampling. A sensitivity analysis showed no major differences between the inclusion or exclusion of potential antimicrobial users. For each model (*tetW/ermB*), three outliers were removed based on the outcome of model diagnostics. A sensitivity analysis showed no major differences between the inclusion or exclusion of outliers.

Our results show clear resistance gene gradients in carcasses along the slaughter line. *TetW* and *ermB* are mostly detected on carcasses after bleeding and partly after scalding (*tetW* > *ermB*) with only small numbers found after evisceration and pluck removal (*tetW*) and within the cutting and deboning area (*tetW* > *ermB*). Consistent with these findings, previous research in pigs has shown that scalding (apart from dehairing) and singeing consecutively reduce the number of resistant bacteria on carcasses,^{22–24} while higher bacterial concentrations may be found after evisceration or pluck removal.^{24,25} The increase in detection and abundance of *tetW* and *ermB* in carcasses after cooling suggests regrowth of resistant bacteria or increased cross-contamination from workers handling meat at the cutting and deboning rooms. Prior studies have reported conflicting effects of chilling (both increases and reductions) on bacterial contamination depending on the pathogen, or the chilling and detection method used.^{25–27} Wu *et al.* (2009)²³ showed a decreasing trend of culturable tetracycline resistant bacteria along the line, yet, in contrast to our study, respectively incalculable or no tetracycline resistant bacteria were found after cooling and cutting, probably related to the higher sensitivity of qPCR, which also detects non-viable bacteria. Rapidly decreasing ARG concentrations on carcasses in the slaughter line and nearly inexistent ARG levels in packed pork chops in our study are encouraging findings with respect to exposure to *tetW* and *ermB* resistance genes of abattoir staff working in a large slaughterhouse, but also of consumers of pork chops derived from Dutch reared and slaughtered pigs. Since 2007, Dutch antimicrobial sales in general and AMU in Dutch pigs have decreased drastically, possibly reducing the resistance levels entering the slaughterhouse and/or potential carcass contamination with resistant bacteria further down the line. However, ARG concentration reduction through scalding and singeing in the current study is likely to be much higher than resistance reduction previously seen in indicator bacteria isolated from Dutch pigs.²⁸

We also found decreasing *tetW* and *ermB* levels along the slaughter line in gloves and air samples. Similar gradients have previously been reported in an MRSA slaughterhouse study applying culture and qPCR (*mecA*, ST398, *SCCmec*).¹² Interestingly, despite no or few *tetW* and *ermB* positive carcasses after singeing, we found high percentages of positive gloves and air samples after singeing, including high numbers of samples >LOQ, especially on gloves. One way to explain this difference between gloves and carcasses is to consider *tetW* and *ermB* abundances on gloves a reflection of resistance found in the worker's environment (e.g. contact with cutting boards, conveyor belts, scaffolds) including meat. Additionally, we could hypothesize that relatively more *tetW* and *ermB* positive gloves compared with carcasses could be caused by frequent hand-meat contact which might result in bacterial, and consequently ARG accumulation on gloves.

Human *tetW* and *ermB* concentrations are lower than in pigs and vary along the slaughter line, yet lack a decreasing gradient similar to the environmental samples. Univariate analysis revealed positive associations between the percentage of *tetW* positive carcasses or gloves and human *tetW* carriage, as well as a positive association between the percentage of *tetW* positive gloves and faecal *tetW* abundance after adjusting for gender, age, AMU, smoking and nationality, but not after adjusting for sampling round. Also, no association with job position remained significant in the adjusted models. This contrasts with findings from culture-based studies where exposure to ESBL-PE and MRSA at certain slaughter positions (mainly live animal contact) was found to be a major determinant for, respectively, faecal ESBL and nasal MRSA carriage, although similar model adjustments as in our study were or could not always be applied.^{10–13}

Microbial composition is interrelated with the resistome.^{14,29} Additionally, tetracycline and macrolide resistance genes (including *tetW* and *ermB*) are known to dominate the gastrointestinal resistome of healthy individuals.³⁰ Prior research also identified inter-individual variation in relative resistance abundances or antimicrobial resistance potentials in the resistome with respect to tetracycline and macrolide resistance gene classes, often dominated by significant country resistome differences.^{31–33} These findings make it less likely to pick up additional differences resulting from AMR exposure along the slaughter line, especially in largely heterogeneous slaughterhouse populations potentially also characterized by different diets or other habits (e.g. diet can quickly influence the microbiome).³⁴ This is also reflected within the significant effects of nationality in our models. Part of the slaughterhouse population is originating from Eastern Europe and working temporarily in the Netherlands. Geographic differences in AMR prevalence have been previously established between Northern and Eastern-European countries, mostly suggesting a higher AMR prevalence in Eastern-European countries.³⁵ Interestingly, Dutch nationality is associated with higher *tetW* concentrations compared to other staff, while Polish and Romanian staff, respectively, carry higher and lower *ermB* concentrations. An effect of international travelling (in our case interpreted as long-distance commuting) was previously described in AMR studies,³⁶ but its potential relationship with nationality and/or ARG carriage could not reliably be tested in our study due to too many missing values. Finally, it must be noted that potential differences in the length of exposure to ARGs between employees (misclassification of long-term exposure due to temporary work) or short-term exposure in general, could also have contributed to the absence of a direct association between environmental exposure and human ARG carriage.

Surprisingly, although the same protocols were applied, higher *tetW* and *ermB* human faecal yields were found in samples from 2016 compared with 2015, even after 16S-normalization, less clearly identified within the environmental samples (data not shown). This potentially could be explained by study cohort differences, differences with regard to the temperature during sampling (resulting in different bacterial loads entering the slaughterhouse) or potential unidentified technical differences. A sensitivity analysis excluding data from 2016 confirmed the main significant associations detected in the adjusted models.

Smoking is reported to affect the gut microbiome.³⁷ Within our study, we found a negative effect of smoking on faecal tetracycline/macrolide resistance gene carriage, which, to the best of our knowledge, is not yet described before. Nevertheless, non-significant negative associations with AMR were reported in previous slaughterhouse studies (including the ESBL-PE study using data from 2015).^{12,13}

Limitations of the study

We investigated exposure to and carriage of ARGs as a proxy for tetracycline and macrolide resistance, hence did not measure resistance expression in viable (pathogenic) bacteria, nor exposure to antimicrobial residues in pig faeces,³⁸ and potentially on carcasses. Additionally, our study had a cross-sectional design and took place in only one, however large, slaughterhouse and was based on convenience sampling. Due to low ARG numbers (including samples <LOQ) on carcasses and gloves, we transformed these variables to the percentage of ARG detects. This reduces contrast and statistical power compared to the usage of average exposure per job area. Applying a JEM might also lead to less statistical power and misclassification, although generally, group-average exposure levels result in little bias of risk estimates.³⁹ Other possible bias might have resulted from recalling e.g. past AMU or only sampling active staff ('healthy worker effect'). Finally, we were not able to adjust our model for more covariates (e.g. animal contact, meat consumption) due to missing data.

5.5 Conclusion

Our study shows occupational dermal and respiratory exposure of slaughterhouse workers to tetracycline (*tetW*) and macrolide resistance (*ermB*) along the slaughter line (*tetW* > *ermB*). Exposure to *tetW* or *ermB* resistance through packed pork chops from Dutch reared and slaughtered pigs is low. Positive associations were

found between *tetW* in the work environment and human faecal *tetW* carriage, yet, could not be upheld in adjusted models. No such associations were found for faecal *ermB*. Sampling round, nationality, and smoking are determinants for *tetW* and *ermB* carriage in slaughterhouse workers.

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Competing interests

Vion Food Group was not involved in the statistical analysis, the interpretation of the data, nor the decision to publish.

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Data statement

Part of the human population included in this study (2015 cohort) is largely overlapping with the study population used in Dohmen *et al.* (2017)¹³ in a study involving faecal ESBL-PE testing.

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Supplementary data

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S5.1 Slaughter process and sampling scheme environmental samples

Supplementary Figure 5.1 Flowchart overview of environmental samples collected per (or in between) slaughter step(s)

C = collected carcass samples (Cc = cork borer, Cm = meat pieces), G = collected glove samples, A = collected air samples, P = pig faecal samples. Black area: Step 1-4. Step 1: Pigs were CO₂ stunned. Step 3: Pigs were scalded in a ~60°C immersion bath. Clean area: Starting after singeing to just before cooling. Step 6: Pig faecal samples were collected at the veterinary inspection platform. Step 7: Cooling and organs: Cooling includes the carcass cooling area (carcasses first pass through a spray cool tunnel of 2°C for 1,5 hour) where carcasses hang overnight between 2-3°C and where the carcass samples were collected. Cooling also includes several 'cooling units'. Organs are processed in several 'warm' and 'cold organ units'. Cutting and deboning rooms: Step 8-9. These are separate entities located within the same building as steps 1-7. Pork collar: 'Pork collar' is meat which can be further processed into shoulder pork chops later in the chain.

S5.2 Sample handling

Human faecal samples were refrigerated at the collection day and transported to the lab on dry ice. Pig faeces were transported to the lab at 4°C. Both sample types were subsequently stored at -80°C the same day until DNA extraction. Air filters were transported at 4°C, released from their sampling heads the same day and consequently stored in a sterile petri dish at -80°C until DNA extraction. Carcass samples and gloves were transported and stored at 4°C and processed the next day. Packed meat end-products were successively collected and transported to the lab at 4°C in the morning, followed by cork borer sampling and further preparations in the afternoon.

S5.3 Laboratory preparation steps

S5.3.1 Gloves, carcasses and meat-end products

To the inside of each glove, 25 ml of autoclaved Ringer's solution (1 Ringer's solution tablet dissolved in 500 ml Milli-Q) was added and subsequently stomached (BagMixer®400W, Interscience, France) for 90 seconds. Next, 13 ml of the suspension was transferred to a 15 ml Greiner vial (Greiner Bio-One GmbH, Germany) and left for 5-10 minutes. Next, 1.8 ml supernatant was transferred to a 2 ml Eppendorf vial (Eppendorf AG, Germany) and centrifuged 6 min at (14000g) at 4°C. After decanting the vials, the resulting pellet was stored at -80°C until DNA extraction.

Stomacher bags containing carcass or meat-end product (pork chops) samples were filled with Ringer's solution in a 1:2 ratio (sample weight:Ringer) and stomached for 1.5 min. 1.8 ml supernatant from behind the membrane was transferred to a 2ml Eppendorf vial. Vials were centrifuged at 16100g for 5 min at 4°C. After decanting the vials, the resulting pellet was stored at -80°C until DNA extraction.

S5.3.2 Air samples

Filters were transferred to 15 ml Greiner tubes (Greiner Bio-One GmbH, Germany), after which 5 ml extraction fluid (Aqua B. Braun, Germany + 0.05% Tween 20, Merck, Germany) was added to the tubes before mixing one hour on an end-over-end roller at room temperature. Next, vials were centrifuged for 15 minutes (1000g). After removal of the filters, the tubes were positioned obliquely overnight at -20°C. Subsequently, all tubes were placed in a freeze-dryer (Labconco Freezone 2.5, USA) until all fluid was vaporized. Remaining lyophilizate was stored at -20°C until DNA extraction.

S5.4 DNA extractions

S5.4.1 DNA extraction glove samples

DNA extraction from gloves was performed by means of the modified NucleoSpin® 96 Food kit (Macherey-Nagel, Germany). This modification included a bead beating step. More specifically, after the addition of 550 µl CF Buffer (pre-heated at 65°C) the sample was homogenized and pipetted in a Lysing matrix A tube (MP Biomedicals, USA) and treated in a Tissue Lyser II (Qiagen, USA, at 30 Hz, 3 times 30 sec) before centrifuging (1500g, 6s) and continuation of the rest of the protocol.

S5.4.2 DNA extraction carcass and other meat samples

DNA extraction from meat was performed by means of the modified NucleoSpin® Food kit (Macherey-Nagel, Germany). Modifications included the addition of a bead beating step. After adding 550 µl CF Buffer (pre-heated at 65°C) the sample was homogenized and pipetted in a Lysing matrix A tube (MP Biomedicals, USA) and treated in a Tissue Lyser II (Qiagen, at 30 Hz, 3 times 30 sec) before centrifuging (1500g, 6s) and continuation of the rest of the protocol.

S5.4.3 DNA extraction air samples (filters)

DNA extraction from air samples was performed by means of the modified vacuum NucleoSpin® 8 Plant II kit (Macherey Nagel, Germany). The following modification was performed: After adding 500 µl cell lysis buffer, the mixture was transferred to a bead beating tube matrix E (MP Biomedicals, USA) after which 10 µl RNase A solution was added. After vortexing, the sample was placed in a bead beater (30 sec at position 5.5; Fastprep-24, MP Biomedicals, USA).

S5.5 qPCR assay preparations and outcome calculation

16S qPCR was performed in Poland (National Veterinary Institute, PIWet, Puławy, PL) while *tetW* and *ermB* qPCRs were performed in the Netherlands (Institute for Risk Assessment Sciences, Utrecht, NL). DNA template was diluted with TE-buffer pH8: 1:100 faeces, 1:10 air filters, 1:20 meat and gloves (Thermo Fisher Scientific, USA) to overcome possible PCR inhibition. For all DNA samples, the PCR reaction (10 µl) consisted of 5 µl supermix (IQ SYBR Green Supermix (16S), IQ supermix (*tetW*) or SsoAdvanced™ Universal Probes Supermix (*ermB*) (Bio-Rad, USA)), 3 µl DNA template, as well as primers (16S: 200 nM each; *tetW*: 600 nM each; *ermB*: 400 nM each) and a probe (*tetW*: 200 nM, *ermB*: 250 nM) (Table S1). Primers were diluted with TE-buffer pH8 (Thermo Fisher Scientific, USA), nuclease-free

water (VWR, UK) and 0.05 µl Precision Blue Real-Time PCR Dye (Bio-Rad, USA) for ease of pipetting. A non-competitive internal amplification control (IAC, synthetic DNA coding for the blue fluorescence protein, *bfp*) was spiked to the master mix (*tetW*: meat, air and gloves, *ermB*: all samples) to control for qPCR inhibition. Consequently, we added *bfp* primers (*tetW*: 600 nM, *ermB*: 400 nM) and a probe (*tetW*: 200 nM, *ermB*: 250 nM). Assays were performed in a CFX384 Real-Time System, C1000 Touch thermal cycler (Bio-Rad, Singapore) while using Hard-Shell® 384-Well PCR Plates (Bio-Rad, USA) and Microseal 'B' Adhesive Seal (Bio-Rad, UK). Calibration curves were prepared from synthetic DNA consisting of fused target sequences for the different genes. In addition, per PCR plate we used 8 negative (TE buffer pH8, Thermo Fisher Scientific, USA) and 4 positive control samples (aliquots of mixtures of faecal, meat and air DNA extracts depending on the sample examined). The preparations of the standard curves and positive control samples were centralized in a single laboratory (Institute for Risk Assessment Sciences, Utrecht, NL). All samples and controls were run in two technical qPCR replicates.

Ct values were derived from the CSV exported amplification data by means of the 'R' package *chipPCR*¹. The limit of detection (LOD) was defined based on the number of false-positive (among the negative controls) and false-negative samples (among the calibration curve samples of low concentration not showing amplification) through a receiver-operator curve approach.² Limit of quantification (LOQ) values were determined by defining a tolerable absolute deviation in log copies from true concentrations and by determining the highest Ct value below which absolute deviation was tolerable. The tolerable absolute variation was computed from the joint calibration curve constructed from all PCR plates and defined as 95% of all samples showing a log copy deviation from the true concentration of less than 1 log unit within a 2 Ct window. The following LODs, LOQs and qPCR efficiencies were applied and computed (respectively LOD, LOQ, qPCR efficiency percentage): 16S – 3.11, 3.11, 103%; *tetW* – 0.64, 2.16, 97.1%; *ermB* – 1.52, 2.02, 94.3%. Tolerable limits for the IAC (*bfp* Ct within 2 standard deviations of the mean *bfp* Ct in standard curve samples across all plates) and for the deviation between the two technical replicates (limits defined as retrieved from deviations between the standard curve technical replicates) were defined. Two types of variables were calculated. The first variable of ARG carriage was calculated as the number of log₁₀ copies (*tetW* or *ermB*) per gram faeces (human and pig faecal samples), per glove (glove samples) and per filter (air samples). Furthermore, 16S-normalized amounts of *tetW* and *ermB* genes were separately derived by subtracting the 16S log₁₀ copies from the *tetW* and *ermB* log₁₀ copies.

Supplementary Table 5.1 Overview of qPCR target and internal control sequences

Gene	Sequence - forward	Sequence - reverse	Sequence - probe	Reference	Modifications to the standard protocols and primer/probe concentrations
<i>tetW</i>	CGGCAGGGCAAGAGAAC	CGGGTCAGTATCCGCAAGTT	6FAM-CTGGACGCCTTTACG-TAM-RA-BHQ1	Walsh <i>et al.</i> (2001) ³	<ul style="list-style-type: none">■ Primer and probe concentrations: resp. 600 nM and 200 nM■ Annealing temperature: 59°C, 45 sec.
<i>ermB</i>	GGTTGCTCTTGACACTCAA	CAGTTGACGATATTCTCGATTG	AACTTACCCGCCATACCACAGATGTTCC	Kolke <i>et al.</i> (2010) ⁴	<ul style="list-style-type: none">■ Primer and probe concentrations: resp. 400 nM and 250 nM■ Annealing temperature: 61°C, 1 min.
16S	ACTCCTACGGGAGGCAGCAG (338F)	ATTACCGGGCTGCTGG (518R)	n/a	Flierer <i>et al.</i> (2005) ⁵	<ul style="list-style-type: none">■ Primer concentrations: 200 nM■ Annealing temperature: 60°C, 45 sec.
Internal control	Sequence - forward	Sequence - reverse	Sequence - probe	Reference	Modification to the standard protocol
Bfp	CAAGGTCATATCATGCCGAC	CCGTCTCGATGTTGTGG	TGAAGTTCGCCTTGATGCCGTTCT	de Rooij <i>et al.</i> (2019) ⁶	<ul style="list-style-type: none">■ Primer concentrations: <i>tetW</i> 600 nM, <i>ermB</i> 400 nM

S5.6 Overview of samples

Supplementary Table 5.2 Overview of samples for statistical analysis per ARG target after qPCR quality control based on qPCR duplicate consistency and the internal amplification control (IAC)

Gene target	human faeces	pig faeces	carcasses	gloves	air (filters)	packed meat-end products
<i>tetW</i>	480 (100%)	60 (100%)	167 (25.8%)	300 (71.0%)	32 (78.1%)	189 (0.5%)
<i>ermB</i>	471 (100%)	60 (100%)	141 (20.6%)	268 (68.7%)	29 (86.2%)	166 (0.0%)

The percentage of detects (> LOD: *tetW*: Ct = 0.64, *ermB*: Ct = 1.52) is provided between brackets.

S5.7 Results of environmental samples, additional figures

S5.7.1 Gloves – normalized log₁₀ copies

Supplementary Figure 5.2 Total number of *tetW* and *ermB* 16S-normalized log₁₀ copies on gloves worn by slaughterhouse staff along the slaughter line (N = number of samples > LOD). A box represents the 25th (Q1) to 75th (Q3) percentile, the centreline depicts the median (Q2). Dots represent values larger or smaller than Q1 – 1.5 or Q3 + 1.5 times the interquartile range. Blue dotted lines represent the location of the cooling areas (including the carcass cooling) in the slaughter line. The slaughter line runs from left to right.

S5.7.2 Air samples – normalized log₁₀ copies

Supplementary Figure 5.3 Total number of *tetW* and *ermB* 16S-normalized log₁₀ copies on air filters collected along the slaughter line (N = number of samples > LOQ). A box represents the 25th (Q1) to 75th (Q3) percentile, the centreline depicts the median (Q2). Dots represent values larger or smaller than Q1 – 1.5 or Q3 + 1.5 times the interquartile range. Blue dotted lines represent the location of the cooling areas (including the carcass cooling) in the slaughter line. The slaughter line runs from left to right.

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Determinants for the faecal resistome of farmers and slaughterhouse workers

Chapter 6

The faecal resistome and microbiome of farmers and slaughterhouse workers: a metagenome-wide cross-sectional study

Manuscript in preparation

Supplementary data can be found at the end of this chapter

Supplementary Excel tables can be retrieved by clicking [this link](#) (online version) or upon request

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Abstract

Background

By studying the entire human faecal resistome and associated microbiome, the diversity and abundance of faecal antimicrobial resistance genes (ARGs) can be comprehensively characterized. Prior culture-based studies have shown associations between occupational exposure to livestock and carriage of specific antimicrobial resistant bacteria. Using shotgun metagenomics, the present study investigated 194 faecal resistomes and bacteriomes from humans occupationally exposed to ARGs in livestock (i.e. pig and poultry farmers, employees and family members, pig slaughterhouse workers) and a control population ('Lifelines' cohort) in the Netherlands. In addition, we sought to identify determinants for the resistome and bacteriome composition by applying a combination of multivariate analysis techniques (NMDS, PERMANOVA, SIMPER and DESeq2 analysis).

Results

Pig slaughterhouse workers and pig farmers had a higher total ARG abundance compared to broiler farmers and control subjects. Tetracycline, β -lactam and macrolide resistance gene clusters dominated the resistome of all studied groups. No significant resistome alpha diversity differences were found among the four populations. However, the resistome beta diversity showed a separation of the mean resistome composition of pig and pork exposed workers from broiler farmers and controls, independent of their antimicrobial use. We demonstrated differences in resistome composition between slaughter line positions, pig versus poultry exposed workers, as well as differences between farmers and employees versus family members. In addition, we found a significant correlation between the bacteriome and resistome, and significant differences in the bacteriome composition between and within the studied populations.

Conclusions

We found an increased ARG carriage in persons working in the Dutch pork production chain as compared to poultry farmers and controls. Additionally, we showed significant differences in resistome and bacteriome composition of pig and pork exposed workers compared to a control group, as well as within-population (farms, slaughterhouse) compositional differences. Our results suggest direct or indirect livestock contact as a determinant for human ARG carriage. Future studies should focus on the connection between the human and livestock resistome to substantiate the evidence for livestock-associated resistome acquisition.

6.1 Background

Recent technological advances have resulted in a rapidly increasing number of studies using metagenomic sequencing to investigate the composition and determinants of the human gut resistome (i.e. the full collection of antimicrobial resistance genes (ARGs) in gut microbiota).¹ Studies investigating determinants for the human faecal resistome have reported an increase in ARG abundances after antimicrobial use (AMU) or international travel,^{2–4} whereas large resistome differences have been demonstrated between countries, including significant associations with country-dependent use of human and veterinary antimicrobials.^{5–7} Other studies have described associations between the bacterial community composition and the resistome in human faeces, animals and soil.^{8–10}

Numerous studies have highlighted occupational exposure of humans to AMR bacteria among agricultural or food industry workers (e.g. farmers, slaughterhouse staff or veterinarians) through direct or indirect contact with livestock.^{11–20} However, research has often focused on the transmission of specific resistant bacteria like livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) or extended-spectrum β -lactamase (ESBL) producing bacteria. Studying the entire resistome of occupationally exposed populations could provide a more comprehensive characterization of ARG exposure, offering additional insight into the biological pathways of AMR in these groups.

Our recent studies as part of the European project “Ecology from Farm to Fork Of microbial drug Resistance and Transmission” (EFFORT: <http://www.effort-against-amr.eu/>), have shown clear compositional faecal resistome differences between pigs and broilers, and were able to report on determinants for these livestock resistomes.^{9,21,22} The current study aims to characterize the abundance and diversity of the faecal resistome and microbiome of 194 persons occupationally exposed to AMR in livestock (pig slaughterhouse workers, pig and broiler farmers, their employees and family members working or living in the Netherlands) and ‘nonexposed control’ subjects from the ‘Lifelines’ cohort. Additionally, we sought to identify (dis)similarities between, and determinants for, the faecal resistome and microbiome composition of these four populations.

6.2 Results

Descriptive study results

Pig slaughterhouse workers and pig and poultry farmers were selected within the context of the EFFORT project and were contrasted with a control population selected from the 'Lifelines' cohort (refer to the Materials and methods). Overall, the study population consisted mostly of males (66.8%), especially in the slaughterhouse (91%) (Table 6.1).

Table 6.1 Characteristics of the study population

	Pig slaughterhouse employees (%), from 1 slaughterhouse	Pig farmers (%) from 19 farms	Broiler farmers (%) from 12 farms	Control population (%)	Overall (%)
No. of participants	70/194 (36.1)	54/194 (27.8)	24/194 (12.4)	46/194 (23.7)	194/194 (100)
Gender (<i>female</i>)	6/67 (9.0)	25/53 (47.2)	7/24 (29.2)	25/46 (54.4)	63/190 (33.2)
Smoking (<i>no</i>)	35/66 (53.0)	43/54 (79.6)	22/24 (91.7)	34/43 (79.1)	134/187 (71.7)
Mean age (10-90 th percentile)	37.8 (25.8-51.8)* N=69	44.8 (28.0-61.7) N=54	43.4 (23.7-56.7) N=24	46.6 (37.5-54.0) N=46	42.5 (28-55.0)* N=193
Antimicrobial use (<i>no</i>)	64/70 (91.4)**	47/54 (87.0)**	21/24 (87.5)**	46/46 (100)***	178/194 (91.8)

Missings are excluded from the summary statistics. N = Number of participants in each group for which data with regard to age was present. *One observation was missing. **No antimicrobial use: Participants reported no antimicrobial use within one year before sampling. ***No antimicrobial use: Participants reported no oral or intravenous antimicrobial use up to three months before sample collection.

Participants had an average age of 42.5 years and were mostly non-smokers (71.7%). Within the occupationally exposed populations (farming populations, pig slaughterhouse workers), between 8.6-13% had used antimicrobials of any kind in the past year before sampling. The farming population consisted of farmers, employees, spouses/life partners, and other family members. Hereafter, we will refer to the separate populations as 'pig farmers, employees and family members (PFs)', 'broiler farmers, employees and family members (BFs)', 'pig slaughterhouse workers' (WOs) and 'controls' (COs). To refer to results from the farming subpopulations, the terms 'farmers', 'employees' and 'family members' ('spouse'

and ‘other family’) will specifically be used. Within the farming population, the largest groups were farmers (38.5%) and their spouses (33.3%). All farmers and employees entered the stables and nearly all had daily contact with their farm animals ($\geq 92.6\%$). While the majority of the pig and broiler farmer family members did enter the stables ($\geq 83.3\%$), the majority had no daily animal contact ($\geq 66.7\%$) (Supplementary Table 6.1). The mean reported number of working hours per week on pig farms (32.6 hours) was higher than on broiler farms (23.5 hours), but only when family members were excluded a (borderline) significant difference [Analysis of Variance (ANOVA), $p=0.05$] was noted between the mean number of hours worked by pig farmers and employees (48.5 hours) compared to broiler farmers and employees (37.7 hours) (Supplementary Table 6.1).

Faecal resistome counts were normalized for bacterial counts [Fragments Per Kilobase reference per Million bacterial fragments (FPKM)] and clustered at two levels [90% identity level (ARGs) and the AMR phenotypic drug class level (AMR class cluster level) as defined in the ResFinder database]. Absolute levels of ARG abundance (total FPKM) were significantly higher in both PFs and WOs compared to BF and COs (Figure 6.1a). At the AMR class level, we observed large median differences between these populations for e.g. β -lactam and macrolide resistance clusters (Supplementary Figure 6.1a). At pig farms, the highest median ARG abundances were seen in farmers and employees (Figure 6.1b). At broiler farms, the highest median ARG abundances were found in other family members, closely followed by the farmers. When examining persons from both farming populations together, we found a significant difference in median ARG abundance between farmers and employees compared to family members (Kruskal-Wallis test, $p=0.008$).

Figure 6.1 Overview of the faecal resistomes of the occupationally exposed study populations (incl. controls)

1a: Overall ARG abundance (FPKM) per study population. **1b:** Overall ARG abundance (FPKM) in the farming populations. **1c:** Overall ARG abundance (FPKM) per slaughter position in the slaughterhouse population. **Boxplots:** A box represents the 25th (Q1) to 75th (Q3) percentile, the centreline depicts the median (Q2). Dots represent values larger or smaller than $Q1 - 1.5$ or $Q3 + 1.5$ times the interquartile range.

N = Number of observations in each group. *1a: ‘pig sh worker’ = pig slaughterhouse worker. ‘pig farmer’ and ‘broiler farmer’ categories include employees and family members. The Welch’s Heteroscedastic F Test with trimmed means and Winsorized Variances with trimming rate 0.1 tests differences between the four populations. Values above the arches represent the p-values corresponding to the pairwise tests adjusted for multiple testing (Benjamini-Hochberg procedure). *1b: The Kruskal-Wallis test tests farmers and employees versus family members (the latter include spouses and other family members). *1c: The Kruskal-Wallis test includes all slaughter positions presented on the X-axis. The slaughter line runs from left to right. The cooling area is located between the job position ‘other removal’ and the ‘deboning’ room.

Figure 6.1 Overview of the total faecal AMR abundance per and within study subpopulation(s)

Figure 6.2 Relative faecal resistome and bacteriome abundance per study subpopulation

2ab: Relative resistome abundances (FPKM) are clustered at AMR class (a) and at the ARG cluster level (90%ID) (b) and are scaled to 100%. 2cd: Bacteriome phylum (c) and genus (d) level abundances are corrected for genome length and scaled to 100%. 2BCD: Plots represent the top ten most abundant features across the four study populations and labels are arranged in decreasing order with respect to overall ARG abundance across all populations.

Figure 6.2 Continued

All remaining features are part of the category ‘Other’. Legend labels are arranged with respect to a decreasing total ARG abundance calculated for the overall study population. X-axis: ‘pig sh worker’ = pig slaughterhouse worker (WO); ‘pig farmer (PF)’ and ‘broiler farmer (BF)’ categories include employees and family members. ARG cluster names: oxaz-phen = oxazolidinone & amphenicol, macr-oxaz-phen = macrolide & oxazolidinone & amphenicol.

Tetracycline [i.e. *tet(Q)*, *tet(W)*], β -lactam (i.e. *cfxA6*, *cfxA_clust*), and macrolide [i.e. *mef(A)_3_AF227521*, *erm(F)_clust*] resistance clusters were relatively the most commonly identified ARG clusters across all populations (Figure 6.2a-b). Although the relative contributions of these ARG clusters to the resistome composition were fairly homogeneous across populations, some small differences could be noted [e.g. relative *tet(W)* fraction in WOs < *tet(W)* fraction in other populations; *cfxA6* fraction in WOs and PFs > COs > BF]. An overview of the median AMR class or ARG abundance per study population can be found in Supplementary Figure 6.1a-b. Overall, Bacteroidetes (44.2%-53.0%) was the most common bacterial phylum found in faecal samples across all four populations, followed by Firmicutes (18.5-41.9%) and Actinobacteria (10.0%-26.5%) (Figure 6.2c-d). *Prevotella* (15.2%-36.4%), *Cellulomonas* (8.1%-25.0%) and *Bacteroides* (8.6%-22.0%) were the most common bacterial genera.

In WOs, a decreasing mean ARG trend is visible along the slaughter line, whereas the highest mean ARG abundances were found at lairage, evisceration and pluck removal. Significantly different total ARG abundances were found between the slaughter line positions presented in Figure 6.1c (Kruskal-Wallis, $p=0.04$). Additionally, total ARG loads varied significantly between the black area (positions lairage to scalding & dehairing) and the deboning area [Pairwise Wilcoxon rank-sum test (WT), $p=0.006$], and more specifically between lairage and deboning (WT, $p=0.048$). At the AMR class level, a decreasing trend was mainly observed within the (most abundant) tetracycline and β -lactam resistance clusters (Supplementary Figures 6.2a-b). Furthermore, a more fluctuating (up and down) trend along the slaughter line was identified for e.g. the macrolide and aminoglycoside resistance clusters. Relative resistome and bacteriome abundances across the slaughter line are further illustrated in the supplement (Supplementary Figures 6.3a-d).

Faecal resistome and bacteriome richness and evenness

To compute alpha diversity indices, count matrices were rarefied at respectively 5,185 (resistome) and 104,737,719 (bacteriome) counts per sample, retaining respectively 97.4% and 98.5% of all samples. Rarefaction curves are provided in the supplement (Supplementary Figure 6.4). In total, across all human populations, we identified 208 ARG clusters (at 90%ID) of 14 different AMR classes (13 AMR classes in the occupationally exposed populations) of which 38.5% were shared among all populations (Supplementary Figure 6.5). No significant differences in resistome alpha diversity were found between the four study populations (Figure 6.3). In contrast, significant overall differences in mean bacteriome alpha diversity were demonstrated (ANOVA/Welch's test: Richness and Shannon diversity index,

$p \leq 0.0007$). Species richness and Shannon diversity of WO's differed significantly from the three other study populations, while the bacteriome Shannon diversity index of the PFs differed significantly from the COs.

Figure 6.3 Alpha diversity indices per study subpopulation (ARG richness and Shannon diversity index)

Alpha diversity indices were calculated after rarefying the resistome and bacteriome matrices. *3a*: Resistome richness, *3b*: Resistome Shannon diversity index, *3c*: Bacteriome richness, *3d*: Bacteriome Shannon diversity index. 'pig sh worker' = pig slaughterhouse worker, 'pig farmer' and 'broiler farmer' categories include employees and family members. Values above the arches represent the p-values corresponding to the pairwise tests adjusted for multiple testing (Benjamini-Hochberg procedure). Welch's test = Welch's Heteroscedastic F Test with trimmed means and Winsorized Variances with trimming rate 0.1. NA = Pairwise comparisons are not applicable. N = Number of observations in each group after rarefaction. *Boxplots*: A box represents the 25th (Q1) to 75th (Q3) percentile, the centreline depicts the median (Q2). Dots represent values larger or smaller than $Q1 - 1.5$ or $Q3 + 1.5$ times the interquartile range.

Faecal resistome and bacteriome clustering

Clusters were visualized using Non-Metric Multidimensional Scaling (NMDS). Figure 6.4 shows NMDS configurations based on Bray-Curtis dissimilarities for the faecal resistome and bacteriome. The distribution of the resistome population mean (centroid) associated with the BFs and the COs was fairly distinct from those associated with PFs and WOs. Clustering patterns did not largely change after excluding potential antimicrobial users, nor after the exclusion of family members or deboning staff (potential ‘low-risk groups’) from respectively the farming and slaughterhouse populations. The latter resulted in a slight overlap of the PF and BF’s 95% centroid confidence interval (CI, data not shown). Similar compositional differences were observed for the bacteriome, although the population mean (95% CI) of persons sampled at broiler farms was less distinguishable from persons sampled at pig farms (Figure 6.4). Additionally excluding above mentioned lower risk categories resulted in overlapping centroids of pig and pork exposed persons (farmers, slaughterhouse staff) (data not shown).

Figure 6.4 Resistome and bacteriome ordinations (NMDS, Bray-Curtis dissimilarity)

4a: Resistome clustering at ARG cluster level (90%ID). 4b: Bacteriome clustering at the bacterial genus level. ‘pig sh worker’ = pig slaughterhouse worker. ‘pig farmer’ and ‘broiler farmer’ categories include employees and family members. 2D/3D = NMDS in 2 or 3 dimensions. stress = stress associated with the NMDS ordination. Ellipses represent the 95% confidence intervals of the centroid’s standard error per study population. The associated Shepard stress plots can be found in Supplementary Figure 6.7

Determinants for the faecal resistome and bacteriome of humans occupationally exposed to livestock

Compositional differences between the studied populations

Permutational Analysis Of Variance (PERMANOVA) revealed significant differences between the faecal resistome compositions of the four study populations (ARG cluster: $R^2=12.3\%$, Table 6.2; AMR class: $R^2=16.0\%$, data not shown, both $p=0.001$).

Excluding potential antimicrobial users slightly increased the percentage explained variation (ARG cluster: $R^2=13.2\%$, AMR class: $R^2=17.3\%$, both $p=0.001$), while excluding potential low-risk categories (family members, deboning staff) resulted in a more substantial increase in explained variation (ARG cluster: $R^2=19.1\%$, AMR class: $R^2=25.8\%$, both $p=0.001$). Similar findings were observed for the bacteriome (Table 6.2).

We also identified significant within-population dissimilarities (Table 6.2). In particular, small resistome differences were demonstrated distinguishing slaughterhouse staff based on job position (working at the (i) black area, (ii) clean area and (iii) deboning room), which were not found for the bacteriome. Within the farming population, resistome and bacteriome compositional differences were observed between PFs and BF, and between farmers & employees and family members.

The human faecal bacteriome shapes the resistome

The Procrustes analysis revealed a significant correlation ($0.49, p=0.001$) between the faecal resistome and bacteriome recovered from the overall study population (Supplementary Figure 6.6a). The largest range in (dis)similarities between the resistome and bacteriome was seen in the slaughterhouse population (Supplementary Figure 6.6b).

Occupational associations with specific ARGs

NMDS ordinations mainly showed a shift of the mean resistome and bacteriome composition in subjects occupationally exposed to pigs and pork compared to the COs. Therefore, we further examined the compositional dissimilarities between these populations [pig and pork exposed workers (PFs/WOs) versus the control subjects (COs)] using two types of analyses: Similarity Percentages analysis (SIMPER) and DESeq2.

Table 6.2 Faecal resistome and bacteriome compositional differences between occupationally exposed groups incl. and excl. antimicrobial users

Study population	Categories used in the analysis	Resistome (R ²) % explained variation, incl. antimicrobial users (ARG cluster)	Resistome (R ²) % explained variation, excl. antimicrobial users (ARG cluster)	Bacteriome (R ²) % explained variation, incl. antimicrobial users (genus level)	Bacteriome (R ²) % explained variation, excl. antimicrobial users (genus level)
Full study population (4 groups)	(1) Pig slaughterhouse workers, (2) Pig farms, (3) Broiler farms, (4) Controls	12.3 (p=0.001)	13.2 (p=0.001)	14.9 (p=0.001)	15.7 (p=0.001)
Full study population excl. family members (farms) and deboning staff (slaughterhouse) (4 groups)	(1) Pig slaughterhouse workers, (2) Pig farmers & employees, (3) Broiler farmers & employees, (4) Controls	19.1 (p=0.001)	19.3 (p=0.001)	26.1 (p=n.a.)*	25.9 (p=n.a.)*
Pig farms and abattoir workers versus controls (2 groups)	(1) Pig slaughterhouse workers & Pig farms, (2) Controls	9.2 (p=0.001)	9.5 (p=0.001)	13.3 (p=0.001)	13.7 (p=0.001)
Abattoir workers (3 groups)	Workers from: (1) Lairage to dehairing, (2) Evisceration to Cooling, (3) Deboning	7.3 (p=n.a.)**	7.6 (p=n.a.)**	5.4 (p=0.09)	4.2 (p=0.2)
Farms (2 groups)	(1) Pig farms, (2) Broiler farms	5.4 (p=0.004)	6.0 (p=0.001)	4.7 (p=0.008)	4.7 (p=0.01)
Farms (2 groups)	(1) Pig and broiler farmers & employees, (2) Family members working or living on pig and broiler farms	5.6 (p=0.001)	6.8 (p=0.06)	14.3 (p=n.a.)***	17.3 (p=n.a.)***

Results from the PERMANOVA analyses. The lowest possible p-value = 0.001 at 999 permutations. *, **, *** Unless otherwise specified, the assumption of homogeneity of variances was always met (function 'betadisper', p>0.05); p=n.a.: Although these p-values were highly significant (≤0.004), the assumption of homogeneity of variances was not met. Therefore, resistome or bacteriome dissimilarity was retested at a higher cluster level (AMR class or bacterial class level): *Bacterial class, including potential antimicrobial users: R²=22.4, p=0.001; Bacterial class, excluding potential antimicrobial users: R²=22.8, p=0.001. **AMR class, including potential antimicrobial users: R²=8.9, p=0.002; AMR class, excluding antimicrobial users: R²=8.8, p=0.01. ***Bacterial class, including potential antimicrobial users: R²=13.5, p=0.001; Bacterial class, excluding potential antimicrobial users, R²=19.7, p=0.001.

Results Similarity Percentages analysis (SIMPER)

Highly abundant AMR class clusters, tetracycline [*tet(Q)*, *tet(W)*] and β -lactam (*cfxA6*, *cfxA_clust*) resistance clusters, contributed most to the overall resistome dissimilarity between PFs/WOs and the COs as visualized in the NMDS (up to a ~64% cumulative contribution, Supplementary Excel Table 6.3a). The overall bacteriome dissimilarity is mainly resulting from the joint contributions of the following bacterial genera in decreasing order (up to a ~70% cumulative contribution): *Prevotella*, *Bacteroides*, *Cellulomonas*, *Oscillobacter*, *Roseburia*, *Blautia* and *Alistipes* (Supplementary Excel Table 6.3b).

Results differential abundance analysis (DESeq2)

A more in-depth ARG differential abundance analysis between the pig and pork exposed workers and the control population was performed using DESeq2. Here, we found that resistomes of PFs/WOs contained significantly higher levels of 30 ARGs compared to the resistomes of COs (range: 1.9–32.2 times as high, FDR $p < 0.05$), with only one ARG cluster (*VanR-D*) being significantly less abundant in pig/pork workers compared to controls (Supplementary Excel Table 6.4a). Of these ARGs, 22 genes (73.3%) are part of three highly abundant AMR classes previously identified (tetracycline, β -lactam, macrolide resistance).

6.3 Discussion

The present study used metagenomic shotgun sequencing to investigate the abundance and diversity of the faecal resistome and bacteriome of persons occupationally exposed to pigs or poultry in the Netherlands. Our goal was to identify determinants for the composition of these resistomes and bacteriomes. Findings from this study show an increased ARG carriage in pig and pork exposed populations (PFs/WOs) compared to control subjects (COs). No such differences were found between broiler farmers (BFs) and COs.

Overall human resistome composition

Tetracycline [i.a. *tet(Q)*, *tet(W)*], and to a lesser extent β -lactam (i.a. *cfxA6*, *cfxA_clust*) and macrolide resistance clusters [i.a. *mef(A)_3_AF227521*, *erm(F)_clust*], dominate the resistome of both livestock exposed and control populations. Overall, these findings were consistent with previous research in which mostly

tetracycline [e.g. *tet(Q)*, *tet(W)*], but also macrolide [e.g. *erm(B)*, *erm(F)*] and β -lactam (e.g. *cfxA*) resistance clusters were identified in the gut of healthy individuals.^{7,23} Although differences were noted, relative resistome compositions were found to be fairly homogeneous among the studied populations.

Animal exposure as a potential determinant for resistome composition in humans

Overall, higher ARG loads were identified in PFs and WOs compared to BFs and COs. Furthermore, we did not observe significant differences in resistome alpha diversity among the studied populations, but showed differences in mean resistome composition between pig and pork exposed workers and family members (PFs/WOs) and BFs/COs in the ordinations. Using PERMANOVA, we also observed significant resistome compositional differences between these populations. The effect was shown regardless of antimicrobial use, a potential determinant for faecal microbiome composition and ARG carriage.^{2,24,25} In addition, removing potentially lower-exposed persons (farmers' family members, slaughterhouse 'deboning' staff) from the analysis increased the explained variation substantially. Further comparison of the resistome of PFs/WOs with the COs revealed higher mean ARG abundances within faecal samples from PFs/WOs compared to those of COs for a limited number of (mainly) highly abundant ARG clusters. Finally, next to between-population differences, we also demonstrated smaller significant within-population resistome compositional differences based on farm type (pigs versus broiler), farm work (farmers and employees versus family members), and job position within the slaughterhouse.

To the best of our knowledge, no direct comparisons between persons sampled in slaughterhouses, farms or the general population are currently available regarding resistome composition or overall ARG loads. Prior culture-based studies in occupationally exposed populations have shown increased carriage rates of specific resistant bacteria [nasal LA-MRSA, faecal ESBL-producing Enterobacteriaceae (ESBL-PE)]. For instance, an overall higher MRSA prevalence was reported in pig farmers compared to pig slaughterhouse staff or broiler farmers, with the two latter populations in turn carrying a higher prevalence compared to the general population.^{18,19,26–28} This contrasts with reports on overall faecal ESBL carriage, which was described to be higher in broiler farmers compared to pig farmers and pig slaughterhouse workers employed at early positions in the pig slaughter line, while the latter two populations again carried higher faecal ESBL loads in comparison to the general population.^{17,29,30} Possible explanations for these different carrier rates among occupationally exposed

populations include differences in bacterial prevalences found between livestock species (e.g. pigs, broilers, veal calves) to which farmers or slaughterhouse workers are exposed to. Resistome compositional differences have been reported between pigs and poultry, while higher mean ARG loads were identified in pigs compared to broilers,⁹ levels which were confirmed for the pooled pig and broiler faecal samples from the Dutch farms included in the current study: the mean faecal ARG load of pigs was 4352 FPKM (10-90th percentile: 3084-6472 FPKM) versus a mean ARG load of 1880 FPKM of broilers (10-90th percentile: 1061-2814 FPKM).

Culture-based studies have also described positive associations between nasal or faecal AMR carriage and on-farm working hours, intensive on-farm livestock contact (especially with MRSA or ESBL-positive livestock), and working in the stables.^{13,15,29–32} Similarly, having contact with live animals or working at early slaughter positions in the slaughter line have been associated with AMR carriage in slaughterhouse staff.^{17,18,26} Within the present study, we did not find significant mean differences between pig and broiler exposed persons with respect to daily animal contact, on-farm working hours and stable entry. Therefore, animal exposure only unlikely explains all differences in ARG abundances or resistome dissimilarities between the pig and broiler farming populations. However, the lower percentages of daily farm animal contact and lower number of on-farm working hours of (pig) farmer family members compared to farmers and employees could potentially explain their lower faecal ARG loads, including the significant resistome compositional differences between pig and broiler family members and pig and broiler farmers and employees. Alternatively, the overall potential for ARG transmission, might be higher at pig farms than broiler farms. Although we did not measure the intensity of individual animal contact in this study (e.g. recording of specific on-farm activities), conventional Dutch farrow-to-finish farmers in the Netherlands tend to engage in a higher degree of individual animal contact compared to conventional Dutch broiler farmers, as they are involved in various activities such as inseminating, handling of piglets (e.g. applying ear tags, tail cutting), weaning piglets or individual medicine treatments, activities which are not part of conventional broiler farming.

Resistome differences within the context of the slaughterhouse

We demonstrated relative resistome dissimilarities between pig slaughterhouse staff. In particular, we identified a decreasing trend in overall faecal ARG loads along the slaughter line, with significantly higher overall ARG loads in staff working at earlier slaughter line positions (black area, lairage) compared to persons

working at the deboning room. Additionally, we found significant resistome compositional differences with regard to job location in the slaughterhouse [(i) black, (ii) clean, or (iii) deboning area]. These findings confirm results from prior culture-based research in pig slaughterhouse employees, where higher nasal MRSA and faecal ESBL carriage were found to be associated with live animal contact or working at early slaughter line positions.^{17,18} Our recent qPCR study³³ tested associations between slaughter line job position and *tet(W)/erm(B)* human environmental exposure to these ARGs and human faecal *tet(W)/erm(B)* carriage. The latter study identified decreasing ARG loads [*tet(W)* and *erm(B)*] in the environment along the slaughter line, but no robust significant differences were found between workers with respect to job position along the line, nor an association was found between environmental ARG exposure and faecal ARG carriage after elaborate adjustment for confounding.

The role of the bacteriome

Prior research has highlighted the importance of the microbiome in shaping the resistome.^{8,10} Correspondingly, we found a significant correlation between bacteriome and resistome compositions of the overall population. We also observed significant differences between bacteriome alpha diversity of WOs and other populations, as well as a significant difference between bacteriome alpha diversity in PFs and COs (mainly due to evenness: linearly extrapolated from a significant difference in the Shannon index and a non-significant difference in ARG richness). Similar to the resistome, bacteriome compositions of pig and pork exposed populations were shifted from the controls. In addition, we found within-farm bacteriome dissimilarities depending on farm type or farm work (farmers and employees versus family members). Interestingly, the highest explained bacteriome variation was found when excluding potentially lower-exposed family members and slaughterhouse deboning staff, which seems to suggest an effect of livestock exposure.

The healthy microbiome is known to be heavily influenced by environmental factors like lifestyle and diet.^{34–36} The majority (~85%) of the slaughterhouse population consisted of Eastern European workers (mostly Polish and Romanian nationalities), often temporarily residing in the Netherlands, which are potentially exposed to different diets, habits and environments (e.g. through long-distance commuting). The latter might explain differences in gene richness compared to the other three farming and control populations assumed to be Dutch or more permanently residing in the Netherlands. To the best of our knowledge, the role of frequent and/or close human-livestock contact in shaping

the adult gut microbiome has not been investigated before, yet, exposure to pets was previously shown to influence the infant gut microbiome.³⁷ Others have suggested that factors such as cohabitation with dogs, living in urban versus rural areas, the type of occupation (dairy farmers versus non-farmers) and contact with pig barn indoor-air could affect the human skin or nasal microbiome.^{38–41}

Overview of future study improvements

Our cross-sectional study provides first insights into the composition and determinants of the faecal resistome and microbiome of populations occupationally exposed to ARGs in livestock. In contrast to previous culture-based studies, we did not study ARG expression in viable (pathogenic) bacteria, but instead provided a comprehensive overview of ARGs in total community DNA. Within our study, we shotgun sequenced and analysed a relatively large dataset of nearly 200 human faecal samples. The increasingly lower costs of sequencing and the continuous development of improved analysis techniques will provide ample future opportunities to further examine other large AMR exposed populations and additional subpopulations (e.g. within the slaughterhouse), preferably using a longitudinal design or an assembly-based metagenomic strategy to better understand cause-effect and microbiome-resistome relationships.

Based on our study results, we hypothesize that the faecal resistome of persons occupationally exposed to livestock might be influenced by direct contact with livestock or indirectly through contact with the farming or slaughterhouse environment (e.g. indirect contact with ARGs and AMR bacteria on carcasses, in barn dust and air^{14,16}). However, to further substantiate the former claim, it is recommended to include livestock resistome data or other environmental data into the models.

6.4 Conclusion

We identified higher faecal ARG loads in pig and pork exposed workers compared to broiler farmers and control subjects, as well as significant faecal resistome and bacteriome dissimilarities between and within (farms, slaughterhouse) populations occupationally exposed to AMR. Our results suggest that livestock contact might be a determinant for faecal ARG carriage. Further studies are needed to examine the influence of direct or indirect livestock contact on the human faecal resistome.

6.5 Materials and methods

Study population and data collection

As part of the EFFORT project, we visited 31 Dutch conventional, non-mixed, farrow-to-finish pig (N=19) and broiler farms (N=12) between October 2014 and December 2015. Farm selection criteria were as previously described.⁹ Farmers, their family members and employees living (≥ 1 day/week) or working (≥ 0.5 hours/week) on these farms were asked to hand in a faecal sample. From the pool of participants (N=94), we selected a representative subset of samples (N=78) for DNA extraction and sequencing (54 from pig farms and 24 from broiler farms) from adults (≥ 18 years) and always included a sample from the principal farmer, his or her spouse and employees if present. One sample was removed later in the process regarding technical reasons. Furthermore, in two one-week sampling rounds (June 2015 and July 2016), 483 human faecal samples were collected at the largest Dutch pig slaughterhouse as described before.³³ Overall, pig slaughter was divided into two main phases, slaughter in the black area [successively: lairage, stunning, bleeding, scalding/dehairing and carcass scorching (singeing)] and the clean area [successively: evisceration, pluck removal (removal liver, lungs, heart, oesophagus and tongue), carcass splitting, meat inspection, dressings and carcass cooling], followed by a third phase of cutting and deboning. From the pool of workers we then randomly selected 70 samples for sequencing located at the most important slaughtering steps based on the prevailing AMR risk factor literature (sampled steps are illustrated in Figure 6.1c). All participants (farmers and slaughterhouse staff) filled out a questionnaire regarding personal information and past occupational exposure (e.g. age, gender, smoking, antimicrobial use and animal contact). Antimicrobial use was defined as using any type of antimicrobials in the past year before sampling. When participants had indicated medicine use without any further specification, participants were regarded as potential antimicrobial users. In sensitivity analyses, (potential) antimicrobial users were excluded. In total, 46 human faecal samples, collected between February 2014 and January 2015 were retrieved through the Dutch Lifelines biobank (www.lifelines.nl) which serve as controls. Lifelines participants were recruited through general practitioners in the northern part of the Netherlands (Groningen, Friesland and Drenthe).⁴² Based on the available questionnaire data, we excluded intravenous and oral antimicrobial users ($\leq$ three months before sampling) and all individuals who reported to live on a farm or worked with farm animals. All Lifelines participants were not hospitalized three months prior to the assessment. All but one participant were born in the Netherlands.

Sample handling, DNA extraction and first laboratory preparation steps

Farmers were instructed to collect their faecal sample as close as possible to the collection by the researchers and to keep the sample frozen. Following the farm visit, human samples were transported on dry ice and stored at -80°C the same day. Slaughterhouse workers' samples were collected and stored as described before.³³ Lifelines' faecal samples were kept refrigerated by the participant until collection by the researchers, followed by transportation on dry ice and storage at -80°C until DNA extraction.

Samples were thawed on ice and of each sample, DNA was extracted using the modified QIAamp® Fast DNA stool mini kit (Qiagen, cat. no. 51604) as previously applied and demonstrated to preserve microbiome diversity.^{9,43} DNA was subsequently stored at -80°C until further processing. Frozen DNA was consecutively shipped on dry ice to Wageningen Bioveterinary Research (Lelystad, the Netherlands) for library preparation. Libraries were prepared with the amplification poor NEBNext® Ultra DNA Library preparation kit (3-10 cycles, New England BioLabs® Inc., UK). Multiplexing and sequencing were performed using the Illumina HiSeq4000 platform (2x150bp paired-end sequencing) targeted at 40 million PE clusters (80M reads) per sample (GenomeScan, Leiden, the Netherlands).

Metagenomic processing and analysis

Bioinformatic processing was performed as described before.⁹ Briefly, quality controlled and adapter-cleaned read-pairs were mapped to the NCBI bacterial genome reference database and the ResFinder reference resistome database. Raw counts matrices were preserved for certain analyses (see below), while the bacteriome count matrix was corrected for genome length. To normalize the resistome counts, Fragments Per Kilobase reference per Million bacterial fragments (FPKM) were computed. The bacteriome was classified at the taxonomic phylum, class and genus level, whereas the resistome was clustered at the AMR class and the 90% identity cluster level (ARG cluster, CD-HIT-EST).^{9,44}

Database management and statistical analysis

Questionnaires were entered and checked for consistency in EpiData 3.1 before importing into 'R' v.3.5.1⁴⁵ for data cleaning and statistical analysis (an overview of the used 'R' packages, including references for each package, is provided in the supplement). The most important potential ARG determinants were

selected from the questionnaire for further use in the analysis when at least 95% of the questions were answered and if the question still provided enough contrast. Slaughterhouse participants were asked to indicate the slaughterhouse position(s) where they worked the most (maximum of three) from a list of slaughter line positions. In case workers had indicated multiple job positions, slaughterhouse staff was assigned to the last position in the slaughter line as previously described.^{33,46} For all analyses, we initially grouped and compared the four study populations based on the expected intensity of animal contact (PFs > BFs > COs), animal species (pigs versus poultry), and the occupational setting (farms versus slaughterhouse), followed by within-population analyses (farms, slaughterhouse).

Alpha diversity

Overall richness (observed number of species) and the Shannon diversity index were calculated after rarefying the raw resistome count matrix and the genome length corrected species matrix (multiplied by 10^6 before transforming to integers) while retaining at least 95% of all samples (90% per group) ('R' *vegan* function 'rrarefy'). Subsequently, overall statistical differences and pairwise comparisons between the different populations were computed followed by adjustments for multiple testing [False Discovery Rate (FDR), Benjamini-Hochberg procedure].

Clustering of and determinants for the human resistome and bacteriome (NMDS, PERMANOVA, Procrustes analysis)

Before ordinating the faecal resistome (FPKM, at AMR class and ARG cluster level) and bacteriome (genome length corrected and normalized by the sum of genome length corrected counts at the genus level), BC dissimilarities were calculated. NMDS was performed in a 2-dimensional configuration ('R' *vegan* function 'metaMDS'). In case of stress levels greater than 0.15, NMDS was repeated in three dimensions. Additionally, differences in overall resistome or bacteriome composition and/or relative abundances between groups were tested within a PERMANOVA (BC-dissimilarities, 'R' *vegan* function 'adonis' at 999 permutations).⁴⁷ PERMANOVA was applied to identify possible determinants for between and within-population resistome and bacteriome dissimilarities. For each PERMANOVA test, the assumption of homogeneity of variances was checked ($p > 0.05$) using the 'R' *vegan* function 'betadisper'.

The association between the resistome (90% ID clustering) and bacteriome (genus level) was determined by superimposing both resistome and bacteriome

NMDS outcomes (2D) within a symmetric Procrustes analysis.⁹ A p-value was retrieved through the 'R' *vegan* function 'protest' at 999 permutations.

Bray-Curtis dissimilarity decomposition (SIMPER) and differential abundance analysis (Bioconductor: DESeq2)

The most interesting resistome or bacteriome pattern of dissimilarity identified within the NMDS and PERMANOVA analyses was further investigated by means of a SIMPER analysis ('R' *vegan* function 'simper') to assess the contribution of the individual ARGs or bacterial genera to the overall dissimilarities displayed in the beta diversity ordinations.⁴⁸ SIMPER uses the input as described for the NMDS, and defines the contribution of each ARG cluster in explaining at least 70% of the overall dissimilarities between populations as visualized within the NMDS (resulting from variation in feature abundances and differences among groups). Specific differences between groups were further detailed within a differential abundance analysis (Bioconductor *DESeq2* 'R' package). *DESeq2* uses empirical Bayesian shrinkage for dispersion estimation and a negative binomial distribution to estimate log fold changes of features between groups.⁴⁹ For our study, we used the raw resistome data, and applied no further shrinkage for log fold change estimation, while size factors (total bacterial pairs divided by the minimum number of total bacterial pairs)⁹ were provided for normalization purposes. FDR corrected p-values were obtained as previously described.⁹

6.7 Declarations

Ethics approval and consent to participate

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 populations (Protocols 14-346/C, 14-403/C). 'Lifelines' research operated according to the protocols approved by the Medical Ethics Review Board of the Medical Center Groningen (NL) (Protocol METc2007/152). All participants gave written consent. EFFORT participants were financially compensated (25 euro: slaughterhouse employees, 100 euro: per farm family which includes efforts for participation in previous studies).⁹

Data disclosure and availability of data and materials

This research uses datasets from three different study populations. Participants from the farming population (EFFORT project) were selected from Dutch pig and poultry farms sampled within the scope of Munk *et al.*, 2018.⁹ The slaughterhouse population (EFFORT project) was partly analysed for ESBL in Dohmen *et al.*¹⁷ (cohort 2015) and tested for specific ARGs (*tetW* and *ermB*, cohort 2015 and 2016) in Van Gompel *et al.*³³ The Dutch control population is selected from the Dutch Lifelines cohort.⁴² The 194 metagenomes presented in this paper have not been published before.

The DNA sequences (reads) from the 194 metagenomic samples are deposited in the European Nucleotide Archive (EGA) under project accession number EGAS00001003944. All data access should follow the informed consent regulations as stipulated within EFFORT and Lifelines. Access to the metadata from the control group ('Lifelines' cohort: e.g. age, gender, antimicrobial use, animal contact) was purchased from Lifelines for a period of 6 months (data access agreement OV19_0483) and can only be retrieved through www.lifelines.nl/researcher. This excludes the variable gender which can be retrieved through the EGA repository. Nevertheless, Lifelines metadata was only used to select a suitable control group and was not used in the analyses. Finally, due to the potential of tracing back individuals at the slaughterhouse based on the combination of job position, age and nationality, open-access metadata from this group is restricted to the variables directly used in the main analysis (job position, antimicrobial use) and the additional variable sampling round.

Competing interests

Vion Food Group (owner of the sampled slaughterhouse) was not involved in the statistical analysis, the interpretation of the data, nor the decision to publish.

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Supplementary data

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S6.1 Supplementary figures and tables

Supplementary Table 6.1 On-farm livestock contact of persons living or working on a farm

Type of contact	Pig farms (pig contact)			Broiler farms (poultry contact)		
	Farmers & employees	Family members	Total pig farms	Farmers & employees	Family members	Total broiler farms
Number of persons entering stables (%) [*]	27/27 (100)	22/25 (88.0)	49/52 (94.2) [°]	11/11 (100)	10/12 (83.3)	21/23 (91.3) [°]
Persons having daily contact with farm animals (pigs or poultry) (%) [*]	25/27 (92.6)	8/24 (33.3)	33/51 (64.7) [°]	11/11 (100)	3/11 (27.3)	14/22 (63.6) [°]
Mean/median on-farm working hours/week ^{**} (10-90 th percentile)	48.5 / 50.0 (27.6-62.0)	15.5 / 10.0 (0.0-40.0)	32.6 / 39 ^{oo} (1.0-50.0)	37.7 / 40 (20.0-54.4)	9.3 / 4.0 (0.1-24.5)	23.5 / 20 ^{oo} (0.0-60.0)

^{*}Family members' include spouses/life partners and other family members. Data regarding contact with pigs (broiler farms) or poultry (pig farms) could not reliably be analysed due to too many missings. Of those who filled out these questions, only two persons (pig farmers) reported daily contact with 'backyard' (N=5-15) chickens.

^{**}Missing values were excluded. ^{oo}Two missing values excluded. [°]No significant differences were found between pig and broiler farms regarding the number of persons entering the stables or regarding persons having daily farm animal contact (X²-test with Yates' continuity correction, $p \geq 0.9$). ^{oo}No significant difference was found between pig and broiler farms regarding the number of on-farm working hours (Kruskal-Wallis test, $p=0.18$), however, after removing of family members, a borderline significant difference was found between pig and broiler farms (ANOVA, $p=0.0504$).

a

Supplementary Figure 6.1 Overview of the total faecal AMR abundance per study subpopulation clustered by AMR class (6.1a) and at the ARG cluster level (6.1b)

Faecal resistomes clustered by AMR class (a, $\log_{10}(\text{total FPKM} + 1)$) and at the ARG cluster level (b, 90%ID, $\log_{10}(\text{total FPKM} + 1)$). Plot b represents the top ten most abundant features among the four study populations. All remaining features are part of the category 'Other'. Plot sections are arranged with respect to a decreasing total ARG abundance calculated for the overall study population. X-axis: 'pig sh worker' = pig slaughterhouse worker (WO); 'pig farmer (PF)' and 'broiler farmer (BF)' categories include employees and family members. ARG clusters names: oxaz-phen = oxazolidinone & amphenicol, macr-oxaz-phen = macrolide & oxazolidinone & amphenicol. Boxplots: A box represents the 25th (Q1) to 75th (Q3) percentile, the centreline depicts the median (Q2). Dots represent values larger or smaller than $Q1 - 1.5$ or $Q3 + 1.5$ times the interquartile range.

b

Supplementary Figure 6.1 Continued

Supplementary Figure 6.2 Overview of the total faecal AMR abundance in slaughterhouse workers working along the slaughter line, clustered by AMR class (6.2a) and at the ARG cluster level (6.2b)

Faecal resistomes clustered by AMR class (a, log₁₀(total FPKM + 1)) and at the ARG cluster level (b, 90%ID, log₁₀(total FPKM + 1)) level. Plot b represents the top ten most abundant features detected in workers among the slaughter line. All remaining features are part of the category 'Other'. Plot sections are arranged with respect to a decreasing total ARG abundance calculated for the overall slaughterhouse population. X-axis slaughter positions: LA = lairage, HB = hanging & bleeding, SD = positions after scalding and dehairing, EV = evisceration, PR = pluck removal, OR = other types of removal, DB = deboning room. The slaughter line runs from left to right. ARG cluster names: oxaz-phen = oxazolidinone & amphenicol, macr-oxaz-phen = macrolide & oxazolidinone & amphenicol. Boxplots: A box represents the 25th (Q1) to 75th (Q3) percentile, the centreline depicts the median (Q2). Dots represent values larger or smaller than Q1 – 1.5 or Q3 + 1.5 times the interquartile range.

Supplementary Figure 6.3 Relative faecal resistome and bacteriome abundance in slaughterhouse workers working along the slaughter line

6.3ab: Relative faecal resistome abundances (FPKM) are clustered at AMR class (a) and at the ARG cluster level (90%ID) (b) and are scaled to 100%. 6.3cd: Faecal bacteriome phylum (c) and genus (d) level abundances are corrected for genome length and scaled to 100%. 6.3bcd: Plots represent the top ten most abundant features and labels are arranged in decreasing order with respect to overall ARG abundance across the full slaughterhouse population. All remaining features are part of the category 'Other'. The slaughter line runs from left to right. X-axis slaughter positions: LA = lairage, HB = hanging & bleeding, SD = positions after scalding and dehairing, EV = evisceration, PR = pluck removal, OR = other types of removal, DB = deboning room. ARG cluster names: oxaz-phen = oxazolidinone & amphenicol, macr-oxaz-phen = macrolide & oxazolidinone & amphenicol.

Supplementary Figure 6.3 Continued

Supplementary Figure 6.4 Rarefaction curves of the raw resistome and bacteriome count matrices

Rarefaction curves represent the (a) raw resistome and (b) bacteriome count matrix. The bacteriome is corrected for genome-length and represents counts at the bacterial species level. Bacterial counts are multiplied by 10^6 . Each line represents a sample. *X-axis*: Count matrices were randomly subsampled with sample size steps of 1 (resistome) and 1000 (bacteriome). 'sh worker' = pig slaughterhouse worker, 'pig farmer' and 'broiler farmer' categories include employees and family members.

Supplementary Figure 6.5 ARG clusters shared among study subpopulations (Venn diagram)

The dataset was rarefied and clustered at the ARG cluster level (90%ID). In total, 208 ARG clusters were identified across all populations of which 80 ARG clusters were fully shared. 'pig farmer' and 'broiler farmer' categories include employees and family members.

Supplementary Figure 6.6 The human bacteriome shapes the resistome (Procrustes analysis)

Resistome and bacteriome NMDS ordinations (Bray-Curtis dissimilarities) were used as input for the Procrustes analysis. 6.6a: Procrustes superimposition in two dimensions. The bacteriome is rotated to maximum similarity with the resistome as such that arrowheads point towards the resistome configuration. 6.6b: Procrustes residuals plot. Horizontal lines respectively represent from top to bottom the 25% (dashed), median (solid) and 75% (dashed) quantiles of the residuals. The p-value was generated by the function `protest` ('R' *vegan*, at 999 permutations). Shepard stress plots corresponding to the NMDS configurations can be found in Supplementary Figure 6.7.

Supplementary Figure 6.7 Shepard stress plots corresponding to NMDS ordinations Figure 6.4 and Supplementary Figure 6.6

6.7a: Shepard stress plot corresponding to the NMDS ordination in Supplementary Figure 6.4a (resistome, 3D). 6.7b: Shepard stress plot corresponding to the NMDS ordination in Supplementary Figure 6.4b (bacteriome, 2D) and used as input for the Procrustes analysis (Supplementary Figure 6.6). 6.7c: Shepard stress plot corresponding to the NMDS ordination (resistome, 2D) used as input for the Procrustes analysis (Supplementary Figure 6.6).

S6.2 An overview of used 'R' packages and datasets

R version 3.5.1. was used for all analyses.¹ This paragraph contains an overview of all main 'R' packages and datasets.

S6.2.1 General 'R' packages for basic analyses

The following 'R' packages were used to create basic figures, structure data and perform analyses:

car,² cowplot,³ dplyr,⁴ data.table,⁵ ggplot2,⁶ knitr,⁷ markdown,⁸ tidyverse,⁹ venn¹⁰.

S6.2.2 Specific 'R' packages and datasets

Supplementary Table 6.2 Specific 'R' packages and datasets used to perform the following analyses: alpha diversity, NMDS, PERMANOVA, Procrustes and SIMPER

Topic	'R' packages	Resistome dataset	Bacteriome dataset
Alpha diversity	vegan ¹¹	Raw resistome counts dataset: Supplementary Excel Table 6.5a	Raw bacteriome counts dataset, genome length corrected (species level): Supplementary Excel Table 6.5b
NMDS, PERMANOVA, Procrustes, SIMPER	vegan ¹¹	FPKM dataset, 90% ID level (includes metadata): Supplementary Excel Table 6.6a	Genome length corrected dataset, genus level (includes metadata): Supplementary Excel Table 6.6b
DESeq2	DESeq2 ¹²	Raw resistome counts dataset, AMR class and 90% ID level: Supplementary Excel Table 6.7a (AMR class) Supplementary Excel Table 6.7b (90%ID)	

S6.2.3 References to 'R' packages

The titles of packages are provided as directly retrieved from 'R'.

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Chapter 7

General discussion

After the rise of antimicrobial resistance (AMR) in the last decades, it seems fitting that AMR has become politically fashionable.¹ In light of previous reports on the burden of AMR imposed on society, AMR has squarely entered the political agenda. This burden has often been expressed in terms of human deaths attributed to AMR.^{2–4} Although the political discussion about AMR is therefore fuelled from a perspective of anthropocentric urgency, the epidemiology of AMR itself must be approached by taking into account more than only the human side of the AMR story. It should be approached holistically, by integrating the presence and spread of AMR within and between different ecologies, i.e. the interplay between humans, livestock, companion animals, wildlife, aquaculture and all types of biological environments including soil, (waste)water and air.⁵ Over the years, accumulating evidence points towards antimicrobial use (AMU) in animals as one of the causes for the worldwide spread of AMR.⁶ However, based on the outcome of an infectious disease model, it has also been advocated that only curtailing animal AMU will be insufficient to decrease AMR levels in humans.⁷

AMR in livestock: a source of AMR in humans?

The direct attribution of human AMR carriage to livestock, though established for certain types of resistance, is not generally certain and subject to some controversy. For instance, although reported to have a human ancestral origin,⁸ the zoonotic potential of certain livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) clones seems rather clear due to frequent reports of these clones colonizing the nares or throats of humans occupationally exposed to livestock (e.g. LA-MRSA Sequence Types ST398 or ST9),^{9,10} while human-to-human transmission of LA-MRSA was found to be less likely.^{11–13} In contrast, intestinal carriage of community-acquired extended-spectrum β -lactamase producing *E.coli* (ESBL-PE) and plasmid-mediated AmpC producing *E.coli* was recently largely attributed to human-to-human transmission in the open community,¹⁴ contrary to earlier reports, which pointed to the food chain

as a likely source of ESBL.^{15,16} Nevertheless, association studies have also shown ESBL-carriage in livestock farmers to be linked to direct and indirect livestock contact.^{17,18}

Source attribution studies and microbial risk assessments are key in battling antimicrobial resistance

Source attribution studies are only one piece of the puzzle to inform risk managers on AMR risks, for instance by allowing to identify and prioritize intervention strategies.¹⁹ To properly address the risk of AMR for public health, an integrated risk analysis has to be executed, for which different guidelines have been proposed [e.g. Codex Alimentarius approach or the OIE International Animal Health Code (Covello-Merkhofer system)].²⁰ An essential step of these guidelines is microbial risk assessment (MRA), which is defined as the evaluation of *“the level of exposure and the subsequent risk to human health due to a specific organism or particular type of resistance”*, which – depending on the pathogen involved – might necessitate a farm-to-fork approach.^{19,21} Performing an MRA in the field of AMR is challenging, due to the complex nature of the hazard and the human health risk involved (e.g. including potential resistome related risks). Furthermore, high-quality epidemiological data are lacking on, for instance, dose-response relationships, but also on resistome variability and the potential for ARG exchange.¹⁹

This thesis focuses on the epidemiology of AMR in livestock and humans occupationally exposed to livestock. Within this thesis (i) determinants are identified for AMR carriage in animals and humans, and (ii) human exposure to AMR is investigated. In this work, the role of persons occupationally exposed to livestock is assessed. Workers are essential within AMR epidemiology as they might not only be exposed to higher resistant loads through livestock contact, but also because of their pivotal role in serving as a potential point of entry for resistant bacteria or antimicrobial resistance genes (ARGs) to gain access to the community. Although by far not sufficient to execute an adequate (or part of an) MRA, certain findings from this thesis, might be fed into one of the stages of a qualitative or potentially quantitative MRA in the future. In general, though some good examples of MRAs concerning AMR are available,¹⁹ the field of AMR needs much more high-quality data to increase future risk assessment efforts.

Is the new 'metagenomic' kid on the block also an asset to AMR epidemiological research? Let's find out.

Focusing on single bacterial species is likely to underestimate the overall AMR exposure and thus the risk posed by AMR. With the rise of next-generation sequencing (NGS) techniques such as shotgun metagenomics, AMR can be studied in all types of bacteria, not just the culturable fraction. This means that the full spectrum of ARGs, the resistome, can be targeted. For example, when farmers or slaughterhouse managers want to implement intervention measures to reduce the risk for AMR carriage, it is helpful to know how to tackle all relevant types of AMR and not just resistance in one particular bacterial species. A focus on the resistome also allows gaining insight into the genetic determinants and transmission of AMR. Within this thesis mainly ARG detection methods are used for risk factor analysis. But does studying the resistome or certain ARGs generate additional practical knowledge and scientific evidence compared to solely studying pathogenic or indicator bacteria? Are we moving forward?

The current chapter reflects on determinants for AMR/ARG carriage in livestock and persons occupationally exposed to livestock identified in prior studies and those described in this thesis. It also reflects on the benefits and possible limitations of using state-of-the-art metagenomic technology within the field of AMR surveillance.

7.1 Determinants for the faecal resistome of livestock

While the human resistome has been the subject of intensive study for many years, the investigation of the animal resistome has only gradually gained traction in the past decade. At present, the 'usual suspects', i.e. pigs^{22–25}, cattle^{26–31} and poultry^{32–34}, have been studied most often through the use of high-capacity qPCR or NGS techniques. From an epidemiological perspective, it is unfortunate that the majority of these studies only have an explorative nature, include relatively few animals, are bound to certain countries (mainly European countries or the USA), mainly focus on the diversity and abundance of ARGs in relation to AMU, seldom contain longitudinal observations, and have an ecological or cross-sectional study design incapable of inferring causality.

Impact of AMU on the faecal resistome in livestock

The epidemiology of AMR, including ARG carriage, has a complex and multifactorial nature. Not only are a variety of environmental factors at play, changes in the resistome are likely and greatly defined by the microbiome, while the latter in turn is also influenced by factors such as age, sex, diet and host genetics.^{24,35} Microbiome-resistome correlations have previously been defined,^{26,36,37} and were confirmed in this thesis for the resistomes of pigs and broilers.^{32,38} Moreover, using metagenomic assembly, prior studies have linked antimicrobial-induced alterations of the bacteriome to changes in resistome abundances.³⁴ ARGs harboured by human or livestock gut microbiota are established soon after birth, even in the absence of antimicrobial exposure.^{39–41} However, clinical trials investigating the impact of AMU on the livestock microbiome have demonstrated elevated levels of faecal ARG abundances and altered ARG diversity resulting from AMU.^{22,28,42} Yet, results from these or other studies are ambiguous, e.g. sometimes associations were not found or findings indicate simultaneous ARG increases and decreases in relation to AMU, or co- or cross-selection.^{25,29,34,43,44} Within this thesis, the faecal resistomes of more than 350 pig and broiler herds from nine European countries were explored (**chapter two**),³² including their relation with on-farm and country-level veterinary AMU (**chapters two and three**).⁴⁵ Using linear mixed-effects modelling, significant positive associations were demonstrated between country-level or average on-farm AMU, and total ARG abundance in pigs. For broilers, only country-level AMU was significantly associated with an increased AMR abundance (**chapter two**).³² In addition, a random-effects meta-analysis by country revealed exclusively positive associations between detailed on-farm class-specific AMU and components of the pig resistome (**chapter three**).⁴⁵ This meta-analysis approach made it possible to thoroughly screen all possible combinations of ARGs and AMU classes, providing a comprehensive picture of direct and co- and cross-selection of ARGs.

Impact of other farm characteristics on the faecal resistome in livestock

Prior studies have reported on the presence of high ARG baseline levels or a high diversity of ARGs in the livestock gut microbiome in the absence of antimicrobial selective pressure.^{22,24,46} This raises the question which other determinants might be responsible for this resistome accumulation. Results presented in this thesis demonstrate clustering of the faecal resistome of pigs and broilers by country (**chapter two**),³² likely resulting from different country-level on-farm AMU, but

potentially also related to diverging management practices. Depending on the animal and bacterial species studied, observational culture-based studies have produced long lists of associations between resistant bacteria and specific on-farm characteristics and management practices (e.g. hygiene measures, specific farm and stable configurations, the presence of other animals at the farm, housing temperature and crowding)^{47–58} and/or livestock herd characteristics (e.g. herd size, age, origin, breed, health status, AMR status of the dam).^{47–49,54,55,57,59–64} However, some of the aforementioned studies have also specified counterintuitive or conflicting results with regard to the impact of hygiene or biosecurity measures on carriage of AMR (e.g. positive associations between high hygienic practices and AMR).^{49,51,55,59,65} To explain these associations, some of these studies have speculated on the potential for reversed causality or pointed towards confounding e.g. correlations with other – more intuitive – hygiene and biosecurity measures. Nevertheless, it might be expected that the effects of intervention measures might differ depending on the investigated AMR bacterium, which makes it all the more enthralling to see if cross-bacterial AMR preventive measures could be identified within resistome studies.

As discussed in the introduction of this thesis, modelling high-dimensional metagenomic data can be challenging, in particular when handling a small number of observations which prevents several covariates from being investigated simultaneously. In addition, the road to the epidemiological eureka moment is likely clouded by gene interactions/correlations and microbiome interferences, complicating the discovery of additional variation. Consequently, the expediency of management practices has not largely been substantiated within resistome studies. Most research, commonly using dimension reduction techniques (DRT), has examined just a handful of covariates or have – if even possible within DRTs – failed to control for confounding, including AMU. The composition and diversity of the resistome have been reported to differ between calves versus adult cattle (potentially driven by age-dependent taxonomic differences),⁶⁶ beef feedlot versus dairy cattle,^{30,66} and conventional versus organic (i.e. ‘raised without antimicrobials’) or ‘household farming’ systems.^{30,33} Another study used multivariable linear and logistic regression to study the occurrence of seven ARGs (qPCR: *tetW*, *tetO*, *tetM*, *ermB*, *ermF*, *sul1*, *sul2*) in pig faecal samples collected at the slaughter line.²⁵ Here, the authors identified a positive association between *tetO* and the number of on-farm slaughter pigs, a negative association between *tetM* and other age groups present at the farm, and both positive associations between *sul2* or *ermF* and the number of farms pigs were originating from (as a proxy for the degree of mixing and transportation of pigs). Furthermore, resistome studies have suggested heavy metals and antibacterial

biocides (Quaternary Ammonium Compounds) to provide co-selective pressure for AMR.⁶⁷ Studies applying metagenomics have shown correlations between ARGs and metals in livestock manure or soil, or have shown differential expansion of ARGs as a consequence of heavy metal supplementation (e.g. copper, zinc, arsenic, mercury).^{23,42,68} Within this thesis, a comprehensive, yet simple, random-effects meta-analysis modelling approach was chosen in which a list of more than one hundred farm, herd, and biosecurity measures (condensed into biosecurity scores) was related to all ARGs detected within the faecal microbiome of 176 European pig herds close to slaughter (**chapter three**).³⁸ By doing so, the previously proposed counterintuitive message that cleaning and disinfection might be positively associated with AMR was confirmed. Since we have not quantified factors such e.g. biocide or metal resistance genes (BMRGs) in pig faecal samples, the probability of BMRG/ARG co-selection or any other possible explanation for these findings remains to be elucidated.

Persistence of the livestock resistome and its long-term dynamics

Let us assume that after AMU and by applying or discontinuing certain on-farm practices, a resistome is formed within the gut microbiome, what happens next? Not much is known about the persistence of the livestock resistome after AMU or its long-term dynamics. A resistome trial has, for instance, demonstrated that the pig gut resistome diversity had largely recovered two weeks after the end of a prior three-week course of in-feed AMU.⁴⁶ In addition, findings from this thesis showed no direct associations between AMU during the piglet suckling or weaning phase and components of the resistome found in the grown-up fattening pigs, while clear associations between AMU and AMR in fatteners were demonstrated, indicative for a possible transient effect of AMU on ARGs (**chapter three**).⁴⁵ Nevertheless, a longitudinal study design is essential to study long-term dynamics of the resistome, and preferably includes a thorough investigation of microbiome-resistome interactions. Such studies are currently still scarce. However, recent research in a commercial pig farm has shown no marked changes in high faecal ARG abundances in sows during a five-month period following short-term AMU, as part of a partial farm depopulation in which all younger pigs were removed.⁴⁴ Preceding this depopulation phase and a reduction of overall on-farm AMU, the removed younger pigs had received high-levels of AMU over a long period of time which was claimed by the authors to not affect the AMR gene abundance or diversity of the pig resistome. A history of high-level AMU, however, could potentially have been responsible for the establishment of high ARG loads and diversity in pigs on this farm. Although the used study design was

hardly ideal to study resistome persistence and associations with AMU (as a much more controlled environment is preferred), the authors stressed the importance of preventing AMU, as ARGs which are established within the microbiome may likely only decay slowly following antimicrobial withdrawal.

Lessons learned from livestock resistome studies to reduce AMR in livestock

Based on the current uncertainties with regard to on-farm measures to prevent carriage and dissemination of ARGs among livestock apart from AMU, the limited number of studies adjusting for confounding, and some of the contradicting and counterintuitive evidence generated from livestock resistome studies, we seem to be left with nothing more than the precautionary measure of limiting on-farm AMU, and to still follow recommendations based on results from phenotypic studies. It would be tempting to ask ourselves the question: “Are we needlessly overcomplicating our lives including our search for determinants of AMR by spinning the question metagenomically?” However, until we have exhausted all our options, have performed even larger scale and more detailed in-depth livestock resistome studies, have thoroughly checked for covariates and confounders and finally, once we have performed longitudinal studies investigating these factors, there is still hope to further disentangle resistome, microbiome and environmental influences to identify additional, possibly better livestock farm management interventions having a broader AMR-mitigation capacity. Ultimately, NGS techniques are capable of studying AMR more extensively, while insights can be gained into ARG co- and cross-selection or resistome-microbiome interactions, potentially even uncovering novel ARGs. Despite that, it must be noted that only a subset of metagenomically derived ARGs in combination with their bacterial host will be of direct public health relevance, unless bacteria harbouring these genes indirectly act as a reservoir for horizontal gene transfer (HGT) events.

The livestock resistome: a potential reservoir for AMR in humans

Apart from functional metagenomics, metagenomic studies are not designed to study functional expression, but they do allow to further investigate genetic AMR determinants including the role of mobile genetic elements (MGEs) in the spread of resistance between livestock and humans. Horizontal gene transfer is considered the most common mechanism through which pathogens acquire new ARGs.⁶⁹ Livestock resistome studies have reported co-enrichment of ARGs

and MGEs, including correlations between these in manure/faecal samples.^{23,33,70} In addition, the occurrence of AMU induced enrichment of ARGs of which the majority have been found on MGEs,⁴⁶ provide a strong indication for HGT as a means of ARG transmission between bacteria, but possibly also a means of ARG transmission from livestock bacteria to human pathogens. Therefore, the study of HGT-elements in faeces of pigs and broilers would be an interesting future complement to the findings of this thesis, in order to unravel AMR transmission from livestock to humans, resulting in human AMR carriage. The perfect bridge to the next paragraph: What determines human faecal AMR/ARG carriage?

7.2 Determinants for AMR and the faecal resistome in humans occupationally exposed to AMR in livestock

Determinants of the human microbiome and resistome

A study in 124 European Nordic and Mediterranean individuals estimated the human gut microbiome to harbour around 1000 to 1150 bacterial species, and of those present in an individual, a large amount is shared among others.⁷¹ Although claimed to demonstrate a certain degree of stability over time,⁷² the gut microbiome has also shown great plasticity and is highly affected by diet, lifestyle and environmental factors.^{73–75} These factors have been associated with more than 20% of the inter-person microbiome variability, while host genetics are suggested to play only a minor role in shaping the microbiome.⁷⁶ Antimicrobials, in particular, can have pervasive effects on intestinal microbial communities. For example, human studies have observed diminished bacterial diversity and shifts in community composition as a consequence of antimicrobial perturbation, while post-treatment microbial recovery might take years.^{77–79}

The human resistome has been the subject of more elaborate study compared to the livestock resistome. Previous human resistome studies have noted an increase in intestinal ARG abundances in response to AMU,⁸⁰ including the potential for a long-term increase of specific ARGs.^{77,78} In addition, prior research observed elevated levels of ARGs after international travel,^{81,82} and differences in relative ARG abundances between countries that coincided with country-specific human and veterinary antimicrobial (sales) data.^{83–85} Although some of the latter studies also investigated other explanatory variables, such as age or BMI,⁸⁵ the ecological study design and the indirectness of the proposed associations with e.g. country-level veterinary AMU renders this type of inferences rather speculative. More detailed resistome studies investigating the

link between livestock contact or AMU in livestock and human ARG carriage are needed to substantiate this claim further.

Determinants for AMR carriage in occupationally exposed workers: findings from prior research

To examine the causal effect of AMU or AMR in livestock on human ARG carriage, a highly controlled experimental environment is preferred, which is difficult to achieve in relevant occupational settings (e.g. farms, abattoirs). It ideally also includes the study of AMR carriage in animals and humans throughout the whole animal food production cycle (farm-to-fork approach). The research contained within this thesis zooms in on occupational exposure of workers in contact with livestock, a ‘natural experiment’, in which humans highly exposed to AMR in livestock are investigated. The presented research explores human carriage of AMR bacteria and the resistome of farmers and pig slaughterhouse workers using both phenotypic and genotypic approaches including metagenomics.

Apart from the research presented in this thesis, our knowledge on AMR carriage in occupationally exposed workers (e.g. farmers and slaughterhouse employees) seems to be solely derived from culture-based studies. Although not consistently,^{86–88} higher faecal or nasal/oropharyngeal AMR prevalences have been suggested in farmers and occupational meat handlers compared to a control or the general population.^{10,17,86,87,89–98} However, it is challenging to compare carrier rates among occupational populations since prevalences differ among the type of AMR bacteria involved, the investigated countries or occupational setting (e.g. farm versus slaughterhouse, type of livestock involved), and the intensity of livestock contact.^{18,99–103} Only a few studies have a complex scope and report on human carriage of multiple resistant bacterial species (e.g. ESBL and MRSA), include various occupational settings, or study AMR carriage in several types of livestock farmers.^{88,99,104–109} Nevertheless, observational studies identifying risk factors for AMR carriage in farmers and slaughterhouse workers have mainly demonstrated (direct) livestock contact as the main driver for carriage of AMR bacteria (e.g. the number of hours working on a farm as a proxy for livestock contact, Table 7.1). Furthermore, the farm or slaughterhouse environment is known to be contaminated with for instance LA-MRSA or ESBL-PE (e.g. AMR in ambient air, dust, on soil surfaces),^{18,89,110–113} and positive associations have been demonstrated between respectively MRSA and ESBL-PE contamination of farm/stable dust and nasal MRSA or faecal ESBL-PE carriage in farmers.^{18,114}

Determinants for AMR/ARG carriage in pig slaughterhouse workers

Within this thesis, human occupational exposure to AMR in livestock was investigated. Two studies were conducted in the same Dutch pig slaughterhouse in partly overlapping human populations. Both the prevalence of ESBL-PE and tetracycline (*tetW*) and macrolide (*ermB*) resistance genes were studied in abattoir workers, together with the association between occupational exposure and faecal ESBL-PE or ARG carriage (**chapters four and five**).^{103,120} Overall levels of ESBL-PE found in slaughterhouse staff were similar to those in the Dutch general population, however, significantly higher faecal ESBL-PE loads were found in staff employed at earlier positions in the slaughter line (i.e. lairage to cooling), in line with previous LA-MRSA findings (**chapter four** and Table 7.1). Secondly, although employees are exposed to clear and decreasing environmental tetracycline and macrolide resistance gene concentrations along the slaughter line, an association between environmental ARG contamination and human faecal ARG carriage could not be established after elaborate adjusting for confounding (**chapter five**). Lastly, tetracycline and macrolide resistance genes were nearly absent in samples from pig meat end-products, suggesting a low exposure of pork consumers to ARGs (**chapter five**).

The association between faecal ARG carriage and environmental ARG exposure has not yet been studied before, and studies investigating determinants for human faecal AMR carriage within the slaughterhouse context are rare. Therefore, it is difficult to say if ARG methods (qPCR) have an added value compared to phenotypic screening in a slaughterhouse setting, or even offer a stronger and wider means of AMR screening. It should be noted that ARGs selected for qPCR within the scope of AMR exposure assessment need to (i) exhibit a high probability of being detected, (ii) should preferably have a public health relevance and (iii) provide enough between-sample contrast, (iv) while all selected qPCR signals together should preferably represent a diverse set of relevant AMR classes. Consequently, the selection of the 'right' gene is vital. Recently, a study was performed within a Spanish pig slaughterhouse which assessed the resistome recovered from environmental surface swabs (including swabs from carcasses) by shotgun metagenomic sequencing.¹²¹ Here, the highest ARG richness and diversity was detected in the lairage and stunning areas, whereas other areas showed lower to absent ARG abundances in line with findings from this thesis. In the latter study, however, *tetW* was not recovered from the environment, possibly due to a lower sensitivity of sequencing methods compared to the qPCR in our study. Macrolide resistance genes were only minimally recovered, compared to ARGs coding for e.g. antimicrobial inactivation

Table 7.1 Previously reported determinants for AMR carriage among humans occupationally exposed to AMR in livestock

Occupationally livestock exposed population		
	Persons living or working on farms	Slaughterhouse staff
Determinants for human nasal/oropharyngeal LA-MRSA carriage	Factors related to direct or indirect exposure to animals: <ul style="list-style-type: none">■ Number of hours working with livestock or working on a livestock farm (e.g. pigs, veal calves) (+)^{10,54,90,100,106,109,113,115–117†}■ Performing specific livestock-related tasks (e.g. pigs, veal calves) (+)^{58*,90,113,117}■ The presence or percentage of MRSA positive livestock on the farm (e.g. MRSA positive pig or veal farms) (+)^{58*,115,118}■ AMU in livestock (e.g. veal calves) (+)^{57*,58*}■ Contact with or the presence of companion animals on the farm (+,-)^{58*,106,113,115}	Factors related to direct or indirect exposure to animals: <ul style="list-style-type: none">■ Contact with live broilers (+)⁹⁴■ Working in the lairage, scalding and dehairing area of a pig slaughterhouse (+)^{112*}, working with live pigs (+)^{95*}
	Factors related to other farm characteristics, biosecurity measures, or potential protective factors: <ul style="list-style-type: none">■ The use of protective clothing (+,-)^{90,115}■ Farm size (+)¹⁰⁹■ On-farm cleaning activities (+)¹¹³	Factors related to the slaughterhouse: <ul style="list-style-type: none">■ Slaughterhouse stunning method (conventional electric stunning showed a higher risk compared to CO₂ stunning) (+)⁹⁴
	Factors specifically focused on household members of farmers or persons living on a farm without occupational livestock contact: <ul style="list-style-type: none">■ Having a household member with occupational livestock contact or living with an MRSA-positive farmer (+)^{100,116,117}■ Private visits to farms (+)¹⁰⁰	
	Factors related to the sampled individuals and possible confounders: Gender (male, +) ^{54,117} Age (+) ^{54,90,113,117}	
	Factors related to the farm environment: <ul style="list-style-type: none">■ Level of MRSA in farm dust (on pigs and veal calves farms)¹¹⁴	

Table 7.1 Continued

Occupationally livestock exposed population		
	Persons living or working on farms	Slaughterhouse staff
Determinants for human faecal ESBL-PE/resistant <i>E.coli</i> carriage	<p>Factors related to direct or indirect exposure to animals:</p> <ul style="list-style-type: none"> ■ Number of hours working on the pig farm or in the pig barn (+)^{18,102,119} ■ Performing specific tasks involving direct livestock contact (e.g. pigs)¹¹⁹ (+) ■ Presence of ESBLs or CTX-M-gr1 in pigs (+)^{18,102} ■ Antimicrobial use in pigs (+)¹¹⁹ <p>Factors related to the sampled individuals and possible confounders:</p> <ul style="list-style-type: none"> ■ Personal use of antimicrobials (+)¹¹⁹ <p>Factors related to the farm environment:</p> <ul style="list-style-type: none"> ■ Presence of CTX-M-gr1 in farm dust on pig farms (+)^{18*} 	<p>Factors related to direct or indirect exposure to animals:</p> <ul style="list-style-type: none"> ■ Working from lairage to cooling [<i>chapter four of this thesis</i>] (+)¹⁰³

This table does not represent the result of a thorough systematic review and focuses on studies using multivariable regression techniques unless otherwise specified with an asterisk (*). From these models, only determinants were selected from the presented final multivariable model(s) ($p < 0.05$). (*) An asterisk represents determinants identified in univariate or bivariate analyses. (+) A plus-sign represents a positive association. (-) A minus-sign represents a negative association. Risk factors presented in this table are sometimes combined into one overarching factor. †'Number of hours working with livestock or working on a livestock farm' for instance also includes # hours working in stables, # days spend on the farm, working with livestock on a daily basis, having regular, intensive or recent livestock contact.

enzymes or aminoglycoside resistance. Although this resistome study only provided a 'snapshot' and was executed in Spain where pigs are likely subjected to differing AMU regimes compared to those applied in the Netherlands (ESVAC;¹²² EFFORT non-anonymised AMU data, data not shown), this study illustrates the challenges of selecting the right antimicrobial resistance gene(s) for qPCR. It raises the question whether or not (i) the most suitable ARGs were picked for exposure assessment in this thesis (the influence of the qPCR signal is masked by other determinants, or the indicator is not at all suitable) or (ii) whether a relation between environmental AMR exposure and AMR carriage is not at all present (the association does not exist). The latter is rather unlikely when considering the evidence gathered within phenotypic studies (Table 7.1). Therefore, the first alternative is probably more plausible: the influence of the qPCR indicator is either masked or the signal is not suitable. Tetracyclines and macrolide resistance genes, including *tetW* and *ermB*, are widespread across environments and highly abundant in the healthy human gut (**chapter six**),^{83–85,123} hence, likely controlled

by a variety of host-specific and potentially by other environmental factors independent from the slaughterhouse environment (e.g. nationality, smoking as also specified in **chapter five**). Furthermore, resistome-based source attribution studies could be highly instrumental in guiding the selection of ARGs possessing a high public health relevance, which subsequently might be better suited within the framework of qPCR-based exposure assessments.¹⁹ A resistome-based source-attribution study is currently being executed within the EFFORT project which applies machine learning to eventually identify and predict potential livestock sources (i.e. livestock ARGs) of the resistome of persons occupationally exposed to livestock (hierarchical random forest supervised classification model, data in preparation).

Determinants for the resistome of farmers and slaughterhouse workers

In contrast to qPCR screening, metagenomic sequencing is better suited to map overall AMR by screening the full resistome. Metagenomic sequencing of faecal samples from farmers and slaughterhouse workers, as applied within this thesis, is a first attempt to study the resistome and microbiome composition of workers occupationally exposed to AMR in livestock (**chapter six**).³⁸ Here, higher ARG loads were found in pig exposed workers compared to broiler farmers and control subjects. Furthermore, compositional resistome and microbiome shifts related to occupational exposure were highlighted and within-population compositional differences were presented related to job position (slaughterhouse workers), the type of farm (broiler versus pig farmers) or related to the type of livestock contact (farmers and employees versus family members). In addition, a microbiome-resistome connection was established. Within this thesis, a combination of dimension reduction techniques, permutation, and differential abundance analysis was applied. However, these methods are essentially designed to include only a small set of explanatory variables which, combined with a limited sample size, resulted in only a few confounding or explanatory variables to be tested within the scope of this thesis.

Although providing a first insight into the resistome of occupationally exposed groups, these results, based on metagenomic sequencing, generate plenty of follow-up questions and room for future studies. (i) Although this thesis suggests compositional resistome and bacteriome differences between occupational groups resulting from specific livestock exposure, it is imperative to study these human resistomes in conjunction with the livestock resistome to further strengthen the evidence for livestock-associated resistome acquisition. (ii) Even though no robust link was captured between environmental ARG

exposure and human *tetW* and *ermB* faecal carriage in the slaughterhouse context (**chapter five**), environmental influences on the resistome/microbiome remain to be investigated. A recent microbiome study has previously attributed as much as 52% of the farmer's nasal microbiome to the microbiome of pigs present on the farm.¹²⁴ This and studies from Table 7.1 demonstrate that the role of the environment might be essential as a vehicle for zoonotic ARG transmission. (iii) The chosen metagenomic strategy does not yet allow to study dynamic microbiome-resistome intertwinements. Therefore, it is recommended to study the interaction between ARGs and their taxonomic carriers by means of assembly-based strategies.¹²⁵ (iv) What are the long-term dynamics of the resistome acquired through occupational livestock exposure? Prior studies investigating human faecal ESBL/pAmpC carriage have estimated varying median rates of acquiring and losing AMR carriership depending on the ESBL/pAmpC gene involved,¹²⁶ while the persistence and duration of faecal ESBL colonization can vary considerably.^{127,128} As mentioned before, human AMU can induce long-term shifts in the gut resistome. However, less is known how the gut resistome behaves after livestock-mediated ARG acquisition. (v) And finally, what are the health consequences of acquiring ARGs from livestock? Longitudinal studies are essentially required to study possible health-related outcomes of livestock-associated resistome acquisition.

7.3 The application of metagenomic sequencing within AMR surveillance systems

With the increasing popularity of AMR NGS studies, it is often suggested that genome or metagenomic sequencing could be a powerful tool for use within AMR surveillance and monitoring, possibly even replacing traditional methods within this field in the future. Surveillance in an AMR context is defined as *“The systematic, continuous or repeated measurement, collection, collation, analysis, interpretation and timely dissemination of AMR data from defined populations. These data are then used to describe AMR hazard occurrence and to contribute to planning, implementation and evaluation of AMR risk mitigation actions”*.ⁱ Performing the same activities without a pre-defined risk mitigation plan is called monitoring.ⁱⁱ Surveillance networks support AMR risk analysis schemes and are

i-ii The definitions of ‘surveillance’ and ‘monitoring’ are adapted from those proposed by the Risk-based Animal Health Surveillance Systems (RISKSUR) consortium (www.fp7-risksur.eu).¹⁵⁰

essential for early detection of emerging resistance patterns.¹²⁹ In Europe, the basis for AMR surveillance and monitoring of zoonotic and indicator bacteria is laid down in EU legislation (Directive 2003/99/EC and Decision 2013/652/EU). Current national AMR surveillance programs in livestock of EU member states have been suggested to produce heterogeneous data.¹³⁰ Furthermore, at present, only a few EU countries report on the genetic identification of ARGs essential for extensive epidemiological analysis.¹³⁰ In addition, there is much potential for improvement of the availability and timeliness of surveillance reporting as part of national and EU-wide surveillance programs (e.g. yearly publishing of online reports, real-time surveillance).¹³⁰

Traditional AMR surveillance is based on the isolation of indicator organisms and the phenotypic characterization of isolated strains. Compared to phenotypic methods, sequencing methods can provide an elaborate genetic characterization of isolates (i.e. WGS) and are able to study ARGs in complex microbial communities without the need for culturing [i.e. whole metagenome sequencing (WMS)].¹²⁹ Sequencing methods can detect emerging genetic AMR mechanisms, as well as allelic time trends, whereas WGS or metagenomic assembly can provide ARG contextual information relevant to the study of AMR dissemination.

In order for WMS, as applied within this thesis, to be used as part of an international surveillance approach several requirements have to be met. (i) WMS should not only produce similar results as already existing methods, but it also should at least have an added value. For example, next to the advantages of WMS as mentioned above, a recent study in ten Danish pig herds concluded that the applied metagenomic approach was superior compared to traditional phenotypic methods when testing the ability to predict AMR in these herds.¹³¹ (ii) The chosen method should be easy to use and relatively cheap in order to be suitable for application within low-resource environments. This might be the Achilles heel of metagenomics, which is relatively expensive when considering all phases of a metagenomic workflow. For global AMR surveillance, the use of domestic sewage sampling or even sampling of the septic tanks of long-distance flights has been presented as a practical way to monitor AMR in populations across the world.^{132,133} In addition, the study which included sewage sampling identified possible intervention measures by describing associations of AMR with specific societal trends based on a dataset of 79 samples gathered in 60 countries. Findings from this type of studies can indeed provide baselines for and function as indicators for global ARGs patterns, providing both a cheap and easy sampling alternative (i.e. sewage sampling) compared to individual sampling which is subject to legal restrictions regarding e.g. data protection. However, in order to soundly describe and model epidemiological trends and to reliably

create actionable AMR intervention plans, protocols should be additionally standardized and sample sizes, including biological replicates, should be strongly increased, while such studies should imperatively include detailed population metadata. (iii) The chosen method for surveillance should provide a high degree of accuracy and precision. Within this thesis, it was shown that *bla*_{CTX-M'}, although expected to be present, was not detected in all pig samples (**chapter two**). This indicates a lower sensitivity of the applied metagenomic sequencing method. Sequencing depth is known to have a significant effect on ARG richness,^{134,135} while the limit of detection of metagenomic sequencing is *de facto* unknown,¹³⁶ making it difficult to determine if a gene is present or absent. Depending on the ambition to detect rare ARGs, other genotypic methods (e.g. WGS, qPCR) might provide a more accurate image. In particular, qPCR is known to outcompete metagenomics with regard to the detection of rare ARGs.²⁸ Furthermore, non-bacterial DNA (e.g. host DNA) can greatly reduce ARG recovery from e.g. meat samples,²⁶ samples which are often tested as part of traditional AMR surveillance schemes. However, a new form of targeted metagenomics was recently presented (ResCap) which promises to greatly enhance the sensitivity and specificity of metagenomic methods.¹³⁷ Additionally to the low sensitivity of WMS, novel emerging ARGs might still be missed using WMS methods, which could be resolved by using phenotypic methods in combination with WGS or metagenomic assembly. (iv) Finally, perhaps the most critical element of an effective surveillance network, all data collection and analysis efforts should be integrated and harmonised, consequently ensuring the highest possible level of reproducibility, compatibility and interoperability amongst datasets.¹³⁸ At present, the lack of harmonization is of great concern within the field. The jungle of AMR metagenomic studies represents an arcade for those who like to experiment, but might scare off those interested in comparing results. Studies have used various preparation and analysis strategies throughout all stages of the sequencing and bioinformatics process. For instance, next to the already discussed differing sequencing depths, differing normalization strategies can greatly affect inferred ARG abundance distributions.¹³⁵ In addition, there are currently at least 47 open-access bioinformatic resources for *in silico* AMR detection requiring different levels of expert-knowledge and employing several different tools and algorithms.¹³⁹ Despite using identical bioinformatic protocols, the number of reported ARG abundances can significantly vary depending on the reference database used knowing that databases reflect different scopes and functions.¹⁴⁰ Therefore, reference databases should be harmonized and adopt a long-term curation and future-proof data sharing strategy,^{141,142} to facilitate rapid collection of epidemiological data. While stipulated to be challenging, there is also

a need for benchmarking exercises to compare and test bioinformatic tools,^{133,143} as well as external quality assurance systems and elaborate proficiency testing.¹⁴⁴ In conclusion, WMS currently seems not yet ready for optimal use within surveillance networks, even when neglecting barriers such as potential legal or trade implications with regard to genomic data sharing or the current costs of WMS that prevent its implementation within low-resource environments (e.g. costs regarding sequencing, setting up bioinformatic pipelines, training of staff and data storage).

Within the EFFORT project, whose results are partly presented in this thesis, a standardization of questionnaires and several sampling and lab protocols was achieved for future use in an international surveillance context. Here, a harmonized pig and broiler sample collection approach across Europe was demonstrated,^{32,45} including the in-depth sampling of humans on farms and slaughterhouses.^{38,120} More manuscripts are currently prepared, including standardized protocols for the sampling of other animal species. Furthermore, within this thesis, the following aspects were standardized and/or applied: calculation of AMU treatment indices^{45,145,146} and biosecurity scores;^{45,147} protocols for DNA extraction¹⁴⁸, qPCR¹²⁰ and sequencing³² (although lab efforts were centralized); the development of an international bioinformatics pipeline (although centralized in one country)^{32,38,45} and a read normalization and alignment strategy.^{32,38} Ultimately, a simple and transparent approach to analyse metagenomic data was successfully developed,⁴⁵ while several multivariate techniques and a differential abundance analysis were shown to produce insightful outcomes.^{32,38} All in all, despite its current need for harmonization, and the above-stated limitations, the results from this thesis in combination with the benefits of WMS as mentioned above, seem promising and provide a great outlook for use within surveillance networks in the far future, albeit most likely as a complement to phenotypic methods.

7.4 Challenge accepted? An epilogue

AMR may be ancient,¹⁴⁹ but the research presented in this thesis surely is not. Many unanswered questions remain, or shall we say 'room for more interesting future studies'? This work shows associations between AMU and the livestock resistome, while identifying exposure to and possible determinants for occupational exposure to AMR in livestock. However, at the intersection of metagenomics and AMR, there is still lots to unravel, especially regarding the long-term dynamics of the resistome. Modelling metagenomic data is challenging, and even after our findings were final, we still found ourselves contemplating the suitability of the model assumptions, wondering about causality and resistance expression. The complexity of the metagenomics field is high and its modelling solutions and algorithms neither evident nor intuitive. This requires an interdisciplinary approach in which microbiologists, epidemiologists, statisticians, bioinformaticians, computer scientists, mathematicians and many others are required to collaborate. It also makes the field subject to constantly reinventing the wheel, especially within academic environments, exemplified by the maze of previous studies all using different model set-ups which currently hampers the stable set-up of WMS fuelled surveillance systems. However, AMR is a serious issue and more than anything else, the field needs practical solutions and capabilities, which requires research to work towards real-world solutions. The research in this thesis represents an honest effort trying to simplify approaches while laying the first bricks of a pioneering exercise. Future researchers hopefully will be able to build a stable house on this - the future is bright.

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Addendum

Summary (in English and Dutch)
Acknowledgements
Affiliations of co-authors
About the author

Summary (in English and Dutch)

English summary

It has existed for aeons and entered the spotlight when humans discovered a new medicine to treat human bacterial infections. *Its* subsequent emergence and spread coincided with the discovery that animals grew faster when given the same treatment. When we realized many people got ill or died as a consequence of *its* existence, the subject gained political traction. Politicians started to ban the use of the treatment for animal growth promotion hoping to reduce *its* prevalence. Then, in 2014, the European Union commissioned a consortium of researchers to investigate *its* molecular occurrence, ecology and transmission routes through the European food chain, with the ultimate aim of informing risk managers how to continue their battle to limit *its* spread.

As a consequence of human and animal antimicrobial use (AMU), *antimicrobial resistance* (AMR), the ‘it’ in our story, has emerged, and consequently wriggled its way into the European EFFORT project (<http://www.effort-against-amr.eu/>) and subsequently this thesis. [...] Telling a captivating, accessible tale is challenging by itself, let alone when it concerns science. So I’ll stop trying right here and adjust my writing to the complexity of the intricate and multifactorial topic at hand: the epidemiology of AMR.

AMR has an intrinsically complex nature. AMR bacteria, including antimicrobial resistance genes (ARGs) are ubiquitous in our living environment: they may be present in or on the food we eat, the water we drink, the air we breathe, and the humans, animals or objects we encounter. To study AMR, it is therefore imperative to consider different types of ecologies and to approach its epidemiology holistically. Previous AMR research has underlined the role of AMU and AMR in livestock as a potential source for AMR in humans. Not only can humans be directly exposed to AMR bacteria in livestock, livestock bacteria could potentially also serve as a reservoir for horizontal gene transfer through which human pathogens can acquire ARGs. Studies investigating determinants

for livestock and human AMR carriage should therefore essentially include the study of ARGs. However, to date, most studies are culture-based and focus on a single bacterial species. In contrast to ARG research, these studies, through their narrow, individual scope, and by nature limited to culturable bacterial species, likely underestimate the overall exposure of humans to and the risk posed by AMR.

This thesis concerns the epidemiology of AMR in the food chain. It explores how AMU and AMR in livestock affect AMR in humans and focuses on identifying determinants for AMR at the genomic level. It elaborates on how the livestock gut resistome, i.e. the full collection of ARGs in the gut of livestock, is affected by AMU, farm management and on-farm biosecurity practices. Molecular techniques like qPCR and sequence-based metagenomics are applied in order to try to identify an umbrella of on-farm AMR mitigation measures targeted at the livestock resistome, while generating data regarding the genetic characteristics of resistance (e.g. co- and cross selection). This work specifically focusses on AMR in, and the resistome of, human populations occupationally exposed to AMR in livestock, who might not only be highly exposed but also serve as entry points for spread of AMR bacteria and ARGs to the general population. Finally, metagenomic methods are discussed as future potential means to support or potentially replace phenotypic methods within AMR surveillance networks.

Chapter one describes the history and molecular characteristics of AMR and the (dis)advantages of existing antimicrobial susceptibility methods, and presents a range of data analysis options to extract potential AMR determinants from metagenomic data.

Part I presents the metagenomic analysis of the faecal resistome of livestock. It starts with the description of the pig and broiler resistome as part of a large European multi-country study (**chapter two**). Higher AMR loads are observed in pig herds compared to broiler herds, while the overall composition and diversity of the resistome are shown to be more homogeneous among pigs than broilers. For both the pig and broiler faecal resistome a significant country effect is observed, which is larger for pigs than for broilers. The microbiome is demonstrated to shape the resistome in a country-dependent manner. In addition, country-level AMU is associated with both the pig and broiler resistome. Given the large country effect present in both the AMU and AMR data, a straightforward epidemiological analysis approach is proposed to study detailed on-farm determinants for the pig resistome in these countries: a random-effects meta-analysis by country (**chapter three**). This analysis shows positive associations between on-farm class-specific AMU and components of the faecal resistome of slaughter pigs. Using this meta-analysis approach, direct

ARG selection, as well as cross- and co-selection of ARGs is demonstrated. High AMU during early pig rearing phases is not associated with AMR in fattening pigs, suggesting short-term selective pressure. Additionally, on-farm cleaning and disinfection measures are found to be positively linked to specific ARGs, which confirms the counterintuitive, positive, effect of higher hygienic practices on AMR previously noted in phenotypic studies. This finding potentially supports previously suggested genetic linkages between ARGs and biocide and metal resistance genes.

Part II analyses exposure to and faecal carriage of AMR in pig slaughterhouse staff. **Chapter four** illustrates that overall faecal carriage rates of extended-spectrum β -lactamase producing *E. coli* (ESBL-PE) in slaughterhouse staff – working in the Netherlands – are similar to those in the Dutch general population. However, higher odds of ESBL-PE carriage are registered for pig slaughterhouse staff employed from lairage to cooling in the slaughter line. Partly within the same slaughterhouse population, environmental exposure to and human carriage of tetracycline (*tetW*) and macrolide (*ermB*) resistance genes is analysed (**chapter five**). In this chapter, clear and decreasing ARG environmental trends are demonstrated along the slaughter line. Although dermal and respiratory ARG exposure is confirmed in accordance with phenotypic AMR research, no robust link is found between occupational exposure to environmental tetracycline and macrolide resistance genes and faecal ARG carriage in pig slaughterhouse workers after elaborate adjustment for confounding. Instead, nationality and smoking are found to be determinants for human faecal ARG carriage.

Part III uses metagenomic shotgun sequencing to investigate and describe the faecal resistome and microbiome of persons occupationally exposed to AMR in livestock, i.e. in pig and broiler farmers and pig slaughterhouse workers, as compared to a control population (**chapter six**). A higher ARG carriage is found in pig exposed workers compared to broiler farmers and control subjects. Within and between populations, compositional human resistome and microbiome differences are revealed which might suggest livestock contact as a determinant for human faecal ARG carriage. Similar to what was shown for the livestock resistome, the human gut resistome is found to be significantly correlated with its taxonomic composition, highlighting the importance of studying microbiome-resistome entanglements.

Finally, **chapter seven** reflects on all previous chapters by interweaving evidence gathered within this thesis with pre-existing knowledge on determinants for the livestock and human resistome, and discusses the benefits and limitations of sequence-based metagenomic approaches for potential future application within AMR surveillance networks.

The findings in this thesis offer a firm starting point for future epidemiological metagenomic investigation and analysis of human-livestock resistome interactions in the livestock food production chain. Future studies should focus on implementing larger scale, preferably longitudinal and metagenomic assembly-based study designs to investigate long-term resistome and resistome-microbiome dynamics. AMR is a real-world issue requiring immediate action and practical solutions – this thesis underlines the strengths and comments on the limitations of epidemiological research focused on ARGs to combat AMR, while calling for an urgent harmonization of metagenomic approaches to bolster their future use within surveillance networks.

Nederlandse samenvatting

Zij bestaat al eeuwenlang en nam een vlucht toen een behandeling voor humane bacteriële infecties werd ontdekt. *Haar* wereldwijde verspreiding viel vervolgens samen met de toepassing van dezelfde behandeling als groeibevorderaar bij dieren. Toen we ons realiseerden dat mensen ziek werden of stierven als gevolg van *haar* bestaan, kreeg *zij* politieke aandacht. Politici begonnen het gebruik van de behandeling voor groeibevordering bij dieren te verbieden in de hoop *haar* prevalentie te kunnen verminderen. In 2014 gaf de Europese Unie de opdracht aan een wetenschappelijk consortium om *haar* epidemiologie, ecologie en transmissieroutes door de Europese voedselketen te onderzoeken, met als uiteindelijk doel risk managers te informeren en te helpen *haar* verspreiding te beperken.

Als gevolg van antimicrobieel gebruik (AMG) bij mensen en dieren, is *antimicrobiële resistentie* (AMR) – de ‘zij’ en protagonist in dit verhaal – opgedoken en vervolgens het onderwerp geworden van het Europese EFFORT-project (www.effort-against-amr.eu/) en uiteindelijk dit proefschrift. [...] Een boeiend en toegankelijk verhaal vertellen is op zichzelf al een uitdaging, laat staan dat het een wetenschappelijk onderwerp betreft. Daarom zal ik mijn schrijfstijl nu aanpassen aan de complexiteit van dit multifactoriële onderwerp: de epidemiologie van antimicrobiële resistentie (AMR).

AMR is intrinsiek complex. AMR-bacteriën, inclusief antimicrobiële resistentiegenen (ARG's) zijn alomtegenwoordig in onze leefomgeving: ze kunnen aanwezig zijn in of op het voedsel dat we eten, het water dat we drinken, de lucht die we inademen en de mensen, dieren of objecten die we tegenkomen. Om AMR te bestuderen is het daarom noodzakelijk om diverse ecosystemen te bestuderen en de epidemiologie van AMR holistisch te benaderen. Eerder AMR-onderzoek heeft de rol van AMG en AMR in vee als potentiële bron voor AMR in mensen benadrukt. Niet alleen kunnen humane populaties rechtstreeks worden blootgesteld aan AMR-bacteriën afkomstig van vee, vee-bacteriën vormen tevens een mogelijke bron voor horizontale gen-overdracht waarbij humane pathogenen ARG's kunnen verwerven. Binnen het onderzoek naar determinanten voor humaan of dierlijk AMR-dragerschap is het derhalve van belang om ARG's te bestuderen. Tot op heden richtten de meeste AMR-studies zich echter op humane pathogenen en indicatorbacteriën. Door de beperkte reikwijdte van dit soort onderzoek – van nature beperkt tot specifieke en kweekbare bacteriën – wordt de blootstelling aan AMR naar grote waarschijnlijkheid onderschat.

Dit proefschrift gaat over de epidemiologie van AMR in de voedselketen. Zo wordt onderzocht of AMG en AMR in vee AMR in mensen beïnvloedt en

worden er determinanten geïdentificeerd voor AMR op genetisch niveau. Tevens wordt onderzocht hoe het fecale resistoom van vee, dat wil zeggen de volledige verzameling van ARG's in mest van vee, wordt beïnvloed door AMG en bepaalde andere kenmerken van agrarische bedrijfsvoering zoals o.a. bioveiligheidsmaatregelen. Moleculaire technieken zoals qPCR en metagenome analyse worden toegepast om overkoepelende boerderij-technische maatregelen te identificeren om het fecale resistoom van vee te reduceren. Daarnaast worden gegevens gegenereerd met betrekking tot de genetische eigenschappen van resistentie (bijv. co- en kruisselectie). Dit proefschrift richt zich tevens specifiek op het resistoom van personen die beroepsmatig contact hebben met vee. Deze bevolkingsgroepen worden niet alleen sterk blootgesteld aan AMR, maar fungeren mogelijk ook als punt van overdracht voor AMR-bacteriën en ARG's naar de algemene bevolking. Tenslotte wordt het gebruik van metagenome methoden besproken ter ondersteuning of vervanging van fenotypische AMR-methoden binnen het kader van een AMR-surveillance netwerk.

In **hoofdstuk één** worden de geschiedenis en moleculaire kenmerken van AMR omschreven, naast de voor- en nadelen van bestaande AMR-detectiemethoden. Vervolgens worden data-analysemogelijkheden besproken om potentiële AMR-determinanten te identificeren met behulp van metagenome data.

Deel I omvat de metagenome en epidemiologische analyse van het fecale resistoom van vee. **Hoofdstuk 2** begint met de beschrijving van het resistoom van varkens en vleeskuikens als onderdeel van een groot Europees meerlandenonderzoek. Een hogere AMR-belasting wordt waargenomen in varkens in vergelijking met vleeskuikens, terwijl de samenstelling en diversiteit van het varkensresistoom homogener blijkt te zijn dan dit van vleeskuikens. Voor zowel het fecale varkensresistoom als het resistoom van vleeskuikens wordt een significant landeffect waargenomen, dat groter is voor varkens dan voor vleeskuikens. Daarnaast wordt aangetoond dat het microbiom op een landafhankelijke manier gecorreleerd is met het resistoom. Als laatste worden er verbanden omschreven tussen landspecifiek AMG en zowel het varkens- als vleeskuiken-resistoom. Gezien het grote landeffect dat aanwezig is in zowel de AMG als AMR-data, wordt vervolgens een transparante epidemiologische analyse uitgevoerd waarbij gedetailleerde veehouderij-gerelateerde kenmerken en determinanten worden onderzocht in relatie tot het varkensresistoom: een *random-effects meta-analysis* per land (**hoofdstuk drie**). Deze analyse toont positieve verbanden aan tussen specifiek AMG op varkenshouderijen en componenten van het fecale resistoom van slachtvarkens. Met behulp van deze meta-analysebenadering worden zowel directe ARG-selectie als kruis- en

co-selectie van ARG's aangetoond. Hoog AMG in jonge varkens kon echter niet direct in verband gebracht worden met AMR in slachtvarkens, hetgeen duidt op een korte selectieve druk van AMG in jonge varkens. Verder worden hygiënische maatregelen, zoals reiniging en desinfectie op een varkenshouderij, positief in verband gebracht met het voorkomen van bepaalde ARG's. Dit contra-intuïtieve, positieve effect stemt overeen met bevindingen van voorafgaand fenotypisch AMR-onderzoek en ondersteunt mogelijk een eerder gesuggereerde genetische samenhang tussen ARG's en biocide- en metaalresistentiegenen.

In **Deel II** wordt de blootstelling aan en het fecaal dragerschap van AMR in personeel van varkensslachthuizen gemeten en geanalyseerd. **Hoofdstuk vier** laat zien dat het voorkomen van *extended-spectrum β -lactamase producing E. coli* (ESBL-PE) in – in Nederland werkzame – slachters vergelijkbaar is met dragerschap in de algemene Nederlandse bevolking. Een hogere kans op ESBL-PE dragerschap wordt echter geconstateerd voor slachterijpersoneel dat werkzaam is aan de slachtlijn vanaf de stallen tot aan de koeling. Vervolgens wordt in **hoofdstuk vijf** binnen deels dezelfde slachthuispopulatie de omgevingsblootstelling aan, en het humane dragerschap van, tetracycline- (*tetW*) en macrolide-resistentiegenen (*ermB*) gemeten en geanalyseerd. Hierbij worden duidelijk zichtbare, dalende ARG-omgevingsgradiënten aangetoond langs de slachtlijn (karkassen, handschoenen en lucht). Blootstelling aan AMR via de huid en de luchtwegen wordt daarmee bevestigd in overeenstemming met eerder fenotypisch AMR-onderzoek. Vervolgens kan er echter geen robuust verband aangetoond worden tussen beroepsmatige blootstelling aan tetracycline- en macrolide-resistentiegenen in de slachthuisomgeving en fecaal ARG-dragerschap na uitgebreid gecorrigeerd te hebben voor *confounding*. In plaats daarvan blijken nationaliteit en roken bepalende factoren te zijn voor humaan fecaal ARG-dragerschap.

Deel III geeft een overzicht van het fecale resistoom en microbiom van mensen die beroepsmatig zijn blootgesteld aan AMR in vee, dat wil zeggen varkens- en vleeskuikenhouders en varkensslachthuismedewerkers (**hoofdstuk zes**). Een hoger ARG-dragerschap wordt aangetoond in werknemers die aan varkens zijn blootgesteld in vergelijking tot vleeskuikenhouders en een controlepopulatie. Binnen en tussen populaties worden compositionele verschillen aangetoond in het resistoom en microbiom, hetgeen mogelijk zou kunnen wijzen op contact met vee als een determinant voor humaan fecaal ARG-dragerschap. Daarnaast, zoals aangetoond voor het resistoom van het vee, blijkt het humane resistoom significant te zijn gecorreleerd met diens taxonomische samenstelling, wat het belang benadrukt van het bestuderen van microbiom-resistoom interacties.

Tenslotte reflecteert **hoofdstuk zeven** op alle voorgaande hoofdstukken door onderzoek uit dit proefschrift te combineren met reeds bestaande kennis over determinanten voor het dierlijke en humane resistoom. Daarnaast worden de voordelen en beperkingen van een *sequence-based* metagenome aanpak besproken voor wat betreft de toepassing hiervan binnen een AMR-surveillancenetwerk.

De bevindingen in dit proefschrift bieden een stevig uitgangspunt voor toekomstig epidemiologisch metagenoom-onderzoek en de analyse van resistoom-interacties tussen mens en dier in de voedselproductieketen. Toekomstige studies zijn erbij gebaat zich te richten op de implementatie van grootschaligere, bij voorkeur longitudinale – en op metagenomic assembly gebaseerde – studies, om zo de lange-termijn dynamiek van het resistoom, in samenhang met het microbioom, te kunnen bestuderen. AMR is een prangende kwestie die onmiddellijke actie en praktische oplossingen vereist - dit proefschrift bespreekt de sterke punten en beperkingen van epidemiologisch onderzoek gericht op ARGs in de strijd tegen AMR en roept tegelijkertijd op tot harmonisatie van metagenome analysetechnieken om toekomstig gebruik hiervan binnen surveillancenetwerken te versterken.

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Liese

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Curriculum Vitae

Liese Van Gompel was born in Turnhout (Belgium) on 19 June 1984. In 2006, she obtained a master's degree in political sciences from the Catholic University of Leuven (KU Leuven, Belgium) with a major in public administration and a minor in socio-economic policy. In 2013, she graduated as a Doctor of Veterinary Medicine from Utrecht University (UU, the Netherlands) with a specialization in companion animal medicine. During her studies her interest in the subject of public health grew steadily, and between and after

her studies she worked at the World Health Organization (WHO) HQ in Geneva (Switzerland), the Dutch Food and Consumer Product Safety Authority (NVWA) in Utrecht (the Netherlands) and the European Food Safety Authority (EFSA) in Parma (Italy) on topics related to zoonotic diseases and food safety. In 2014, she left EFSA to pursue a PhD in Epidemiology at the Institute for Risk Assessment Sciences of Utrecht University (the Netherlands). The results of her PhD research on the molecular epidemiology of antimicrobial resistance in the food chain are presented in this work. During her PhD, Liese obtained a master's degree in Epidemiology and joined the European College of Veterinary Public Health (ECVPH) as a resident. Her interest in data analysis led her to join the Amsterdam office of PwC the Netherlands as a senior consultant data analytics at the Cyber, Forensics and Privacy Unit in the years 2018 and 2019. She is currently employed as a permanent researcher at the Institute for Risk Assessment Sciences within the Department of Population Health Sciences. From May 2020 onwards, she will continue her research as assistant professor of veterinary public health (VPH) focussing on the integration of novel data analysis techniques within the field of VPH. Liese currently lives in Utrecht with her partner Roelof.

List of publications

1. **Van Gompel, L. et al.** The faecal resistome and microbiome of farmers and slaughterhouse workers: a metagenome-wide cross-sectional study (Manuscript in preparation).
2. Yang, D., Sanders, P., **Van Gompel, L. et al.**, Association of antimicrobial usage with faecal abundance of *ermB*, *tetW*, *aph3* and *sul2* resistance genes in veal calves in three European countries (Manuscript submitted).
3. Luiken, R.E.C., **Van Gompel, L. et al.**, Farm dust resistomes and bacteriomes in European poultry and pig farms (Manuscript in preparation).
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Supplementary Figures:

2.2, 2.6 and 2.7

Supplementary Figure 2.2 AMR phenotype heatmap

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

Supplementary Figure 2.6 AMR gene heatmap

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

Supplementary Figure 2.7 AMR core gene heatmap

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