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

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ABSTRACT

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

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1. Introduction

Antimicrobial usage (AMU) in livestock farms is indicated for treatment of diseased animals but has clear effects on the development of antimicrobial resistant bacteria (ARB) (Magouras et al., 2017). The relationship between AMU and ARB, mainly in the gut of the animals, has been studied extensively (Chantziaras et al., 2014; Bell et al., 2014). However, development and spread of ARB goes beyond the gut of the animals. Feces with ARB reaches the farm floor, manure pit, farm land and wider environment by run off and aerosolization (Woolhouse et al., 2015; Huijbers et al., 2019; He et al., 2020). Fresh animal feces and manure have been demonstrated to be major (microbiological) sources of farm dust (Winkel et al., 2015; Cambra-López et al., 2011a). Viable (resistant) bacteria, bacterial DNA and antimicrobial resistance genes (ARGs) are transported as part of dust particles through the air (de Rooij et al., 2019; McEachran et al., 2015; Dohmen et al., 2017a; von Salviati et al., 2015; Laube et al., 2013; Liu et al., 2018). Sequencing based studies have shown that ARGs are part of the airborne dust microbiome from different urban and agricultural environments (Li et al., 2018, 2019; Yang et al., 2018). Farm dust in particular has a relatively rich and abundant resistome (Yang et al., 2018; Luiken et al., 2020).

Most probably the role of farm dust in the epidemiology and ecology of antimicrobial resistance (AMR) is complex and multilevel. On one hand it can act as a potential transmission route of resistant bacteria within and between animals and humans (Dohmen et al., 2017a; Bos et al., 2016). On the other hand it is a potential reservoir of ARGs which might be transferred to bacteria, including potentially pathogenic bacteria in other reservoirs, by e.g. horizontal gene transfer (Huijbers et al., 2015, 2019; Scott et al., 2019). Work on the quantification of ARGs in poultry or pig farm dust is, to the best of our knowledge, scarce and either small scale or focused on single pathogens (Li et al., 2019; Bos et al., 2016) and literature on determinants is lacking. Studies on farm dust levels, regardless of AMR, point out the potentially high concentrations indoors, especially in poultry and pig farms (Basinas et al., 2013a), resulting in adverse respiratory health effects in farmers (Basinas et al., 2013a; Bolund et al., 2017) and thus potentially for animals. Demonstrated determinants of high dust concentrations in farms are, among others, a low ventilation rate in pig farms (Preller et al., 1995), age of the broilers (Oppliger et al., 2008), or presence of a slatted floor system in pig farms (Basinas et al., 2013b). Previous research on fecal AMR in livestock mainly focused on the association with AMU (Chantziaras et al., 2014; Bell et al., 2014). Yet some studies also identified farm or animal characteristics, or farm biosecurity measures (taken to reduce the entrance and spread of bacteria) to be related to increased fecal ARB levels, such as decreased farm hygiene (Taylor et al., 2016; Persoons et al., 2011; Dohmen et al., 2017b), herd size (Sorensen et al., 2018), number of suppliers (Sorensen et al., 2018), or straw and flax as litter material (Persoons et al., 2011; van Hoorebeke et al., 2011). It is still unknown whether these parameters are also relevant determinants for AMR in airborne dust.

Our earlier study that used shotgun metagenomics sequencing to quantify ARGs in dust samples (Luiken et al., 2020) gave many new insights in the farm dust resistome, but the sample size hampered a thorough quantitative analysis of risk factors for ARG abundance in dust. The current study describes the presence of four different ARGs in 947 freshly settled indoor poultry and pig farm dust samples from 333 farms in nine European countries. In this large-scale analysis, we used qPCR to quantify ARG levels. We present the distributions of absolute ARG levels in farm dust, representing the levels people and animals might be exposed to. In addition we present the relative abundance (normalized over 16S) of ARGs, representing the levels in the bacterial population. We assume that determinants may contribute differently to these two outcomes and therefore discuss results of associations with determinants for both endpoints. First, we determined the relation with ARGs in what we expected to be the most important source, that is animal feces. Subsequently, associations with AMU and farm and animal related characteristics, including biosecurity levels of the farm, were analyzed.

2. Materials and methods

2.1. Study design

In this cross-sectional study, conventional broiler (names poultry throughout the text) and conventional farrow-to-finish pig farms were visited between 2014 and 2016 in nine European countries (Belgium, Bulgaria, Denmark, France, Germany, Italy, the Netherlands, Poland and Spain). In each country, 20 farms per animal species were visited and indoor farm dust, animal feces and meta-data were collected. The whole farm population and its selection criteria were described before (Munk et al., 2018). Important inclusion criteria were regular/conventional production type, all-in all-out procedures on compartment level and no other farm animals kept at the farm for production goals. The study focused on animals closest to slaughter (broilers and fattening pigs) and is part of the EFFORT-project (http://www.effort-against-amr.eu/).

2.2. Farm dust collection

Dust sampling and lab processing has been described before (Luiken et al., 2020). Dust was sampled with the use of Electrostatic Dustfall Collectors (EDCs) (Noss et al., 2008). These are sampling devices for 'passive' airborne dust sampling, consisting of plastic frame equipped with two (sterilized) electrostatic cloths. The cloths were gamma-radiated before use, to break down as much DNA as possible. Four EDCs were placed in and spatially spread over the poultry house or fattening pigs compartments. The EDCs were positioned horizontally at about 150 cm above ground level, distant from heating or cooling systems.

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

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

2.3. Animal feces collection

During the farm visits, 25 fresh individual fecal samples were collected from animals in the same compartment(s) as the EDCs were placed in. Fresh droppings were collected, evenly divided over the compartment (often multiple compartments for pigs), with a sterile plastic spoon and cup. Feces was immediately stored at 4 °C and transported to the local lab where they were stirred, divided in smaller portions and frozen within 24 h at −80 °C (alternatively at −20 °C for a maximum of 4 days, before transferring to −80 °C) (Munk et al., 2018). From the 25 individual samples, five poultry and seven pig fecal samples were randomly selected for further analysis using qPCR. The random selection of five or seven samples was a result of a balance between practicality, financial feasibility and statistical power. Variation within single poultry stables was deemed smaller than within pig stables with different pens, therefore, a larger number of subsamples was analyzed in pigs. The earlier metagenomic study, which was based on the same samples, used a pooled sample composed of all the 25 individual samples (Luiken et al., 2020).

2.4. Meta-data collection

During the farm visit, information on farm and animal characteristics and antimicrobial usage (AMU) data was collected through a questionnaire filled out together with the farmer. Farm and animal characteristics included age of animals, animal density, type of ventilation systems, feed type and others. AMU data was collected per antimicrobial class and in total (sum of all classes). From these records Treatment Incidence for Defined Daily Dosages (TIdddvet) per 100 animals were calculated (Joosten et al., 2019; Sarrazin et al., 2019). Afterwards farm biosecurity scores were calculated with the Biocheck.UGent scoring system (www.biocheck.ugent.be). Results are expressed on a scale from 0 to 100, with 100 meaning that all possible biosecurity measures are present (100%). See also earlier works using these biosecurity scores (Luiken et al., 2019; Van Gompel et al., 2019).

2.5. Lab processing and DNA extraction

After arrival at the central lab the EDCs were stored up to 6 days in the envelope used for transport, and subsequently opened in a flow cabinet. Electrostatic cloths were removed from the folder, transferred to a sealable bag and frozen at −80 °C. Maximally three EDCs per farm were selected for further processing. EDCs showing traces of water damage or other signs of unintended contamination were excluded from further processing. In some cases farmers did not return EDCs or corresponding records were missing and therefore, in total, 947 samples from 333 farms were included in the analysis (500 samples from 174 poultry farms and 447 samples from 159 pig farms). An overview of all sample numbers can be found in Supplemental Table 1.

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

Fecal samples were sent from local labs to the central lab on dry ice and processed as described earlier (Munk et al., 2018). DNA was extracted using a modified protocol of the QIAamp Fast DNA Stool Mini Kit (Qiagen) with an additional bead beating step (3 × 30 s at 30Hz with TissuelyserII) (Knudsen et al., 2016). DNA was stored at −80 °C until further processing.

2.6. qPCR protocol

qPCR was performed to quantify the abundance of the antimicrobial resistance genes *tetW*, *ermB*, *aph(3′)-III* and *sul2*, coding for tetracycline, macrolide, aminoglycoside and sulfonamide resistance, respectively, along with the bacterial *16S* rRNA gene. Target choice was guided by metagenomic results of samples of a pilot study on 4 farms, combined with limited qPCR testing based on the following principles: 1) sufficient abundance to be measured in >25% of the samples as predicted from the metagenomic outcome, 2) representation of resistance to different antimicrobial classes, 3) lack of strong correlation between the qPCR targets chosen, and 4) public health relevance, defined as e.g. inclusion in the WHO list of critically important antimicrobials (WHO, 2018). Although a range of resistance genes of expected high relevance were considered for inclusion (e.g. extended spectrum beta lactam resistance genes, carbapenem resistance genes, colistin resistance genes and vancomycin resistance genes), they were extrapolated to only be detectable in <25% of the samples (based on the strength of the respective metagenomic or initial qPCR signal) and were thus deselected. qPCR was performed in two labs, namely for *16S*, *aph(3′)-III* and *sul2* in Poland (National Veterinary Research Institute, PIWet, Puławy), and for *tetW* and *ermB* in The Netherlands (IRAS, Utrecht). qPCR was performed with a CFX384 Real-Time System (Bio-Rad, USA). Details on the qPCR-protocol, primers, quality control, calibration curves and LOD and LOQ can be found in the supplemental material and in earlier works (Yang et al., 2020; Van Gompel et al., 2020).

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

2.7. Data analysis

2.7.1. Selection of determinants

ARG levels in feces were analyzed as mean number of gene copies per gram of feces based on 5 (poultry) or 7 (pigs) individual samples per farm. Relevant AMU measures were selected. First, total and antimicrobial class specific group treatments given to the animals sampled in this study, second, total and specific purchased products for the whole farm in the year before sampling. Collected farm and animal related determinants were selected for data analysis based on literature and expert opinion. Out of a total of 105 (poultry) or 150 (pig) questions (including sub questions), roughly 20 individual questions were considered potentially relevant for dust exposure. Biosecurity information was summarized in scores (total, internal and external) and one internal biosecurity sub score ('cleaning and disinfection'). Potential determinants were included in further analysis when the missing value level was <10% and the determinant was present at, at least, 10% of the farms. AMU was tested as a dichotomous variable in case of scarce use of a specific group (use in less than 10% of the farms). This resulted in a collection of around 25 variables divided in several subcategories, as potential determinants or source for ARG levels in farm dust (see Supplemental Table 4 for full list).

2.7.2. Statistical analysis

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

For the determinant analysis a linear mixed effect model was used to account for within farm and within country effects by including a nested random effect (R package *nlme* (Pinheiro et al., 2020)). All model results are presented as regression coefficients with accompanying 95% confidence intervals. In the main text we choose to present only results from determinants presenting significant associations with at least two genes within one outcome. All results, including *P* values, are included in the supplemental material.

All data handling and analyses were done in R software (version 4.0.2) (R-Core-Team, 2017). All graphs were created with R package *ggplot2* (Wickham, 2016).

3. Results

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

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

	mean	median	10th – 90th percentile
Poultry farm size (n broilers present on the farm)	70,715	50,000	23,000–150,000
Age of broilers at sampling (days)	34	35	26–42
Number of broilers present in sampled barn	25,386	24,558	14,000–36,801
Pig farm size (n pigs, of all age categories, present on the farm)	4571	3000	1350–9600
Age of fatteners at sampling (days)	180	173	135–259
Number of fatteners present on the farm	562	300	100–1021

poultry house or pigs compartments which were sampled.

Antimicrobial resistance genes (ARGs) and the *16S* rRNA gene were quantifiable in almost all samples (Fig. 1). The percentage of samples below LOQ or LOD was below 0.01% for all genes except for *aph(3')-III* (<LOQ: 10%, <LOD: 3%) and *sul2* (<LOQ/LOD: 19%) in poultry dust. Field and procedural blanks contained only traces of the ARGs investigated. Field blanks had ARG abundances which were about 10,000 (pigs) till 500 (poultry, probably more but limited by LOQ) times lower than actual samples (Supplemental Figure 1) indicating that contamination of samples because of transport, field and laboratory procedures did not likely occur.

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

1. Fecal ARG levels

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

2. Antimicrobial usage

Absolute and relative abundance of ARGs in dust were significantly positively associated with AMU in pigs (Table 3). To test for a direct effect of AMU on dust ARG levels, thus next to mediation through feces, we adjusted the model for fecal ARG levels. The associations became slightly weaker, but a statistically significant effect remained for *tetW, aph(3')-III* and *sul2*. More frequent tetracycline treatments in fatteners was associated with higher absolute and relative *tetW* abundances in pig farm dust (Supplemental Table 4). For poultry these associations were weaker and mainly seen for the relative abundance with *P* values just above 0.05 (Table 3 and Supplemental Table 4).

3. Total dust levels

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

4. Other determinants

a. Animal and farm related parameters

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

b. Biosecurity

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

4. Discussion

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

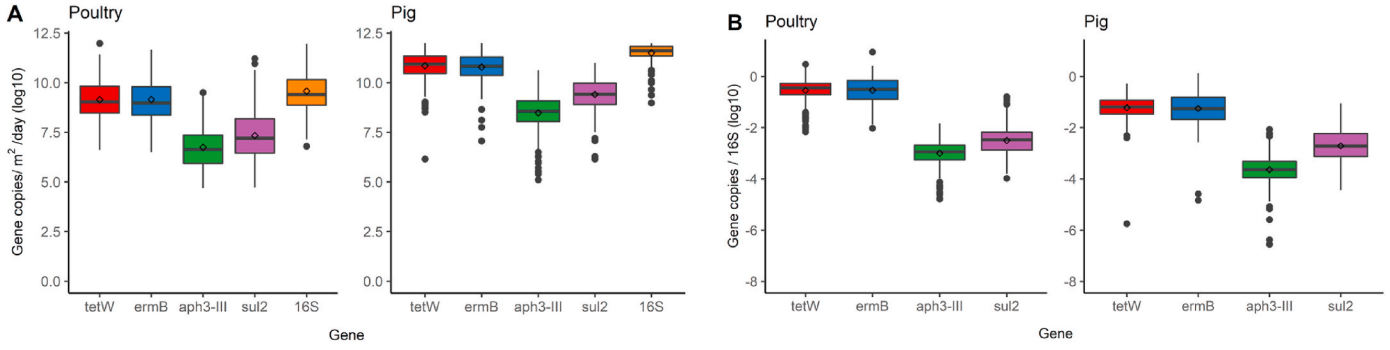

Fig. 1. Farm dust ARG abundances in poultry and pig farms from nine countries. A) Absolute abundance per m^2 per day of four ARGs and 16S. b) Relative abundance (normalized over 16S) of four ARGs. The middle line in the (25–75 percentile) boxplot represents the median, the diamond the mean. Abundances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

Table 2
Results of regression* between ARG abundances in dust and animal feces from the same compartment.

	Absolute abundance (log10 ARG copies/m^2/day)					Relative abundance (log10 ARG copies normalized over 16S)			
	tetW	*ermB*	*aph(3′)-III*	*sul2*	*16S*	*tetW*	*ermB*	*aph(3′)-III*	*sul2*
	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)
Poultry									
	0.12 (−0.16–0.40)	**0.27 (0.04–0.50)**	0.18 (−0.09–0.44)	0.15 (−0.09–0.39)	0.02 (−0.40–0.44)	0.13 (−0.00–0.27)	**0.44 (0.34–0.54)**	0.03 (−0.09–0.15)	**0.22 (0.11–0.33)**
Pigs									
	0.30 (−0.16–0.75)	**0.67 (0.47–0.88)**	0.12 (−0.09–0.33)	**0.31 (0.11–0.51)**	0.11 (−0.20–0.42)	0.13 (−0.10–0.36)	**0.82 (0.68–0.96)**	**0.21 (0.06–0.35)**	**0.30 (0.15–0.44)**

Bold results have $P < 0.05$. *across participating countries analyzed using a mixed model nested by country and farm. Est = estimate, 95% CI = 95% Confidence Interval.

Table 3
Results of regression* between absolute and relative ARG abundances in dust and total AMU administered as group treatment to the sampled animals in their life (poultry) or fattening phase (pigs).

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

Bold results have $P < 0.05$. *analyzed across participating countries using a mixed model nested by country and farm. Est = estimate, 95% CI = 95% Confidence Interval. An overview of all model results, including other AMU measures, can be found in the Supplemental Table 4.

Table 4
Results of regression* between absolute and relative ARG abundances in dust and total dust levels.

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

Bold results have $P < 0.05$. *analyzed across participating countries using a mixed model nested by country and farm. Est = estimate, 95% CI = 95% Confidence Interval.

complementary insights into the dynamics of dust in the epidemiology of AMR in the livestock farm.

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

AMR studies involving airborne or settled dust are still relatively scarce but the available evidence indicates that ARGs are omnipresent in dust (McEachran et al., 2015; Yang et al., 2018; Xie et al., 2019). Having assessed ARGs in all dust samples in the current study is therefore not a surprise, however the relative levels of these genes are not very different from other (AMU exposed) samples such as animal feces or waste water from treatment plants (Rodriguez-Mozaz et al., 2015). This could be explained by the fact that animal feces is very likely the most important source of dust through aerosolization of fecal particles. Indeed, our data shows a positive relation between ARG levels in feces and dust, which was more pronounced for relative ARG abundances. However, although feces is an important source for farm dust, it is not the only organic/microbiological source (Cambra-López et al., 2011a). Other sources, such as skin, mucus, feed, litter and outdoor air or soil are additional sources of bacterial DNA and potentially ARGs. The contribution of each source to the dust composition depends on animal species and the

Table 5
Results of regression* between absolute and relative ARG abundances in dust and animal and farm related determinants, including biosecurity, for a) poultry and b) pigs.

a) Poultry

		Absolute abundance (log10 ARG copies/m2/day)					Relative abundance (log10 ARG copies normalized over 16S)			
Variable	Category	*tetW*	*ermB*	*aph(3′)-III*	*sul2*	*16S*	*tetW*	*ermB*	*aph(3′)-III*	*sul2*
		Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)
Animal and farm related parameters										
Broilers present in the stable (per 10,000)		**0.24 (0.08–0.39)**	**0.27 (0.11–0.43)**	**0.27 (0.10–0.44)**	**0.23 (0.03–0.43)**	**0.21 (0.03–0.39)**	0.03 (−0.03–0.09)	0.05 (−0.02–0.12)	0.08 (0.01–0.15)	0.03 (−0.06–0.12)
Bedding material (ref = sawdust)	other	−0.36 (−0.83–0.11)	−0.28 (−0.76–0.19)	−0.25 (−0.78–0.28)	−0.58 (−1.17–0.00)	−0.42 (−0.95–0.12)	0.03 (−0.16–0.21)	0.05 (−0.16–0.27)	0.03 (−0.18–0.25)	−0.25 (−0.52–0.01)
	shredded_straw	**−0.68 (-1.22–-0.14)**	**−0.84 (-1.38–-0.29)**	−0.51 (−1.11–0.09)	**−1.16 (-1.84–-0.47)**	**−1.00 (-1.62–-0.38)**	**0.27 (0.06–0.49)**	0.17 (−0.08–0.42)	**0.28 (0.03–0.52)**	−0.22 (−0.52–0.08)
Season (ref = winter)	autumn	0.20 (−0.15–0.54)	0.07 (−0.28–0.43)	0.13 (−0.25–0.51)	−0.09 (−0.55–0.36)	−0.04 (−0.45–0.37)	0.23 (0.09–0.37)	0.12 (−0.04–0.29)	0.12 (−0.04–0.28)	−0.12 (−0.32–0.09)
	spring	−0.26 (−0.66–0.14)	−0.33 (−0.74–0.08)	−0.38 (−0.82–0.06)	−0.49 (−1.01–0.03)	−0.49 (−0.96–−0.02)	0.22 (0.06–0.38)	0.16 (−0.03–0.34)	0.09 (−0.10–0.27)	−0.01 (−0.24–0.23)
	summer	**−0.45 (-0.84–-0.07)**	**−0.48 (-0.87–-0.08)**	**−0.62 (-1.04–-0.20)**	**−0.51 (-1.01–-0.01)**	**−0.62 (-1.07–-0.17)**	**0.18 (0.03–0.33)**	0.13 (−0.04–0.31)	−0.13 (−0.30–0.04)	0.07 (−0.15–0.29)
Length ventilation present (ref = no)	yes	0.15 (−0.16–0.46)	0.19 (−0.13–0.51)	0.17 (−0.17–0.51)	−0.02 (−0.42–0.38)	0.01 (−0.35–0.37)	**0.13 (0.01–0.25)**	**0.15 (0.01–0.28)**	0.13 (−0.01–0.27)	−0.03 (−0.20–0.14)
Biosecurity measures										
Biosecurity external (per 10 pts)		0.013 (−0.160–0.187)	0.055 (−0.127–0.237)	0.060 (−0.125–0.245)	0.101 (−0.125–0.327)	0.125 (−0.073–0.324)	**−0.099 (-0.167–-0.030)**	−0.041 (−0.123–0.041)	−0.064 (−0.144–0.016)	−0.001 (−0.099–0.097)
Biosecurity internal (per 10 pts)		0.009 (−0.103–0.121)	0.010 (−0.106–0.126)	0.051 (−0.070–0.171)	0.067 (−0.079–0.212)	0.084 (−0.045–0.213)	**−0.057 (-0.101–-0.013)**	**−0.051 (-0.0102–-0.000)**	**−0.052 (-0.102–-0.001)**	−0.010 (−0.072–0.053)
Biosecurity in total (per 10 pts)		0.017 (−0.159–0.192)	0.047 (−0.136–0.231)	0.083 (−0.103–0.269)	0.124 (−0.105–0.353)	0.154 (−0.045–0.354)	**−0.113 (-0.182–-0.044)**	−0.069 (−0.150–0.013)	**−0.085 (-0.165–-0.005)**	−0.008 (−0.107–0.091)
Internal biosecurity sub score: cleaning and disinfection (per 10 pts)		0.018 (−0.073–0.110)	0.014 (−0.082–0.110)	0.035 (−0.064–0.133)	0.058 (−0.061–0.177)	0.089 (−0.015–0.194)	**−0.051 (-0.087–-0.015)**	**−0.050 (-0.092–-0.008)**	**−0.064 (-0.105–-0.023)**	−0.016 (−0.068–0.035)

b) Pigs

		Absolute abundance (log10 ARG copies/m2/day)					Relative abundance (log10 ARG copies normalized over 16S)			
Variable	Category	*tetW*	*ermB*	*aph(3′)-III*	*sul2*	*16S*	*tetW*	*ermB*	*aph(3′)-III*	*sul2*
		Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)
Animal and farm related parameters										
Animal density (ref = high)	low	**−0.39 (-0.66–-0.11)**	**−0.03 (-0.32–0.26)**	−0.27 (−0.59–0.06)	−0.22 (−0.52–0.08)	−0.25 (−0.47–−0.03)	−0.08 (−0.25–0.10)	0.25 (−0.01–0.50)	−0.09 (−0.32–0.15)	0.07 (−0.17–0.31)
Feed type (ref = dry)	wet-dry	**−0.52 (-0.91–-0.13)**	−0.36 (−0.76–0.05)	**−0.79 (-1.24–-0.34)**	−0.36 (−0.76–0.05)	**−0.52 (-0.82–-0.22)**	0.06 (−0.18–0.31)	0.20 (−0.14–0.55)	−0.28 (−0.59–0.04)	0.27 (−0.05–0.59)
	wet	−0.10 (−0.34–0.13)	−0.09 (−0.33–0.15)	**−0.37 (-0.64–-0.10)**	−0.08 (−0.33–0.16)	−0.02 (−0.20–0.15)	−0.11 (−0.24–0.03)	−0.09 (−0.29–0.11)	−0.36 (−0.54–−0.18)	−0.05 (−0.23–0.14)
Other farm in 500mbuffer around the farm (ref = no)	yes	**0.27 (0.07–0.48)**	0.18 (−0.03–0.39)	**0.30 (0.05–0.54)**	**0.24 (0.03–0.46)**	**0.25 (0.10–0.41)**	0.00 (−0.12–0.12)	−0.06 (−0.24–0.11)	0.07 (−0.10–0.23)	−0.03 (−0.19–0.14)
Season (ref = winter)	autumn	−0.03 (−0.34–0.27)	−0.22 (−0.54–0.10)	0.18 (−0.19–0.54)	0.07 (−0.26–0.39)	0.06 (−0.16–0.29)	−0.06 (−0.24–0.12)	−0.29 (−0.54–−0.03)	0.11 (−0.12–0.35)	0.01 (−0.22–0.25)
	spring	0.06 (−0.28–0.39)	0.09 (−0.26–0.43)	0.06 (−0.34–0.45)	0.09 (−0.26–0.45)	0.11 (−0.13–0.36)	−0.03 (−0.23–0.17)	−0.04 (−0.32–0.24)	−0.08 (−0.34–0.18)	−0.03 (−0.29–0.23)
	summer									

(*continued on next page*)

Table 5 (*continued*)

b) Pigs

Variable	Category	Absolute abundance (log10 ARG copies/m2/day)					Relative abundance (log10 ARG copies normalized over 16S)			
		tetW	*ermB*	*aph(3')-III*	*sul2*	*16S*	*tetW*	*ermB*	*aph(3')-III*	*sul2*
		Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)	Est (95% CI)
		−0.31 (−0.62–0.00)	**−0.35 (-0.67–-0.03)**	−0.36 (−0.73–0.00)	−0.07 (−0.40–0.26)	**−0.27 (-0.50–-0.05)**	−0.00 (−0.18–0.18)	−0.12 (−0.38–0.14)	−0.13 (−0.37–0.11)	0.19 (−0.05–0.43)
Different ventilation system then all other ventilation system options (ref = no)	yes	0.27 (0.00–0.55)	−0.04 (−0.32–0.24)	0.28 (−0.04–0.59)	0.24 (−0.04–0.53)	0.07 (−0.14–0.28)	**0.17 (0.01–0.33)**	−0.12 (−0.36–0.11)	**0.25 (0.03–0.47)**	0.17 (−0.05–0.39)
Valve ventilation system (ref = no)	yes	−0.15 (−0.40–0.11)	0.03 (−0.24–0.29)	−0.15 (−0.45–0.15)	**−0.31 (-0.57–-0.05)**	**−0.24 (-0.44–-0.05)**	0.14 (−0.01–0.29)	**0.31 (0.09–0.52)**	0.13 (−0.08–0.33)	0.05 (−0.15–0.25)
Biosecurity measures										
Biosecurity external (per 10 pts)		−0.090 (−0.187–0.008)	−0.018 (−0.117–0.081)	−0.102 (−0.209–0.005)	−0.073 (−0.176–0.030)	**−0.090 (-0.165–-0.016)**	0.018 (−0.039–0.074)	**0.087 (0.005–0.169)**	0.007 (−0.071–0.084)	0.021 (−0.056–0.099)
Biosecurity internal (per 10 pts)		0.011 (−0.071–0.094)	0.069 (−0.012–0.150)	−0.026 (−0.118–0.066)	0.057 (−0.028–0.143)	−0.039 (−0.101–0.023)	**0.054 (0.006–0.102)**	**0.111 (0.042–0.179)**	0.022 (−0.044–0.088)	**0.084 (0.018–0.149)**
Biosecurity in total (per 10 pts)		−0.041 (−0.145–0.062)	0.046 (−0.058–0.149)	−0.075 (−0.188–0.038)	0.005 (−0.104–0.114)	**−0.083 (-0.160–-0.005)**	0.053 (−0.008–0.113)	**0.138 (0.051–0.224)**	0.022 (−0.062–0.105)	0.079 (−0.004–0.162)
Internal biosecurity sub score: cleaning and disinfection (per 10 pts)		0.012 (−0.039–0.064)	0.039 (−0.012–0.091)	−0.016 (−0.074–0.043)	0.021 (−0.032–0.075)	−0.024 (−0.063–0.015)	**0.037 (0.007–0.066)**	**0.060 (0.017–0.103)**	0.016 (−0.025–0.057)	0.037 (−0.003–0.077)

Bold results have $P < 0.05$ * analyzed across participating countries using a mixed model nested by country and farm. NB Only results with at least two genes significantly associated are shown. Est = estimate, 95% CI = 95% Confidence Interval. An overview of all model results can be found in the Supplemental Table 4.

farming system (Cambra-López et al., 2011b; Zhao et al., 2014) and therefore, imaginably, differs per ARG. This might explain the different coefficients between feces and dust seen in this study per resistance gene.

Higher total AMU in fattening pigs from the fattening unit sampled for dust was positively associated with resistance gene abundances in dust. This was observed earlier for the summed abundance of all ARGs (the resistome) present in dust, determined with metagenomics, in a selection of the same pig and poultry farms (Luiken et al., 2020). A significant association was also observed between tetracycline usage and *tetW* dust levels, despite the smaller association between fecal and dust *tetW* levels. It is expected that at least a part of the relationship between AMU and dust is mediated by the effect of AMU on fecal resistance genes. After adjustment for ARG levels in feces, a significant positive effect remained for all genes except one: *ermB*. Fecal *ermB* concentrations had the largest association with dust *ermB* concentrations. These results seem to point towards an independent effect of AMU on dust resistance genes in addition to the feces pathway. Another related issue here could be the excretion of antimicrobial residues via feces or through the administration route, which is mainly by feed and water (Joosten et al., 2019; Sarrazin et al., 2019), that may lead to local environmental selective effects (Filippitzi et al., 2019; Larsson et al., 2018). The association between corresponding usage and resistance genes for other classes than tetracycline (e.g. macrolide use and *ermB*) was hampered by limited AMU in fatteners or broilers in the sampled animals. Interestingly for poultry we found consistently lower coefficients (than for pigs) and borderline significant associations. This might partly be explained by a relatively small number of sampled poultry batches in which antimicrobials were used.

Differences between the two studied animal species, with known different farming systems, was observed often in this study. Biosecurity is the domain of all measures possibly taken to reduce the influx and spread of bacteria and other microorganisms on the farm (Gelaude et al., 2014) and thus possibly also affects the bacterial composition of the farm environment (i.e. dust). On poultry farms, higher internal biosecurity, including specifically a higher 'cleaning and disinfection score', and external biosecurity led to lower relative ARG abundances (mainly *tetW* and *ermB)* in dust. In pig farms, however, the opposite was observed. This opposite effect has been seen before for ARG in pig feces in the EFFORT project (Van Gompel et al., 2019). This underpins that interpreting biosecurity scores in relation to AMR, rather than to specific pathogens, is challenging, and the role of specific farm practices for AMR might deserve further research (Davies and Wales, 2019). Some negative associations with fecal levels of certain bacteria and biosecurity measures have been already shown (Dohmen et al., 2017b; Mo et al., 2016), however, an effect of biosecurity on bacterial/ARG levels in airborne dust has not been observed earlier. None of the associations with biosecurity (except one) was statistically significant when ARGs were expressed as absolute levels (or 16S*)* in dust for both farm types. This suggest that biosecurity scores such as those used in this project are currently not capturing airborne dust forming processes. Of all other investigated determinants, associations differed between relative and absolute ARG levels, confirming that there are pathways increasing dust ARGs through processes influencing the total level of dust generation (absolute ARG levels), and processes affecting the bacterial microbiome (relative ARG levels). For example, we observed a reduction in absolute ARGs abundance in the summer season compared to winter for poultry and pig farms. These results are in accordance with studies reporting reduced ventilation in winter (due to cold weather) which in turn led to higher dust levels in the stables (Basinas et al., 2013a; Banhazi et al., 2008). Some associations found in this study have not been described before. For example, significant associations in poultry stables between bedding type and dust ARGs, however with an opposite direction of the relation for absolute and relative levels. Another interesting finding is that other livestock farms in a 500m buffer around pig farms resulted in higher absolute ARGs abundance in the dust.

This study was performed with data from nine different countries and identified determinants are thus important across countries. Nevertheless, ultimately the local AMR and AMU situation determines the relevance of animal and farm related drivers for AMR in the farm environment. Therefore, results need to be confirmed with sufficiently powered studies in each country, or for example through intervention studies. Although this study involved a sufficiently high number of farms, it was complicated by between country differences, which required a tailor made analysis leading to some loss of power. Tested determinants in this study were not an ideal aggregation of variables potentially relevant for investigating dust formation, due to the fact that the broader project was not only set up for the objectives of the current study. For example, while effects of ventilation techniques and intensity are also expected to influence dust formation (Basinas et al., 2013a) and therefore absolute ARG levels, it was difficult to collect ventilation related determinants in a practical matter during field work, because it is time consuming, costly and expertise is needed. Across genes, no clear relations with absolute ARG levels were seen, possibly because the ventilation levels rather than the applied technique (tested here) determine aerosolization and dust formation. Since this study was set up as hypotheses generating research no multiple testing adjustment was applied.

The exact relevance of ARG transmission via farm dust is complex, still largely unknown but expected to play a role next to other transmission pathways (Manaia, 2017; Tiedje et al., 2019). Livestock farms are an important reservoir of ARGs and ARBs and a source for environmental AMR (He et al., 2020). Exposure to airborne AMR from farms will probably be more relevant for persons working in and around farms (Dohmen et al., 2017a; Bos et al., 2016) compared to the general population, which is, at least for specific resistant bacteria, dominated by human-human contact (Mughini-Gras et al., 2019). While the general population is probably considerably lower exposed to airborne dust from farms, exposure to resistance genes frequently occurring in animal feces is however possible in the vicinity of farms (de Rooij et al., 2019). Transmission of ARGs and ARBs through air and dust between animals is most likely to occur as well (Crombe et al., 2013). With the methods used here we were able to not only detect, but also quantify ARG dust levels in farms. The use of EDCs enabled us to do large scale sampling with relatively little effort (compared to air sampling using pumps), while keeping relevance, as EDCs collect freshly, airborne, settled dust. We however do not know the ARG fraction that was part of viable bacteria nor do we know the magnitude of potential transmission to other bacteria. Determinants of ARGs in farm dust can guide future research and potentially farm management policy. Clearly any dust related intervention needs to be animal specific due to the different dynamics uncovered on the currently studied farms.

5. Conclusion

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

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Declaration of competing interest

The authors declare they have not conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.112715.

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