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

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ABSTRACT

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

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

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

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

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

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1. Introduction

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

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

In the farm, an important source of dust is animal feces (Cambra-López et al. 2011). Particles are continuously aerosolized, and this process is influenced by many factors like stable design, hygienic measures, ventilation, animal activity, type of feed and bedding material, and climate conditions (Basinas et al., 2015). Although the animal fecal resistome and bacterial microbiome have been described previously (Holman et al. 2017, Kers et al. 2018, Munk et al. 2018, Waite and Taylor 2014), and some first attempts to describe the farm dust resistome and bacterial microbiome have thus been undertaken, studies including both dust and fecal samples and addressing their relation in the same environment across multiple farms are absent.

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

Antimicrobial resistance (AMR) is recognized as a problem which needs a One Health approach as the way to assess and tackle the problems that arise from the presence of antimicrobial resistant bacteria (Robinson et al. 2016). Within the European EFFORT (Ecology from Farm to Fork Of microbial drug Resistance and Transmission) project, the animal resistome (Munk et al. 2018) and farmers' resistome and bacterial microbiome (Van Gompel et al. 2020) have been described. The present study aims to address the resistome and bacterial microbiome of airborne dust, as determined by shotgun metagenomic sequencing. Specifically, we describe the dust resistome and bacterial microbiome, compare it with the poultry and pig fecal resistome and bacterial microbiome, and associate it with on-farm antimicrobial usage (AMU) in poultry and pig farms from nine European countries. In addition, the relation between the resistomes of dust, animal feces, and farmers' stool on Dutch farms is explored.

2. Materials and methods

2.1. Study design and farm population

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

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

2.2. Farm dust collection

During farm visits indoor farm dust was collected by use of Electrostatic Dust fall Collectors (EDCs) (Noss et al. 2008) from compartments with broilers or fattening pigs close to slaughter age. The electrostatic cloths were sterilized and gamma irradiated (50 kGy) to remove possible bacterial contamination before the EDCs were assembled and packed in re-sealable bags. Per farm three EDCs were horizontally placed in the compartment at a height of about 150 cm above the ground, at a location were the air has already passed over the animals, distant from heating or cooling systems. For poultry the compartment consisted of one stable with animals close to slaughter age. For pigs all (with a maximum of four) compartments with pigs close to slaughter age were sampled. The farmer was asked to collect and ship the EDCs after minimally 2 and maximally 7 days in the compartments, the latest before thinning or removing the animals for slaughter. The farmer packed the EDCs and sent them by regular mail to one central lab, from nine countries this took on average 11 days (10th-90th perc.: 2-25 days). One sample that consisted of DNA pooled from three separate DNA extracts extracted from the three samples taken at each farm was included in this study.

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

2.3. Animal feces collection

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

2.4. Farmers and control population and stool collection

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

2.5. DNA extraction and library preparation of farm dust

All EDCs were processed centrally. After arrival at the lab the envelope was stored for maximally 6 days, subsequently opened in a flow cabinet and electrostatic cloths were removed from the folder, folded and put into a small re-sealable bag with sterile tweezers and frozen at −80 °C. Directly before DNA extraction, cloths were thawed, washed in sterile 0.05% Tween20 water (for better dust yield) and blended with a stomacher. Thereafter the remaining fluid was frozen in plastic tubes at −20 °C, subsequently freeze dried for 3–4 days and the remaining material was stored again at −20 °C. After thawing, the dust was weighed and 35 mg (+/-1 mg) was collected for DNA extraction. From each dust sample, DNA was extracted using the Nucleospin 8 plant II kit (Machery-Nagel) using the standard protocol with an additional bead-beating step (30 sec at 5.5G with Fastprep-24). DNA of three EDC's of each farm were pooled for metagenomic analysis in an equi-volume manner and stored at −80 °C until further processing.

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

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

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

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

2.7. Bioinformatics processing

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

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

2.8. Collection of meta-data

Additional information on the farm was collected with the use of standardized field forms. Farm antimicrobial usage (AMU) data were collected through a questionnaire by interviewing the farmer on the day of the visit and/or through the veterinarian as described previously (Joosten et al. 2019, Sarrazin et al. 2019). AMU was expressed as Treatment Incidence of Defined Daily Dosages (TI_{DDDvet}) of either group treatments of the sampled animals or purchased products by the whole farm in the year before sampling. Additional information about the farmers, e.g. age, job type and work hours on the farm, was collected through a personal questionnaire filled out by the participant her/himself.

2.9. Data analysis

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

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

2.9.1. Resistome and bacterial microbiome composition analysis

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

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

2.9.2. Visualization of the resistome and bacterial microbiome

Total relative abundance of the resistome was computed and visualized in boxplots.

Relative abundances of resistance genes clustered per AM class as percentage of the total of resistance genes were computed and visualized per farm and per country with stacked bar charts. The same was done for the abundance of bacteria. The abundance of ARGs in farm

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

2.9.3. Association with AMU

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

3. Results

3.1. The composition of the farm dust resistome

This study included 79 farms with an average size of 77,944 chickens (10–90th perc.: 28840–148400) or 5071 pigs (10–90th perc.: 1682–9339). Total AMR levels in dust of poultry and pig farms were similar and had means of 3,045 and 3,168 FPKM, respectively. This is in contrast to the total levels of ARGs in poultry and pig feces, with poultry feces having mostly lower values than farm dust and pig feces having mostly higher levels than farm dust (Fig. 1).

The resistome composition shows significantly distinct clusters of dust and feces for the two animal species (Fig. 2). Pig and poultry dust

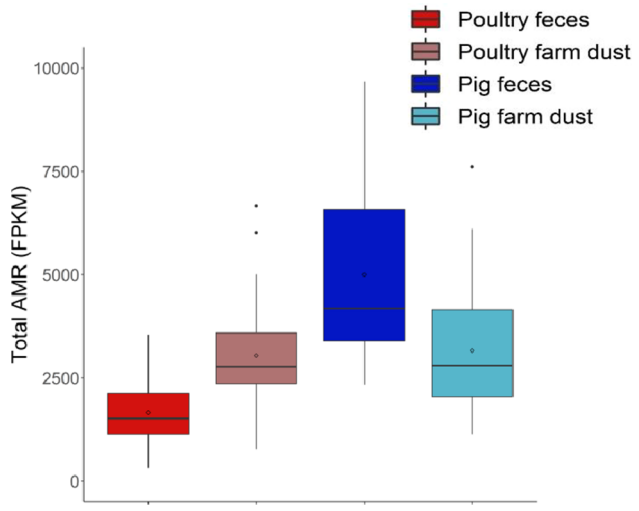

Fig. 1. Total AMR levels of farm dust and animal feces. Boxplots for 35 poultry farms and 43 (feces) or 44 (dust) pig farms from nine countries. The horizontal line in the boxplots depicts the median, the empty circle the mean.

resistomes both cluster closer to feces from their respective species. Pig and poultry dust bacterial microbiomes cluster less distinctly than dust resistomes (Fig. 2), although for both dust resistomes and bacterial microbiomes the variance explained by species is 25% ($p < 0.05$, beta-dispersion $p > 0.05$).

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

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

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

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

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

3.3. The relation between farm dust, animal feces and farmers

Human stool of either poultry and pig farmers or controls have less abundant resistomes compared to farm dust and animal feces (Fig. 5).

Clear clusters consisting of farm dust and animal feces per animal species and a clear human cluster which included all farmers and controls were observed using NMDS analysis (Fig. 6).

The bacterial microbiome composition of farmers, human controls, animal feces and farm dust shows less distinct clustering, in particular poultry and pig farm dust (Fig. 6). Bacterial microbiomes of the

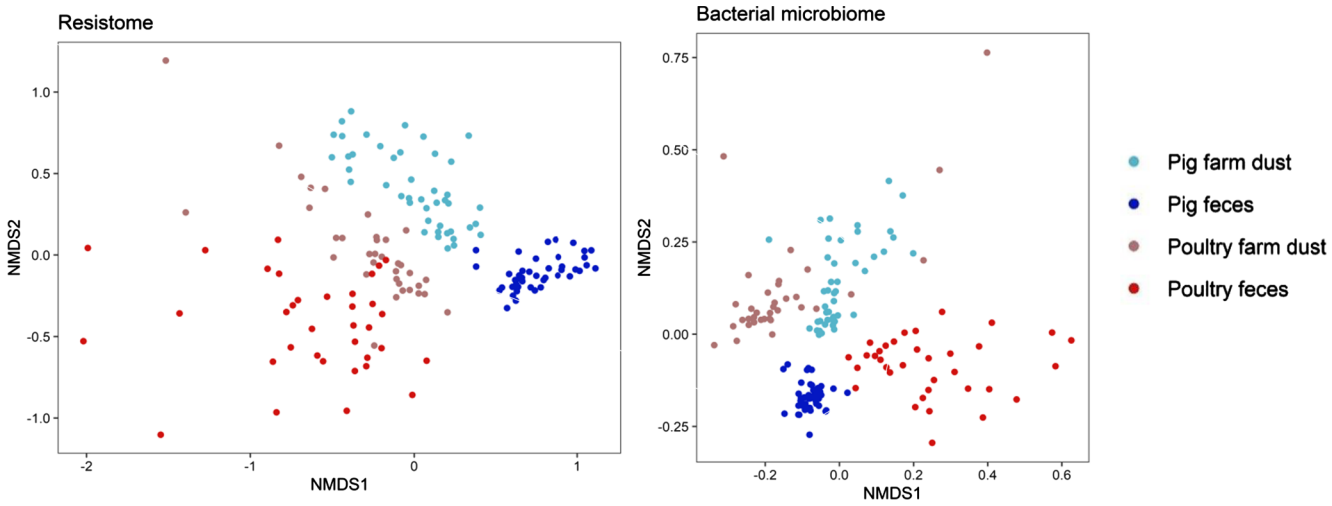

Fig. 2. Compositional differences of the resistomes and bacterial microbiomes of farm dust and animal feces. NMDS plots of 35 poultry and 44 pig farms from nine countries. PERMANOVA results for comparison of dust and feces; Resistome: poultry, R^2 = 0.19, p = < 0.001, beta dispersion p = 0.012. Pig,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

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

Correlation (Procrustes) analyses showed low to moderate correlations between farm (dust and animal feces) and farmers' stool resistome and bacterial microbiome compositions within each farm type; however they were not significant (Table 1C).

The majority of all resistance genes was found to be shared between animal, human and environmental samples, all from one country (Fig. 7). For these analyses, one farmer was included per farm that had most working hours per week in the stables. Dust had the highest number of different resistance genes (i.e. highest richness) and the largest 'unique gene pool': of all dust resistance genes 20% (38/186, poultry) and 26% (49/186, pig) were not found in other sample types included in the study. These unique dust genes code for resistance to a variety of AM classes and have a moderate to low abundance. Examples consist of the *cfr* gene, coding for multi-resistance, and the *blaBRO* gene, coding for beta-lactam resistance, which were measured in dust but not in pig feces nor farmers' or controls' stool (Supplementary data 2).

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

4. Discussion

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

Table 1
Results of Procrustes correlation analysis.

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

Table displays the symmetric Procrustes correlation coefficient (cor), significance level (p-value), the number of countries and farms included in the analysis (n countries, n farms). Bold results have a p-value below 0.05.

* Correlations are plotted in Fig. 3.

^ Stress nearly zero, probably due to a too small sample size.

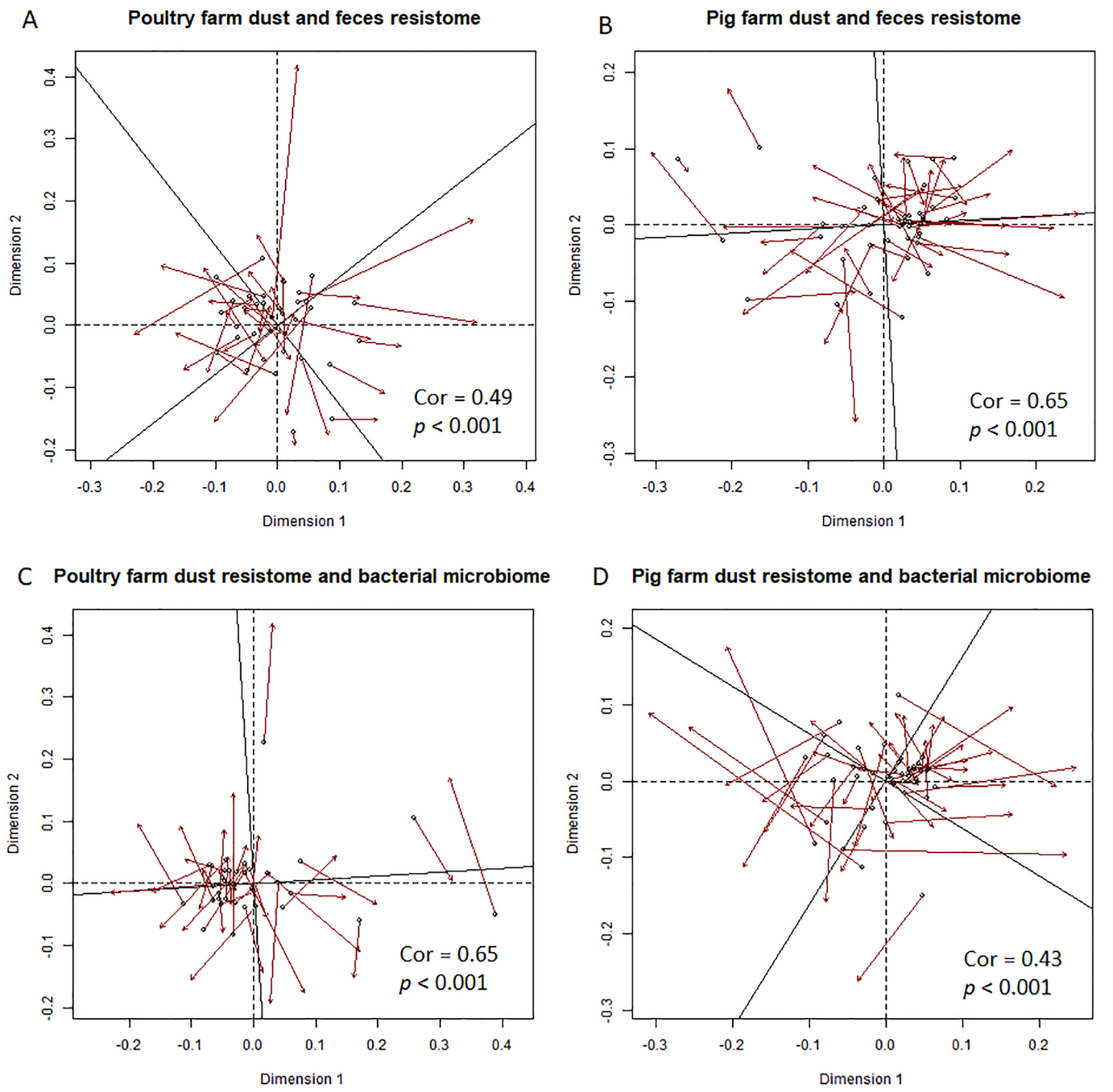

Fig. 3. Correlation between fecal and dust resistome and between dust bacterial microbiome and resistome. Upper: Superimposition plots of Procrustes correlation of feces and farm dust resistomes of poultry (A) and pig (B) farms. Arrowheads point towards the dust ordination. Lower: Superimposition plots of Procrustes correlation of dust bacterial microbiomes and resistomes of poultry (C) and pig (D) farms. Arrowheads point towards the resistome ordination. Corresponding Procrustes error plots in supplemental Fig. 5. Corresponding coefficients in the boxed text and Table 1.

4.1. Farm dust and its relation with feces

To the best of our knowledge, the farm air resistome has only been studied by Yang et al. (2018) and Li et al. (2019), showing that airborne dust in Chinese pig and chicken (laying hens) farms has a high diversity of ARGs compared to a waste water treatment plant, hospital or urban areas. In agreement with these findings, the dust resistome in this study was also found to have the largest richness of ARGs of all sample types (Supplemental Fig. 3). Both pig and poultry farm dust showed 186 different ARGs (after rarefaction), twice as many as farmers' stool and pig feces (results for one country). Part of the dust resistome probably originates from animal feces: 63% and 73% of dust-borne resistance genes are also detected in animal feces from their respective pig and poultry farms (Fig. 7). Also, patterns of fecal and dust resistomes between farms are significantly correlated for both poultry and pig farms (Table 1). Correlation analysis does not inform on directionality of associations, however it is likely that the fecal resistome determines the dust resistome because aerosolization of dried feces results in airborne dust (Cambra-López et al. 2011, Winkel 2016). In turn, dust exposure might also alter animal fecal resistomes.

On the other hand, the higher resistome richness and the large group of non-overlapping ARGs in dust suggest a substantial contribution of microbial sources other than animal feces. Animals and their non-fecal microbiota, such as bacteria stemming from skin, saliva, hairs

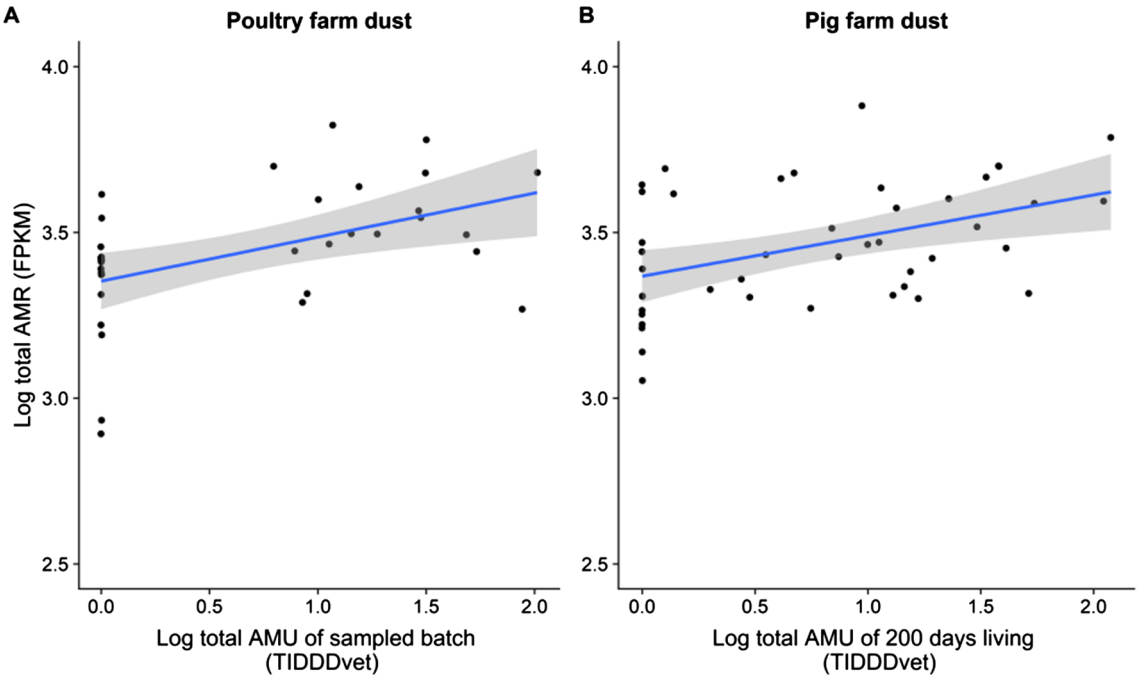

Fig. 4. Relation between total AMU and total AMR in farm dust. A) Scatterplot for poultry farm dust. AMU = total group treatments of sampled chickens, coefficient = 0.13, p = 0.004 in non-adjusted model. B) Scatterplot for pig farm dust. AMU = total group treatments of pigs in standardized 200 days of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

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

The abundant bacterial classes seen in our pig and poultry farm dust samples are consistent with previous studies on farm air, although with a different distribution (Mbareche et al. 2019, Vestergaard et al. 2018, Yang et al. 2018). The significant correlation for both animal species between the dust bacterial microbiome and resistome indicates that the composition of a dust bacterial microbiome mediates the composition of the resistome. The same has been shown in other environments such

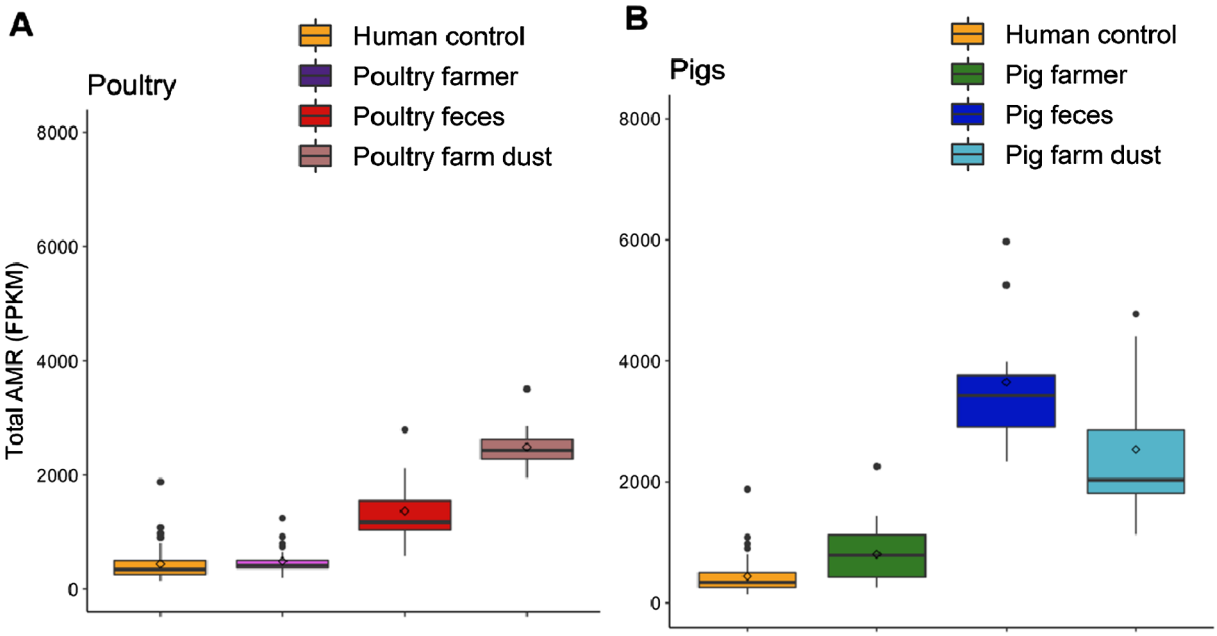

Fig. 5. Total AMR levels of farm dust, animals, farmers and controls. Boxplots for 12 poultry farms (A) and 19 pig farms (B) from one country (the Netherlands). The horizontal line in the boxplots depicts the median, the empty circle the mean.

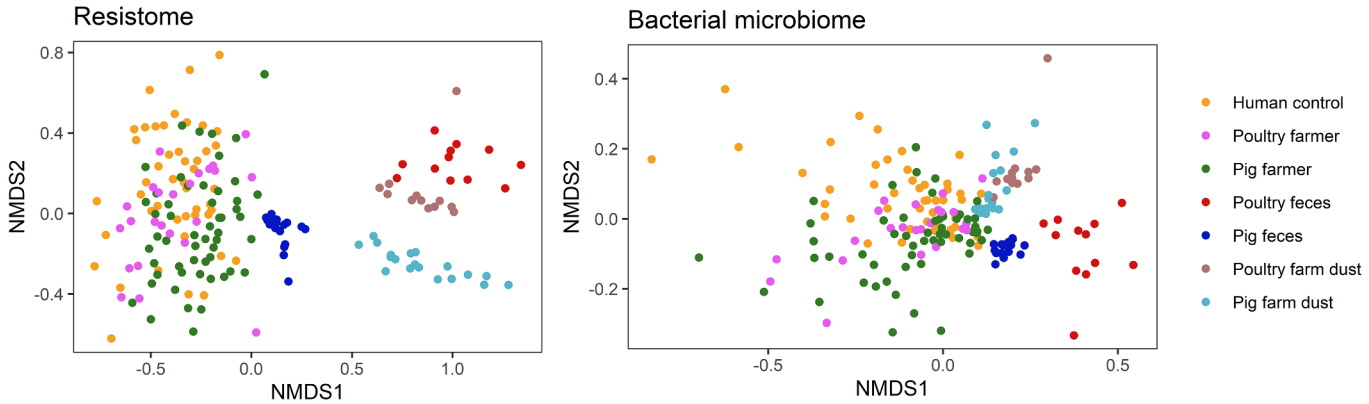

Fig. 6. Compositional differences of the resistomes and bacterial microbiomes of farm dust, animals, farmers and controls. NMDS plots of all samples from the 12 Dutch poultry and 19 Dutch pig farms plus 46 human controls.

as pig and poultry feces, human stool and soils (Forsberg et al. 2014, Munk et al. 2018, Pehrsson et al. 2016).

4.2. The role of antimicrobial usage for resistance in dust

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

4.3. The relation between farm and farmers' resistomes

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

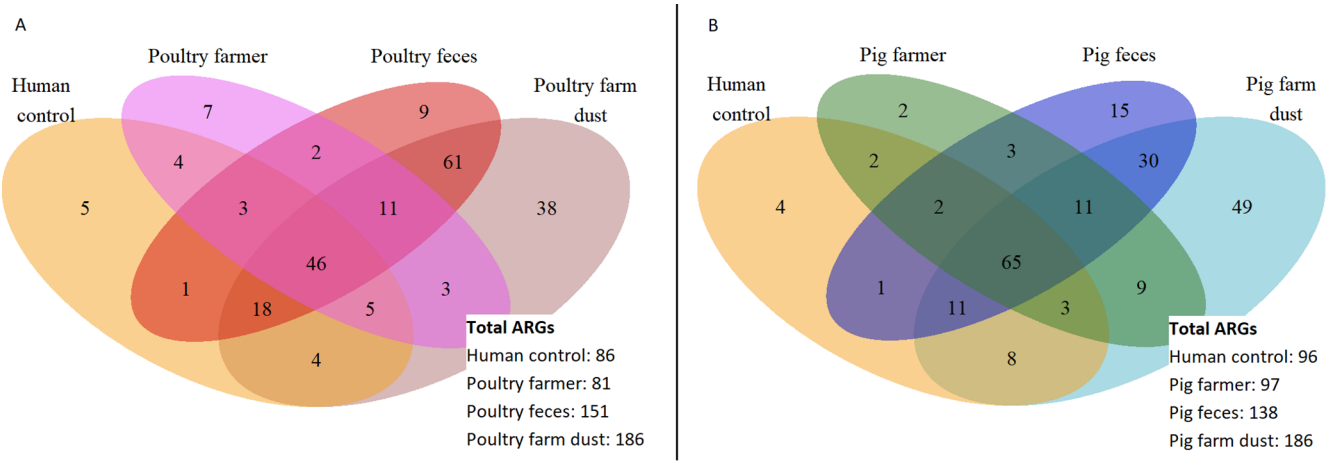

Fig. 7. Overlap in resistomes of the different farm sources and human controls. A – Venn-diagram of 11 Dutch poultry farms, including 11 controls. B – Venn-diagram of 18 Dutch pig farms, including 18 controls. Supplementary data 2 lists the individual resistance genes per sample type per animal species.

4.4. Study limitations

This study is unique in combining high quality data from three different reservoirs, two animal species and nine countries. The inevitable consequence consists of differences in sample processing and DNA extraction between reservoirs, and a relatively small sample size. Confirmation of the overlap and differences we observe between the different farm reservoirs is therefore needed. The bacterial hits seen in blanks can be related by several factors, we however find it most likely to be related to a small degree of cross-contamination during freeze drying, results of the samples were therefore not corrected. While human health hazards are expected to be predominantly determined by the presence of combinations of ARGs in specific pathogens (Bengtsson-Palme et al. 2018), we investigated only ARG distributions. With the short read sequencing methods applied, it was not feasible to determine the bacterial context of ARGs nor their relation with mobile genetic elements which could facilitate their spread between species (von Wintersdorff et al. 2016). Arguably, the transmission of genes and bacteria between different hosts and the environment is complex and therefore difficult to disentangle in a cross-sectional design. To better understand transmission of genes between hosts and environmental reservoirs, a longitudinal design with greater sample size is preferred, and/or other methods like Whole Genome Sequencing of bacterial isolates or long read sequencing can shed light on transmission and the role of mobile genetic elements for resistance gene mobility.

4.5. Conclusions

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

CRediT authorship contribution statement

Roosmarijn E.C. Luiken: Investigation, Data/statistical analysis, Writing - original draft, Writing - review & editing. **Liese Van Gompel:** Investigation, Writing - review & editing. **Alex Bossers:** Data curation, Writing - review & editing. **Patrick Munk:** Data curation, Writing - review & editing. **Philip Joosten:** Data curation, Writing - review & editing. **Rasmus Borup Hansen:** Software, Data curation, Writing - review & editing. **Berith E. Knudsen:** Investigation, Resources, Writing - review & editing. **Silvia García-Cobos:** Investigation, Writing - review & editing. **Jeroen Dewulf:** Conceptualization, Writing - review & editing. **Frank M. Aarestrup:** Conceptualization, Writing - review & editing. **Jaap A. Wagenaar:** Conceptualization, Project administration, Writing - review & editing. **Lidwien A.M. Smit:** Conceptualization, Supervision, Writing - review & editing. **Dik J. Mevius:** Conceptualization, Supervision, Writing - review & editing. **Dick J.J. Heederik:** Conceptualization, Supervision, Writing - review & editing. **Heike Schmitt:** Conceptualization, Writing - original draft, Supervision, Writing - review & editing. **:** .

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Shot-gun metagenomic DNA sequence reads of the dust samples are deposited at National Center for Biotechnology Information (NCBI) under BioProject number: PRJNA623064. The reads of the animal fecal samples are deposited at the European Bioinformatics Institute Nucleotide Archive (ENA) under accession number: PRJEB22062. Original farm dust resistome abundance data (based 90% ID clusters) expressed as FPKM can be found in Supplementary data 2.

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105971.

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