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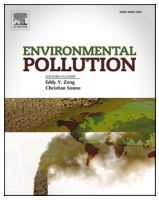

Spatial and temporal distribution of endotoxins, antibiotic resistance genes and mobile genetic elements in the air of a dairy farm in Germany☆

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ABSTRACT

Antimicrobial resistance (AMR) is a serious issue that is continuously growing and spreading, leading to a dwindling number of effective treatments for infections that were easily treatable with antibiotics in the past. Animal farms are a major hotspot for AMR, where antimicrobials are often overused, misused, and abused, in addition to overcrowding of animals.

In this study, we investigated the risk of AMR transmission from a farm to nearby residential areas by examining the overall occurrence of endotoxins, antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs) in the air of a cattle farm. We assessed various factors, including the season and year, day and nighttime, and different locations within the farm building and its vicinity.

The most abundant ARGs detected were *tetW*, *aadA1,* and *sul2*, genes that encode for resistances towards antibiotics commonly used in veterinary medicine. While there was a clear concentration gradient for endotoxin from the middle of the farm building to the outside areas, the abundance of ARGs and MGEs was relatively uniform among all locations within the farm and its vicinity. This suggests that endotoxins preferentially accumulated in the coarse particle fraction, which deposited quickly, as opposed to the ARGs and MGEs, which might concentrate in the fine particle fraction and remain longer in the aerosol phase. The occurrence of the same genes found in the air samples and in the manure indicated that ARGs and MGEs in the air mostly originated from the cows, continuously being released from the manure to the air.

Although our atmospheric dispersion model indicated a relatively low risk for nearby residential areas, farm workers might be at greater risk of getting infected with resistant bacteria and experiencing overall respiratory tract issues due to continuous exposure to elevated concentrations of endotoxins, ARGs and MGEs in the air of the farm.

1. Introduction

Antimicrobial Resistance (AMR) is a critical global health issue that is rapidly increasing worldwide. Currently, 700,000 people die every year due to AMR, and this number is projected to reach 10 million by 2050 if no action is taken (O'Neill, 2016). With high amounts of antibiotics being used in the human and veterinarian medicine, bacteria are developing increasing resistance to these drugs, and new antibiotic resistance genes (ARGs) are being discovered regularly (Hall, 2004). To date, there are more than 10,000 known ARGs, with this number rapidly expanding (Liu and Pop, 2009).

One of the major reasons for the rapid spread of AMR is the exchange of genetic material among bacteria through vertical and horizontal gene transfer (VGT and HGT) (Vikesland et al., 2019; Shi et al., 2022; Shao et al., 2018; Nguyen et al., 2021). VGT involves the inheritance of genetic material from one generation to the next, while HGT refers to the transfer of genetic material from one bacterium to another through transformation, transduction and conjugation. These mechanisms allow bacteria to quickly adapt to new environments, facilitating a swift exchange of ARGs between bacteria and ultimately leading to a rapid spread of ARGs within and between bacterial species. Mobile genetic elements (MGE), such as transposons, integrons, and plasmids, play a significant role in such HGTs and are closely associated with ARG occurrence (Gillings et al., 2015; Ma et al., 2017; Levy et al., 1976; Kruse

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and Sørum, 1994). The presence of ARGs on plasmids often makes bacteria multi-resistant, as multiple ARGs can be found on a single plasmid, resulting in resistance against multiple antibiotics (Alekshun and Levy, 2007; Zhang et al., 2011; Mathers et al., 2015; Carattoli, 2013; Martínez-Martínez et al., 1998).

Hotspots for AMR include hospitals, (dairy) farms, and waste-water-treatment plants (WWTPs). Numerous studies have reported high concentrations of ARGs and AMR bacteria in these locations (Gwenzi et al., 2021; Li et al., 2019; Maharia and Srivastava, 2020; Gilbert et al., 2010; Mao et al., 2015; Xin et al., 2022; Wu et al., 2022; Zhou et al., 2021; Song et al., 2021; Bai et al., 2022; Sancheza et al., 2016; Zieliński et al., 2021; Wang et al., 2022). For example, Li et al. (2019) found 177 different ARGs in air-conditioning systems in hospitals, farms, and residences. Even less prominent ARGs, such as OXA-type genes, are more commonly found in hospitals as well (Gwenzi et al., 2021). These hotspots not only harbor a high abundance of AMR but also act as emission sources, spreading AMR to wider populations. Farms, especially dairy farms, are common hotspots for AMR due to high usage of antibiotics in animals (Xin et al., 2022; Song et al., 2021; Wang et al., 2023). Antibiotics are not only used to treat infections in sick animals but are also used in large quantities for growth promotion. The use of antibiotics as animal growth promotors was permitted in the EU until 2006 and is still allowed in many places worldwide today (Castanon, 2007). Still around 70% of all antibiotics sold globally are used for animals, including for growth promotion (Van Boeckel et al., 2019). For instance, China uses 162,000 tons of antibiotics annually, nearly half of which is used for animals (Song et al., 2021).

ARGs can accumulate in the manure at farms and subsequently contaminate the nearby soil; however, the aerial route should not be neglected. According to Xin et al. (2022), animals consume 3 times more antibiotics than humans. Combined with livestock crowding, animal farms become the perfect enrichment vessel for AMR bacteria that can cause negative health effects to people living nearby and farm workers who spend extended periods on the farm. Studies have shown that living in livestock-dense areas is associated with several negative health effects, such as increased respiratory symptoms (wheezing, cough) and decreased lung function (Bai et al., 2022; Ding et al., 2022; Chang et al., 2001; Gao et al., 2022; de Rooij et al., 2019). This highlights that airborne transmission from livestock farms poses a significant concern for the public health and warrants further investigation.

Another important health risk factor is endotoxins. Endotoxins are parts of the cell wall of gram-negative bacteria, such as *Pseudomonadaceae* or *Enterobacteriaceae,* and are known to cause a number of respiratory tract health issues in exposed individuals (Liebers et al., 2008). Many studies have found high concentrations of endotoxins in farm environments, but their health effects on farm workers and nearby residents are still inconclusive (de Rooij et al., 2019; Rask-Andersen et al., 1989; Basinas et al., 2015). In 1998, a Dutch committee suggested that the exposure level of 50 EU/m^3 should not be exceeded; however, such guidelines were never implemented, and more recent suggestions propose a maximal exposure level of 90 EU/m^3 (Liebers et al., 2020; Sykes et al., 2011). Research toward establishing a clear guideline is still ongoing.

In this study, we investigated the aerial transportation of endotoxins, ARGs, and MGEs from a dairy farm in Germany and created an atmospheric dispersion model to estimate the risk of endotoxins, ARGs, and MGEs being transported via the air to nearby residential areas. The farm was chosen for this study due to its remote location on a plateau with high wind occurrence throughout the year and a small town nearby that could be affected by the endotoxins, ARGs, and MGEs dispersed from the farm. It is a medium-sized dairy farm with 90 cattle and therefore represents an average German dairy farm (the average being 72 cows per farm in Germany in 2022) (Statista, 2022).

2. Material and methods

2.1. Sample collection

To compare seasonal and annual differences of endotoxins, ARGs, and MGEs in the air at a dairy farm and to understand their risks by getting transported from the farm to the nearby surroundings through the air, air samples were collected at a medium-sized dairy farm in Germany during two time periods in 2019 (summer and winter) and one time period in 2021 (summer). The farm houses 90 cows and is located on a plateau near a small town with high wind speeds throughout the year.

Two types of air samplers were used during the sampling periods: a medium-volume air sampler (100 L/min, LY 2050, Qingdao, China) and a high-volume air sampler (1000 L/min, HighBioTrap, Dinglan Tech., Beijing). The medium-volume air sampler collected total suspended particles (TSP) on quartz filters for 24 h (h), except during the summer of 2019 when TSP samples were collected for 8 h during the day and 16 h during the night separately. This variation in sampling time was due to the cows being outside during part of the day and inside during the night. The high-volume air sampler collected particles with aerodynamic diameters less than 2.5 μm ($PM_{2.5}$) and was only used during the winter. It collected samples in mineral oil for 30 min (min) in duplicates each day of sampling. The use of mineral oil allowed for the immersion of the particles into the oil and ensuring the stability of the particles and DNA until reaching the laboratory for further analysis. A detailed summary of the sampling conditions can be found in Table S1.

The farm was naturally ventilated through an open wall side, which was covered with a tarpaulin during the winter and remained open during the summer.

During each day of sampling, the air samplers were placed at different locations inside and around the farm, which were categorized as “Inside”, “Near Window” and “Outside” (S2).

Additionally, manure samples were taken during each time period from various parts of the ground inside the farm.

All samples were kept in −20 °C until further analysis.

2.2. Endotoxin test

The endotoxin test was conducted on all samples to assess the overall burden of gram-negative bacteria in the air at the farm and to evaluate the potential risks associated with airborne endotoxin exposure. For the TSP samples collected on filters, 1/8 of each filter was cut into small pieces, added to 1 mL of double distilled water (ddH_2O) and pretreated with 0.05% Tween 20 by vortexing for 30 min and centrifugation at 10,000g for 1 min. The resulting supernatant was used for the endotoxin test, employing the Chrome-LAL assay from Cape Cod, following the protocol outlined in the method previously published by Yue et al. (2018).

All TSP samples were diluted 1000 times in phosphate-buffered saline (PBS), while the $PM_{2.5}$ samples were diluted 100 times in PBS. The microplate absorbance reader Infinite® M Nano (TECAN, Switzerland) was used for the measurements, and the associated software Magellan (TECAN, Switzerland) was utelized for data analysis and calculation of the endotoxin concentrations.

2.3. DNA extraction

DNA was extracted by using the DNeasy PowerSoil Kit (Qiagen, Germany), in combination with AMPure magnetic beads (Beckman Coulter, USA).

For the DNA extraction from the filters, 1/6 of each filter was cut into small pieces and directly added to the PowerBead tubes. As for the manure samples, 250 mg of manure was added to the PowerBead tubes. To extract the DNA from the mineral oil containing the bacteria and DNA, 1 mL of ddH_2O was added to the mineral oil to separate the

bacteria, DNA, and water from the oil through centrifugation. The DNA was collected in a final volume of 60 μL of ddH_2O. The final DNA concentrations were measured using the microplate absorbance reader Infinite® M Nano (TECAN, Switzerland).

Subsequently, all DNA samples were stored at −20 °C until further use.

2.4. Real-time PCR assays for detection of ARGs

A real-time PCR assay (qPCR) was employed to detect 17 different ARG subtypes that confer resistance to 7 types of commonly used antibiotics, as well as two MGEs and two reference bacterial strains. The primer sets used for the qPCR were previously published by Gao et al. (2018) and Tao et al. (2021). The selection of the tested genes was based on previous research of ARGs and the common practices of antibiotic usage at farms and in order to cover resistance genes to the most common antibiotic classes.

The qPCR analyses were conducted on a BioRad system in a total volume of 20 μL, using SYBR Green. The settings for the qPCR were based on the protocols taken from Gao et al. (2018) and Tao et al. (2021), and the threshold cycle (CT) of 40 cycles was used as the detection limit. Detailed information on all primers and cycling conditions can be found in S3 and S4.

To calculate the relative abundance (R) of the different ARGs, the 16S gene was used as a reference, following equations (1) and (2):

$$R = 2^{-\Delta CT} \tag{1}$$

$$\Delta CT = CT_{ARG} - CT_{16SrRNA} \tag{2}$$

Here, CT_{ARG} and $CT_{16SrRNA}$ represent the threshold cycles for the ARGs and 16S rRNA genes, respectively (Livak et al.). To obtain absolute abundance values for the 16S gene, plasmids with known concentrations of the 16S were used. These plasmids were created from *E. coli* JM109.

2.5. Bacterial 16S rRNA gene sequencing

To investigate the bacterial diversity in the air of the farm, the DNA extracts of all samples collected during the winter of 2019 and of all samples from the summer of 2019 were pooled respectively. This pooling was done to achieve a sufficiently high DNA concentration for performing 16S rRNA bacterial amplicon sequencing using the Illumina MiSeq 2x300bp sequencing platform (Novogene, UK). A sequencing depth of 20,000 reads per sample was targeted.

Similarly, DNA extracts from the manure samples collected during the winter and summer were analyzed using the same approach as for the air samples. This allowed for study of similarities and differences in the bacterial community and diversity between the manure of the cows and the air at the farm.

The V3–V4 region of the 16S rRNA gene was selected for amplification in order to obtain the necessary data for the analysis (see S5). Preparation of the library, PCR, sequencing and bioinformatical analyses were performed by Novogene, UK.

2.6. Statistical analysis

All graphs and statistical test were generated using GraphPad Prism. To assess statistical significance, multiple unpaired t-tests were performed with a significance threshold set at $p \leq 0.05$.

2.7. Dispersion model of endotoxins, ARGs and MGEs

The Graz-Lagrangian model software (GRAL) was used to create a dispersion model of the five most prevalent ARGs, the two MGEs and endotoxins in the air of the farm. The model covered one year (Oct 2018–Sep 2019) and one month (Aug 2021).

To calculate the mean emission rates (E_M) of ARGs, MGEs, and endotoxins from the farm, the following equation was used:

$$E_M = C_M * V_R \tag{3}$$

where C_M is the mean concentration of the specific ARG, MGE, or endotoxin in the summer and winter period respectively, considering all samples from inside the farm and near the windows. V_R represents the ventilation rate for a naturally ventilated farm building, estimated to be 1000 m^3/h per livestock unit (LU) (Fiedler and Müller, 2011).

Meteorological data for the entire year were obtained from U.S. National Oceanic and Atmospheric Administration (NOAA) National Centers for Environmental Prediction (NCEP) Global Forecast System (GFS) (Commerce NC for EPWSSD of and Comm erce NC for EPWSSD of, 2015). During the sampling periods, meteorological data were collected from a mobile weather station installed onsite at the farm. These on-site data were consistent with meteorological data from NOAA. The variables taken into consideration were temperature (indoor, outdoor), relative humidity (indoor, outdoor), wind speed, wind direction, and pressure. Stability classes were calculated using the Pasquil-Gifford-Turner method (The Estimation of The Dispersion, 2023).

To validate and compare the simulation results with measured values at the farm and its surroundings, six receptor points were placed within the simulation domain. These receptor points were located close to the farm, at the nearby factory (NW), at nearby fields (SW), and three points in the nearby town (SE) (in the southern, central and northern part of the town) (see S6).

3. Results & discussion

3.1. Endotoxin concentration

The measured endotoxin concentrations across all analyzed samples ranged from 0.0003 EU/m^3 in a $PM_{2.5}$ sample taken downwind outdoors during winter 2019 to 270 EU/m^3 in a TSP sample taken inside the farm during summer 2019. These results are consistent with findings from other published studies conducted at farms, which also reported endotoxin concentrations ranging from 0.1 EU/m^3 to more than 2000 EU/m^3 (Rolph et al., 2018; Schulze et al.). These values from areas with intensive livestock production are significantly higher when compared to urban areas, indicating that farms play a major role in ambient endotoxin occurrence.

The study's results clearly demonstrate that farms are substantial contributors to the presence of endotoxins in the environment, and the observed concentrations highlight the potential health risks associated with exposure to endotoxins in areas with intensive livestock production.

3.1.1. Endotoxins in TSP vs $PM_{2.5}$

In the winter of 2019, both TSP and $PM_{2.5}$ samples were collected, and a comparison of the results was performed. The endotoxin concentration in the $PM_{2.5}$ samples was significantly lower than in the TSP samples, with mean concentrations of 0.23 EU/m^3 and 17.56 EU/m^3, respectively (Fig. 1a). This indicates that the endotoxin concentration in the $PM_{2.5}$ fraction only accounted for approximately 1.3% of the endotoxin concentration in the TSP fraction.

While one study by Golbabaei and Islami (2000) found higher endotoxin concentrations in the finer particle fraction ($PM_{2.5}$), suggesting that endotoxin tends to stick to finer particles, most studies on similar topics that compared PM_{10} and $PM_{2.5}$ have reported higher endotoxin concentrations in the coarse fraction (PM_{10}) compared to the fine particle fraction ($PM_{2.5}$). The studies have reported minimum concentrations of 6.25 EU/m^3 in $PM_{2.5}$ and maximal concentrations of 125 EU/m^3 in PM_{10} (Maharia and Srivastava, 2020; Yue et al., 2018; Madsen and Nielsen, 2010; Degobbi et al., 2011; Heinrich et al., 2003).

Although the concentration of endotoxin per unit of PM mass was not

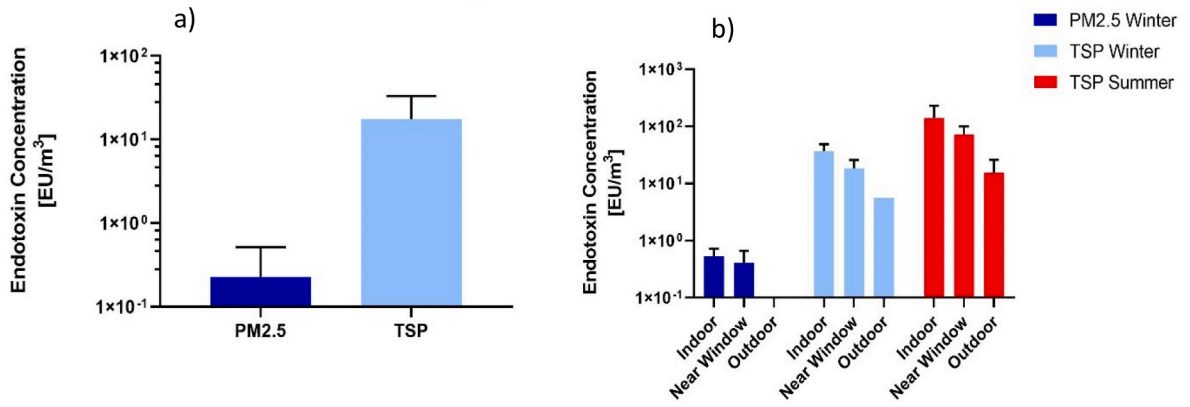

Fig. 1. Comparison of endotoxin concentrations in the TSP and $PM_{2.5}$ a) overall and b) in different seasons of 2019 and at different locations at the farm (indoor, near window, outdoor); error bars indicate the standard deviation.

calculated in this study, it was indirectly concluded that endotoxins at this farm were found in higher concentrations in larger particles than in finer ones, and the endotoxins likely deposited more quickly. This observation aligns with most other published studies on the topic of endotoxin concentrations in particulate matter. However, the finding that a small fraction of endotoxin was still present in $PM_{2.5}$ suggests that some endotoxin can be transported further and may pose health risks to people living in nearby towns. This aspect requires further investigation in future studies.

3.1.2. Concentration gradient of endotoxins

The study found that the endotoxin concentration decreased from the inside of the farm building to the area near the window by about 50%, and decreased even further from there to the outside by about 75%. This trend was observed in both sample types ($PM_{2.5}$ and TSP) (Fig. 1b).

A closer examination of the TSP samples revealed that the highest concentration of endotoxin was found in the middle of the farm building (up to 270 EU/m^3), and the concentration gradually decreased moving towards the windows on both sides of the farm building (Fig. 2). This is of significant importance, as the endotoxin concentration in the summer samples exceeded the suggested occupational health threshold at the workplace of around 90 EU/m^3, posing a health risk to the workers who spend extended time inside the farm building (Liebers et al., 2020). However, the endotoxin concentrations in the outdoor samples, approximately 25 m downwind of the farm building and approximately 8 m upwind, were close to 0, indicating a low health risk for people in the nearby town. The lower values outside can be attributed to the fact that endotoxins were mostly found in the TSP fraction and deposited quickly, with fewer endotoxins found in the fine particle fraction $PM_{2.5}$.

The night samples from the summer of 2019 had slightly higher endotoxin concentrations upwind, while the daytime samples had higher concentrations downwind (Fig. 2b). This shift in concentrations can be explained by the cows spending their daytime outside and the nighttime inside the building. While other studies have shown similarly lower concentrations at up- and downwind outside areas near farms (Rolph et al., 2018), our study is the first to demonstrate this kind of concentration shift based on the location of cows inside and outside of the farm building, indicating that the cows are a major source of aerial endotoxin pollution at dairy farms.

Comparing the endotoxin concentrations in the summer and winter samples, a similar concentrations gradient was observed, but

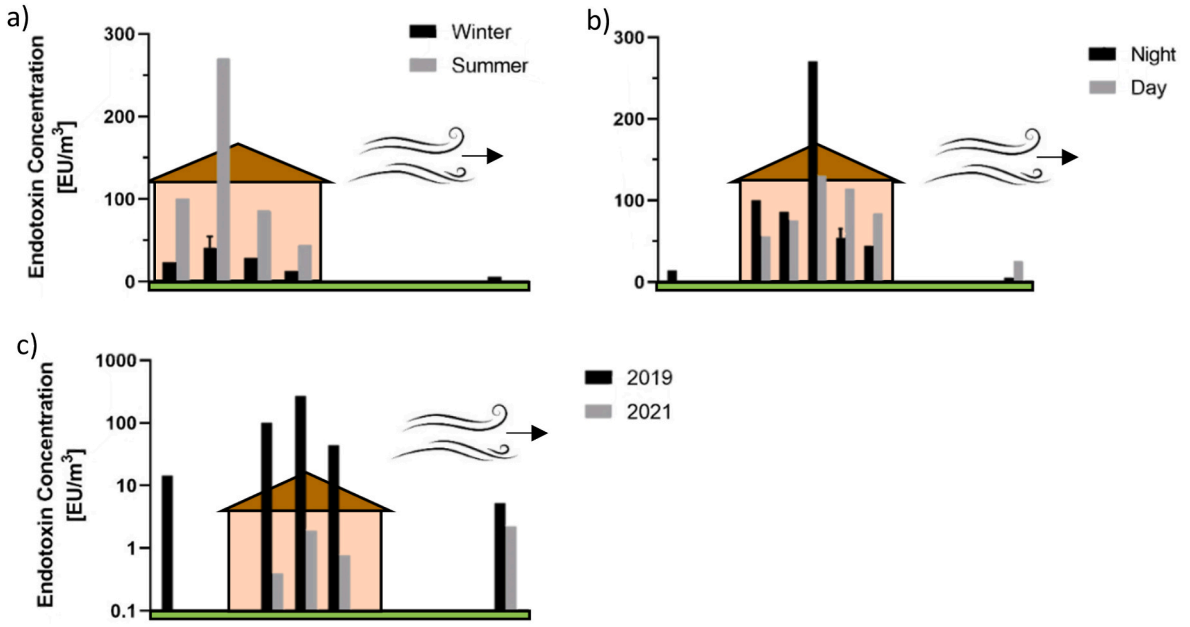

Fig. 2. Endotoxin concentration gradient within the farm building and the surroundings; a) comparing winter & summer, b) comparing night & day, c) comparing summer 2019 & 2021; error bars indicate the standard deviation.

concentrations were significantly higher during the summer, with an increase of about 4-fold (Fig. 2a). This association of higher temperatures and seasonal variation causing higher endotoxin concentrations during the summer has been reported in several other studies (Maharia and Srivastava, 2020; Schulze et al., 2006; Carty et al., 2003). Only one other study demonstrated a lower endotoxin concentration during the summer, despite having a higher total amount of microbes (Purdy et al., 2004).

Comparing the endotoxin concentrations from 2019 to 2021, it can be seen that the trend was similar: the concentrations were the highest inside the farm building and decreased towards the outside (Fig. 2c). However, the concentrations in 2021 were much lower than in 2019. In some samples, the concentration decreased by more than 2 logs. These results can also be explained by higher temperatures, as the climate during the summer of 2019 was warmer than during the summer of 2021. This could lead to a stronger activity of bacterial replication and growth of the amount of microorganisms, subsequently explaining the higher concentrations of endotoxins found in 2019 (Maharia and Srivastava, 2020; Carty et al., 2003; Qiu et al., 2020).

3.2. ARG & MGE abundance

Out of the 17 ARG subtypes that were included in this study, 8 ARG subtypes were detected in air samples from this farm: *aadA1*, *mphA*, *mphA2*, *floR*, *acrA*, *sul2*, *tetG* and *tetW* (S7). This study also detected both MGEs and reference strains, with *intl1* being the most abundant MGE gene found.

Among the detected ARGs, *tetW*, *aadA1* and *sul2* were found with the highest abundance. These genes confer resistance to tetracyclin, aminoglycosides, and sulfonamides, respectively; which are antibiotics commonly used in veterinary medicine (Wang et al., 2016). The farmer confirmed the use of aminoglycoside and fluoroquinolone antibiotics for the treatment of sick animals at the farm. The presence of *aadA1* can be attributed to the use of aminoglycoside; however, fluoroquinolone resistance genes were not detected. Furthermore, although tetracycline commonly used in veterinary medicine and could explain the high abundance of *tetW*, the farmer did not confirm recent usage.

Similar findings have been reported in other studies at cattle farms, where tetracycline, aminoglycoside, and betalactam resistance genes were commonly found (Xin et al., 2022; Song et al., 2021; Wang et al., 2023).

Comparing the relative abundance of the ARGs in the farm building between 2019 and 2021 (summer periods), the same ARGs and MGEs were found, with most genes showing a slight reduction from 2019 to 2021, albeit non-significantly (S7). *Staphylococcus* spp. on the other hand significantly increased in 2021 by more than 2 log. *Staphylococcus* is a common human and animal pathogen often associated with respiratory tract infections (Bai et al., 2022). The increase of *Staphylococcus* spp. abundance in 2021 might indicate an infection wave with that pathogen at the farm during the sampling period in 2021. This finding is of concern, as *Staphylococcus* is a zoonotic pathogen often associated with multi-resistance, leading to difficult-to-treat clinical cases (Xin et al., 2022). An increase of airborne *Staphylococcus*, especially if it becomes multi-resistance, poses a serious threat to farm workers and people living nearby. Other studies have also demonstrated adverse effects on farm workers, such as effects on the oropharynx and gut microbiome (Ding et al., 2022).

3.2.1. Concentration gradient of ARGs and MGEs

Upon closer examination of the summer samples from 2019, it was observed that the concentration of some detected ARGs decreased from the inside of the farm building to the area near the window and further to the outside, as seen for *aadA1*, *sul2,* and *TnpA*/IS26 (Fig. 3). However, for most ARGs and MGEs, there was not a clear concentration gradient, and the differences between locations were statistically non-significant, except for *mphA*2, *floR,* and *TnpA*, which were not detected at all in the outside samples.

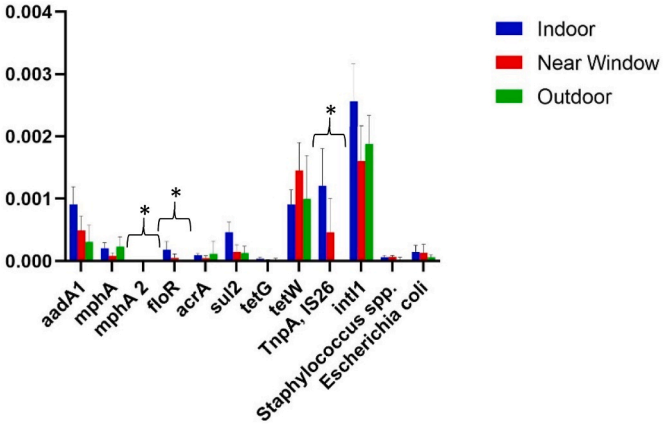

Fig. 3. Comparison of relative abundance of ARGs & MGEs at different locations inside the farm building and its surroundings using the $2^{-\Delta CT}$ method; *: $p \leq 0.05$; error bars indicate the standard deviation.

Overall, there was no universal and clear concentration gradient that applied to all ARGs and MGEs, unlike the clear gradient seen with the endotoxin concentration, and therefore every gene had to be considered individually. One possible explanation for this could be that, according to several studies, ARGs and MGEs are mostly found in the $PM_{2.5}$ fraction. Unlike larger particles like endotoxins, $PM_{2.5}$ can stay in the air for much longer and does not deposit as quickly. As a result, a clear concentration gradient cannot be observed (Zhang et al., 2019; Xie et al., 2019; He et al., 2021).

Another factor to consider is that the cows might be the major but not the only source of ARGs, and therefore, some ARGs might also originate from soil and other environmental bacteria (Noyes et al., 2016). This could contribute to the variability in the distribution of ARGs at different locations within the farm and its surroundings.

Additionally, differences in the degradation speeds of ARGs/MGEs and endotoxins may also play a role in the observed results. These differences in persistence and degradation could further influence the distribution and concentration of ARGs and MGEs in the air.

3.2.2. ARG and MGE abundance in the winter vs summer

Upon comparing the air samples from the winter and summer season of 2019, it was observed that for most genes, the abundance rates were detected at similar levels (S8). This finding contrasts with several other studies that have shown higher abundance of ARGs and MGEs during the winter. Those studies explained the higher abundance during winter by factors such as less ventilation and animals being in a smaller compartment over an extended period, which allows bacterial exchange and enrichment (Song et al., 2021; Wang et al., 2023). However, in this current study, a significant difference between ARG abundance in summer and winter samples was not observed.

In contrast, the relative abundance rates of both MGEs significantly increased in the summer compared to the winter. This increase in MGEs could be explained by the higher temperatures during the summer that allowed for better survival of bacteria. Several studies have shown correlations between higher temperatures and an increase of airborne bacteria and ARGs as well (Wang et al., 2023; MacFadden et al., 2018). They attributed those correlations to the fact that high temperatures facilitate the survival and reproduction of bacteria, leading to an increase in ARGs.

The observed increased endotoxin concentrations during the summer also supports the hypothesis of higher bacterial activity in warmer temperatures. Specifically, *intl1,* mentioned as one of the leading promoters for ARGs spreading in the environment, could facilitate the transmission of genes between bacterial communities. The fact that ARGs were not elevated accordingly might be due to a delayed

observation of ARG transmission by HGT.

3.2.3. ARG and MGE abundance in the day vs night

Comparing the summer samples of 2019 for day and night, it can be seen that for the inside samples, the relative abundance rates of ARGs were rather constant during both the day and night (S9a). For the outside samples, *tetW* was found in higher abundance during the night compared to the day (S9b), although this difference was non-significant. The presence of tracycline resistance genes, such as *tetW*, is commonly observed at farms and is often positively correlated with MGEs like *intl1*. Therefore, it is not uncommon to be found in high abundances at the farm, even though tetracycline was not used recently at this specific farm (Wang et al., 2016; Zhang et al., 2022; Liu et al., 2012).

3.2.4. ARG and MGE abundance in the air vs manure

The finding that all ARGs and MGEs found in the air samples were also present in the manure samples suggest that the cows were indeed the major source of ARGs in the air of the farm. The high abundance of *aadA1*, *sul2,* and *tetW*, as well as *intl1*, in both air and manure samples further supports this idea. In addition, 2 more ARGs were found in manure samples that were not found in the air: *ermA* and *sul1* (Fig. 4). Though the abundance levels of those two ARGs were relatively low.

Relative abundance rates were generally higher in the manure samples taken from the winter compared to summer samples. Attributing factors for this could be that during the winter the cows were inside all the time and shared a much smaller space, facilitating bacterial exchange and better growth conditions for bacteria. The less efficient ventilation during the winter due to the closed wall sidecould also contribute to the higher abundance of ARGs. According to other studies, another explanation could be that during winter months more antibiotics are used on sick animals, which creates a greater environmental pressure of antibiotics and ARGs get selected (Wang et al., 2023). There is however no information about the amount of antibiotics used at this farm.

In general, the relative abundance of ARGs in the manure sample from summer 2021 was in line with the results obtained from the manure sample from summer 2019. The only major difference was that *intl1* was reduced by a factor of 2. Furthermore, *tetG* decreased, while *tetW* increased in abundance from summer 2019 to summer 2021. Similarly to the air sample from 2021, there were significantly more *Staphylococcus* spp. found in the manure sample in 2021 compared to 2019, which strengthened the idea that the cows might have been infected with *Staphylococcus* at that time, which were released from the manure to the air as well.

3.3. Dispersion model

To simulate the dispersion of endotoxins from the farm for one year (2019), the following endotoxin mean emission rates were used: 265.61 μg/h (winter) and 915.42 μg/h (summer).

To similarly simulate the dispersion of ARGs and MGEs, the mean absolute abundance of the three most abundant ARGs and both MGEs from the air samples inside the farm during the summer and winter periods in 2019 were used and can be found in Table S10.

In Fig. 5, it can be seen that the predominant wind direction for 2019 was South-West. Therefore, the concentrations of endotoxin, ARGs, and MGEs were higher in the North-East. Lower concentrations were observed at the nearby factory in the North-West and the town in the South-East. The concentration gradient, however, demonstrated a rapid decrease in concentration as the distance from the farm increased. For example, at the part of the town nearest to the farm, the mean endotoxin concentration already decreased by 99.8% (Fig. 5a). Similarly, ARG and MGE concentrations decreased several folds at the nearest town as well (Fig. 5b).

We used the concentrations of the TSP samples to create these models; however, especially ARGs and MGEs are more frequently found in the finer particle fraction and could potentially be transported much further, causing potential respiratory health risks to people in nearby towns. One study suggested that people living within 500 m of more than 12 animal houses had a 7% lower mean forced expiratory volume in 1 s (FEV1) value compared to a control population with fewer animal houses within 500 m (Rolph et al., 2018). Conducting a similar simulation study with $PM_{2.5}$ samples could provide further information on the dispersion of endotoxin, ARGs, and MGEs and the potential health risk to nearby residential community.

3.4. Bacterial composition

Due to low concentrations of DNA obtained from the air samples, all summer and winter air samples of 2019 were pooled together respectively for sequencing and determining the bacterial diversity. However, the concentration of the pooled winter sample was still too low for sequencing and was not further analyzed.

The top phyla detected in the air sample were: *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* (S11, S12). These results align with findings from other studies conducted at dairy farms (Bai et al., 2022; Macedo et al., 2021; Lopatto et al., 2019).

In the manure samples, the same top phyla were detected, but with less abundance of *Firmicutes* and dominance of *Proteobacteria*. On the

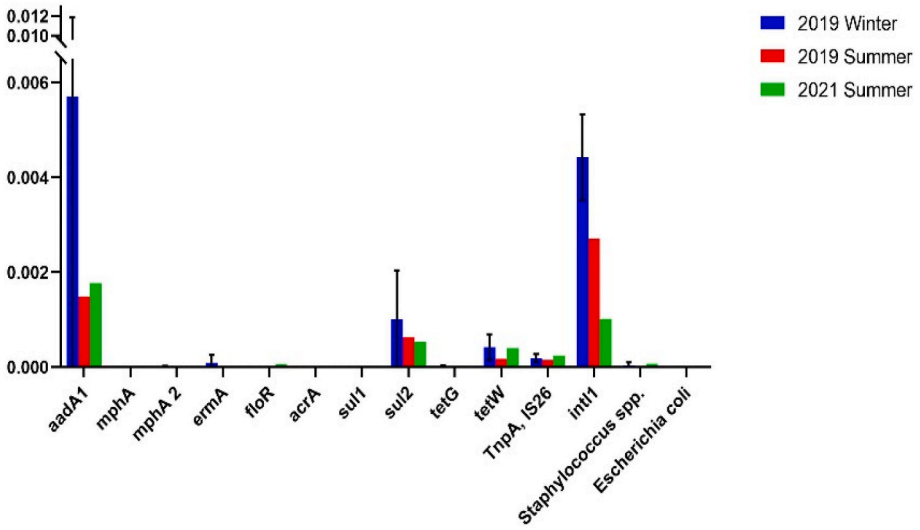

Fig. 4. Relative abundance rates of ARGs and MGEs in the manure samples from 2019 & 2021 using the $2^{-\Delta CT}$ method; error bars indicate the standard deviation.

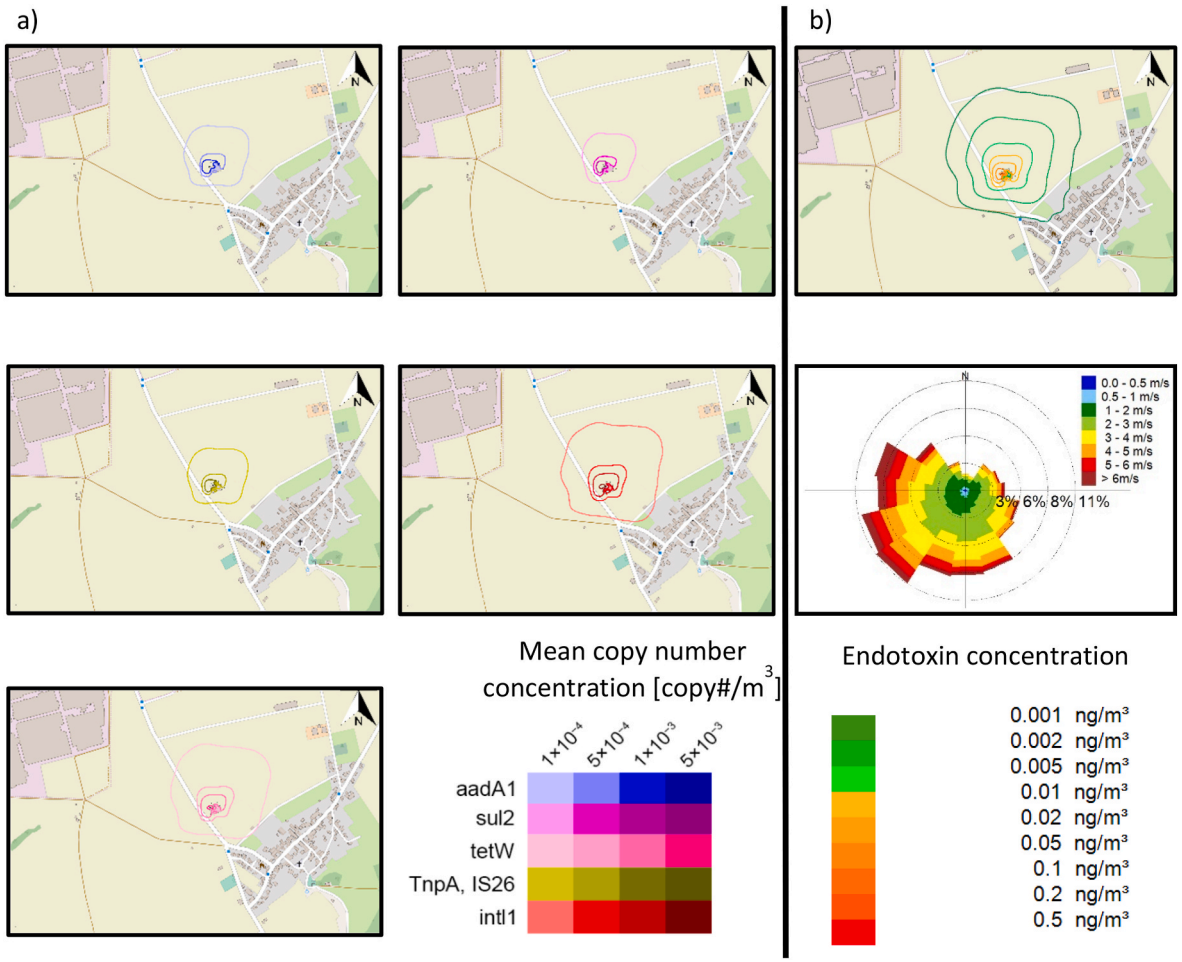

Fig. 5. Dispersion from the farm to the nearby areas of the simulated year Oct 2018–Sep 2019 of a) the mean copy number concentrations of the three most abundant ARGs and both MGE (aadA1, sul2, tetW, TnpA, intl1); b) the mean endotoxin concentration and wind rose.

class level, the air sample showed more diversity than the manure samples with *Gamma-* and *Alphaproteobacteria*, *Clostridia* and *Bacteroidia* being the most abundant.

The most common genera of bacteria found in the air belonged to human and animal gut microbiota, such as *Ruminococcaceae* and *Christensenellaceae,* or to soil bacteria, such as *Solibacillus* and *Dyadobacter* (Fig. 6). However, human and animal pathogens were also found in the air samples, including *Staphylococcus, Bacteroides* and *Acinetobacter*.

The most common genera found in the summer and winter manure samples differed significantly from each other. However, predominantly bacteria belonging to the gut microbiome were found, of which many were pathogenic, such as *Pseudomonas*, *Staphylococcus,* and *Mycoplasma*.

Even though the abundance differed, many human and animal genera found in the manure samples, particularly in the summer, overlapped with pathogenic bacterial genera found in the air samples from the summer season. This suggests that bacteria from the cows can be released into the air and pose significant health concerns for the farm workers. Many of these bacteria are often associated with AMR in the literature, including *Acinetobacter*, *Enterococci*, *Pseudomonas,* and *Staphylococcus*, which further escalates the health risk (Alvarez-Uria and Midde, 2018; Barlow et al., 2017; Coello Pelegrin et al., 2021; Molineri et al., 2021).

4. Conclusion

Our study revealed that endotoxin concentrations and MGEs were higher in the air during the summer compared to winter, likely due to increased bacterial survival and reproduction facilitated by higher temperatures and seasonal variations. However, most ARGs showed similar abundances in both seasons.

We observed a clear concentration gradient of endotoxins based on the location of cows, with higher concentrations inside the farm and lower concentrations outside. In contrast, ARG abundance did not follow such a clear concentration gradient and was relative consistent across different locations. This suggests that endotoxins tend to accumulate in the coarse particle fraction and quickly deposit, while ARGs and MGEs accumulate in the fine particle fraction and remain airborne for longer periods.

The high abundance of *Staphylococcus* in 2021 indicates an infection wave at the farm with this particular zoonotic pathogenic bacterium. However, endotoxin concentrations and the relative abundances of ARGs and MGEs were reduced from 2019 to 2021.

The dispersion models indicated a low risk of transmission towards the nearby town and their residents. However, farm workers might be at higher risk of getting infected with antimicrobial-resistant bacteria and zoonotic pathogens such as *Staphylococcus*. This could potentially hinder proper antibiotic treatment for them.

Credit author statement

Viktoria Agarwal: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Visualization. Yang Yue: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – review &

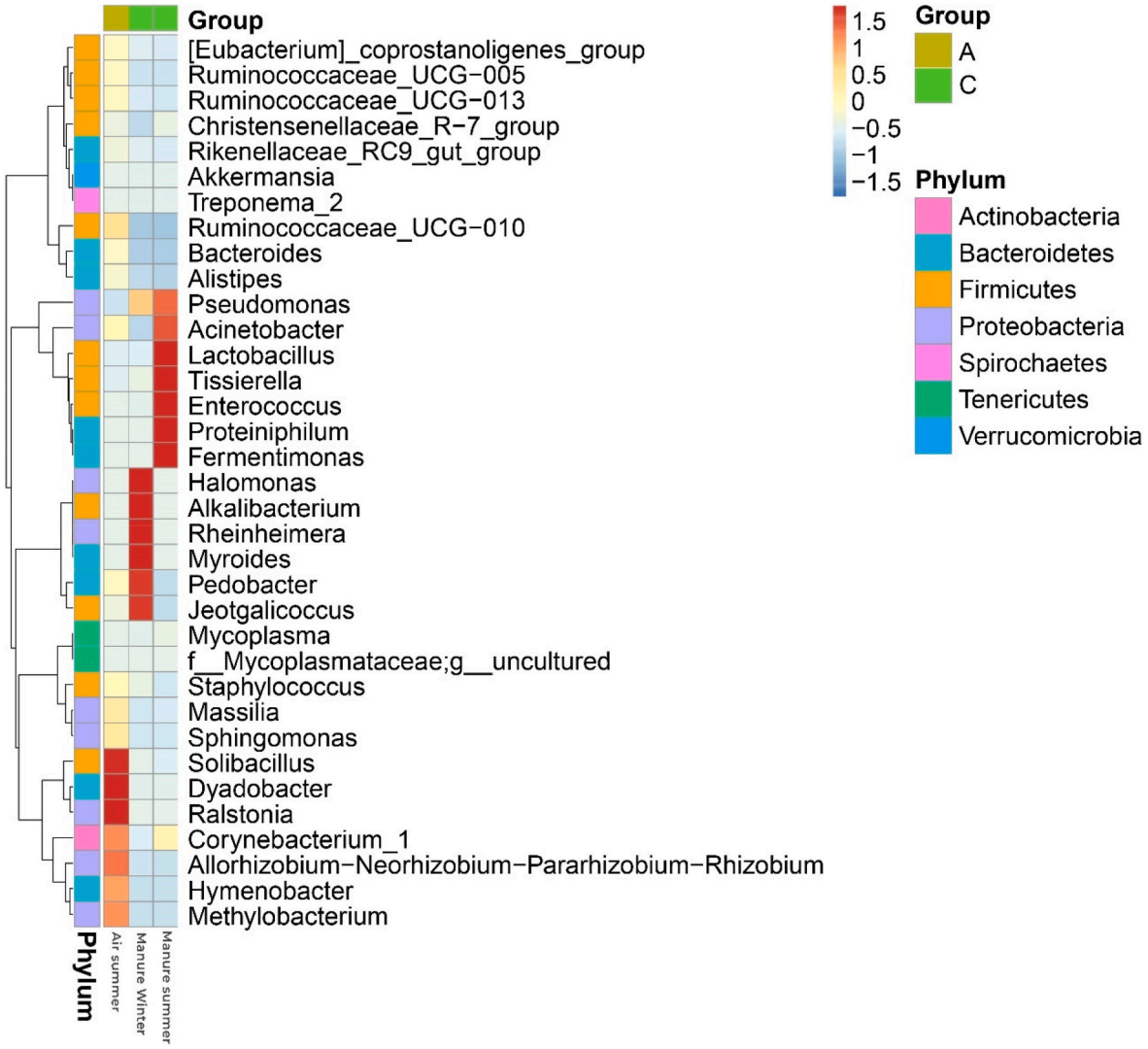

Fig. 6. Heatmap of the top35 genera found in the samples; plotted by sample name on the X-axis and the Y-axis represents the genus; the absolute value of 'z' (color gradient of the heatmap) represents the distance between the raw score and the mean of the standard deviation; 'Z' is negative when the raw score is below the mean, and vice versa.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2023.122404.

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