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Dynamics of antimicrobial resistance in the livestock environment

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Cover: The cover represents the dual nature of this thesis: long hours of data analysis at the laptop balanced by hands-on field and laboratory work. The presence of my pig colleague reflects the farms where this research began and the animals at the heart of the study. The illustration was created using ChatGPT (OpenAI) based on real photographs.

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Abstract

Antimicrobial resistance (AMR) is a major One Health challenge linking human, animal, and environmental health. Livestock production systems play an important role in this context, as they act as reservoirs for antimicrobial resistance genes (ARGs). While antimicrobial use (AMU) is widely recognised as the primary driver of AMR, increasing evidence suggests that AMR dynamics cannot be explained by AMU alone, as environmental and management-related factors may also contribute.

AMR surveillance in livestock relies mainly on phenotypic resistance testing, which captures only a limited fraction of the total resistance reservoir. Shotgun metagenomic sequencing enables comprehensive characterisation of the resistome but has predominantly been applied in high-AMU settings, where strong antibiotic selection may obscure the effects of other drivers. To address this gap, this thesis developed and applied a robust and scalable pipeline for environmental resistome analysis in low-AMU pig farm systems.

The pipeline was optimised by systematically evaluating key methodological steps, including sampling strategy, DNA extraction, sequencing depth and technology, and alternative bioinformatic approaches for assembly and ARG detection. Sock sampling proved to be a practical and standardisable method for farm-level studies, and deep short-read sequencing provided the most sensitive recovery of ARGs in complex farm environments. This workflow was applied in a longitudinal study of ten Swedish farrow-to-finish pig farms followed across a full production cycle.

Despite pronounced temporal shifts in the environmental microbiome, the resistome showed no clear association with AMU, reflecting the low selective pressure in these systems. Resistome diversity and phenotypic resistance in *Escherichia coli* were not correlated, indicating that genotypic and phenotypic metrics capture complementary dimensions of AMR. Different non-antibiotic factors were associated with these outcomes, highlighting the need to integrate metagenomic and phenotypic approaches when studying AMR in livestock environments within a One Health framework. This work provides a methodological foundation for future large-scale, longitudinal resistome studies.

Keywords: One health, pig farm resistome, risk factors, antibiotic, AMR

Antimikrobiell resistensdynamik inom djurproduktionen

Sammanfattning

Antimikrobiell resistens (AMR) är en stor One Health-utmaning som sammankopplar människors och djurs hälsa med miljön. Livsmedelsproducerande djur spelar en viktig roll i detta sammanhang, som reservoarer för antimikrobiella resistensgener. Även om användning av antimikrobiella medel (AMU) allmänt erkänns som den huvudsakliga drivkraften bakom AMR, ökar evidensen för att resistensdynamiken även påverkas av faktorer som rör miljö och djurhållning.

Övervakning av AMR inom djurhållningen baseras huvudsakligen på fenotypisk resistensbestämning, som endast speglar en begränsad del av den totala reservoaren av resistensgener. Så kallad shotgun-metagenomik möjliggör en mer heltäckande karakterisering av resistomet men har främst tillämpats i produktionssystem med hög AMU, där ett kraftigt selektionstryck kan maskera effekten av andra faktorer. I denna avhandling utvecklades och tillämpades därför ett robust och skalbart metodologiskt ramverk för analys av miljöresistomet i grisbesättningar med låg AMU.

Metodiken optimerades genom systematisk utvärdering av provtagningsstrategi, DNA-extraktion, sekvenseringsdjup och bioinformatiska vägval. Sockprovtagning visade sig vara en praktisk och standardiserbar metod för studier på gårdsnivå, och djupsekvensering av korta genfragment gav bäst återgivning av resistensgener i komplexa prov från gårdsmiljön. Arbetsflödet tillämpades i en longitudinell studie av tio svenska integrerade grisbesättningar som följdes under en hel produktionscykel.

Trots tydliga förändringar i mikrobiomet över tid sågs inget tydligt samband mellan resistomet och AMU, vilket speglar det låga selektionstrycket i dessa system. Resistomdiversitet och fenotypisk resistens hos *Escherichia coli* var inte korrelerade, vilket indikerar att genotypiska och fenotypiska mått fångar olika dimensioner av AMR. Resultaten understryker behovet av att integrera metagenomiska och fenotypiska angreppssätt vid studier av AMR i djurmiljöer och att tillämpa ett One Health-ramverk. Arbetet utgör en metodologisk grund för framtida storskaliga longitudinella studier.

Nyckelord: One Health, resistom i grisproduktion, riskfaktorer, antibiotika, AMR

Динаміка антимікробної резистентності у тваринництві

Анотація

Антимікробна резистентність (АМР) є однією з ключових проблем підходу Єдине здоров’я, що поєднує здоров’я людини, тварин і довкілля. Тваринництво відіграє важливу роль у цьому контексті як резервуар генів антимікробної резистентності (АРГ). Хоча застосування антимікробних препаратів (АМП) визнано основним чинником формування АМР, дедалі більше доказів свідчить про роль додаткових екологічних та управлінських факторів.

Традиційний моніторинг АМР у тваринництві базується на фенотиповій резистентності й охоплює лише обмежену частину резистентного потенціалу. Shotgun-метагеномне секвенування дозволяє комплексно охарактеризувати резистом, проте здебільшого застосовувалося в умовах високого використання АМП. У цій дисертації розроблено та застосовано масштабований підхід до аналізу екологічного резистому в свинарських господарствах із низьким рівнем використання АМП.

Методологію оптимізовано шляхом оцінки відбору проб, екстракції ДНК, секвенування та біоінформатичного аналізу. «Шкарпетковий» метод відбору проб і глибоке короткочитне секвенування забезпечили чутливе виявлення АРГ. Підхід застосовано в поздовжньому дослідженні десяти шведських свинарських ферм протягом повного виробничого циклу.

Попри динамічні зміни мікробіому, резистом не демонстрував чіткого зв’язку з використанням АМП. Відсутність кореляції між різноманіттям резистому та фенотиповою резистентністю *Escherichia coli* свідчить про комплементарність генотипових і фенотипових показників АМР. Отримані результати підкреслюють необхідність інтегрованих підходів до дослідження АМР у межах концепції Єдине здоров’я.

Ключові слова: Єдине здоров’я, резистом свинарських ферм, фактори ризику, антибіотики, АМР

Dedication

This thesis is dedicated to my family, for their patience, encouragement, and constant belief in me throughout this journey.

I am deeply grateful to my supervisors for their guidance, expertise, and support, particularly during challenging periods of my PhD journey.

I also wish to thank my colleagues, whose help extended far beyond academic collaboration. This project would have been far more challenging without their willingness to drive to the farms and their support during long days of fieldwork. Through shared experiences, they not only made this work possible but also became close friends. Your generosity, companionship, and good humour made even the most demanding days enjoyable.

Last but not least, I thank the animal collaborators - the pigs - my cutest fieldwork companions, whose participation (sometimes enthusiastic, sometimes less so) made this research possible and ensured that no fieldwork day was ever dull.

Contents

List of publications

Publications included in the thesis

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Ladyhina, V., Sternberg-Lewerin, S., Andersson, L. & Rajala, E. (2024). Antimicrobial resistance among indicator *Enterococcus faecium* and *Escherichia coli* in Swedish pig farms. Acta Veterinaria Scandinavica, 66, 34.

II. Ladyhina, V., Rajala, E., Sternberg-Lewerin, S., Nasirzadeh, L., Bongcam-Rudloff, E. & Dicksved, J. (2025). Methodological aspects of investigating the resistome in pig farm environments. Journal of Microbiological Methods, 230-231, 107103.

III. Ladyhina, V., Sternberg-Lewerin, S., Sannö, A., Bongcam-Rudloff, E., Dicksved, J. & Rajala, E. (2026). Longitudinal investigation of the resistome in Swedish pig farms. (submitted manuscript)

IV. Ladyhina, V., Sternberg-Lewerin, S., Sannö, A., Bongcam-Rudloff, E., Dicksved, J. & Rajala, E. (2026). Risk factors affecting the farm resistome - a longitudinal study of Swedish pig farms. (manuscript)

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The contribution of Valeriia Ladyhina to the papers included in this thesis was as follows:

I. Study design, sampling, supervision of MSc student, data curation and analysis, writing, reviewing and submission.

II. Study design, sampling, laboratory analyses, data curation and analysis, writing original draft, reviewing and submission.

III. Study design, sampling, laboratory analyses, data curation and analysis, writing original draft, reviewing and submission.

IV. Study design, data collection, curation and analysis, writing original draft, reviewing and submission.

Related work

Rajala E., Ladyhina V., Sternberg-Lewerin S. (2026) Factors affecting farm-level antimicrobial resistance among bacteria in livestock – a scoping review. (submitted manuscript)

In this scoping review, we analysed European studies investigating antimicrobial resistance (AMR) in livestock farms. Although associations were identified with antimicrobial use (AMU) and other risk factors, these relationships appear complex. Methodological heterogeneity limits comparability between studies, and current evidence is insufficient to support targeted interventions on specific risk factors. The optimal AMR indicator for risk factor studies remains unclear, highlighting the need for validated methods, adequate sample sizes and robust statistical analyses.

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Abbreviations

AG	Aminoglycosides
AMR	Antimicrobial Resistance
AMU	Antimicrobial Use
APP	*Actinobacillus pleuropneumoniae*
ARG(s)	Antibiotic Resistance Gene(s)
AST	Antimicrobial Susceptibility Testing
BL	Beta-Lactams
CARD	Comprehensive Antibiotic Resistance Database
DNA	Deoxyribonucleic Acid
ECOFF	Epidemiological Cut-off Value
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EU	European Union
FOL	Folic Acid Synthesis Inhibitors
MAG	Metagenome-Assembled Genome
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionisation-Time-Of-Fight Mass Spectrometry
MIC	Minimum Inhibitory Concentration
ML	Machine Learning
MLS	Macrolides-Lincosamides-Streptogramins
NGI	National Genomics Infrastructure
(N)WT	(Non-) Wild Type
ONT	Oxford Nanopore Technologies
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PCU	Population Correction Unit
qPCR	Quantitative real-time PCR
SPF	Specific Pathogen Free
TET	Tetracyclines
WGS	Whole Genome Sequencing
WHO	World Health Organization
WOAH	World Organisation for Animal Health
WMS	Whole Metagenome Sequencing

1. Introduction

1.1 The problem of antimicrobial resistance

The discovery of antibiotics - antibacterial substances - represents one of the most important historical advances in human and veterinary medicine, forming a cornerstone of modern healthcare by enabling effective treatment and prevention of infectious diseases and facilitating the development of many contemporary medical and surgical procedures (Kirchhelle 2018; Hutchings *et al.* 2019). However, while antibiotics revolutionised treatment and significantly reduced morbidity and mortality, their widespread use has also contributed to an increasing global challenge: the emergence and spread of antimicrobial resistance (AMR), whereby bacteria evolve to withstand the effects of drugs designed to kill them, undermining the efficacy of current treatments (Estany-Gestal *et al.* 2024). The spread of AMR causes a decline in the effectiveness of available antimicrobial drugs. This leads to more challenging infection management, reduced safety of surgical interventions, higher mortality rates, and global economic losses via increased healthcare costs both in human and animal medicine, as well as reduced livestock production (World Bank 2017; WHO 2023). In recent years, several large-scale studies have estimated the global burden of AMR, and although the numerical estimates vary, they consistently identify AMR as one of the most pressing global health threats in the 21st century (O'Neill 2016; Murray Ch.J.L., *et al.* 2022; WHO 2023; Mohsen N. *et al.* 2024.)

1.2 AMR as a One Health challenge

Beyond its magnitude as a global health threat, AMR represents a particularly complex challenge with many different players. Among global health challenges, AMR most clearly exemplifies the One Health paradigm, as it involves humans, animals, and the environment, all of which are interconnected components of the AMR ecosystem and linked through direct contact, food production chains, the use and disposal of manure, sewage effluents, and environmental pollution (Velazquez-Meza *et al.* 2022; Woolhouse 2024). The other aspect of AMR as a One Health problem is that effective control and mitigation depend on coordinated approaches that address the factors driving the development, spread, and persistence of resistance across human, animal, and environmental ecosystems (WHO 2015a; Van Boeckel *et al.* 2015). Although this complexity poses a major challenge for AMR control, it also provides opportunities to address the problem through multiple complementary strategies operating across different sectors and levels (Pham & Wozniak 2025).

Importantly, AMR is not a new phenomenon exclusive to the modern era. Numerous studies have shown that antibiotic resistance genes (ARGs) are ancient and widespread across microbial communities, predating the clinical use of antibiotics by millions of years. Genetic evidence from permafrost and natural soils indicates that ARGs have long been part of environmental microbial ecosystems where they likely evolved as adaptive responses to naturally occurring antimicrobial compounds produced by microbes themselves (Davies & Davies 2010; Lupo *et al.* 2012; Xu Y 2025). Although such innate resistance mechanisms existed long before human intervention, the scale and rate at which ARGs are selected and disseminated have been dramatically accelerated by the widespread misuse and overuse of antibiotics in human medicine, agriculture, and animal production. This intensified selection pressure has transformed an ancient and naturally occurring phenomenon into a major global health concern.

Against this background, it is well established that one of the primary contemporary drivers of AMR is the overuse and misuse of antimicrobial drugs, which has led much of current research to focus on human-related aspects of the AMR problem, such as elucidating resistance mechanisms, developing new antimicrobial substances, improving diagnostics, and strengthening stewardship in clinical settings (WHO 2015a; Prestinaci *et al.* 2015). However, antimicrobial usage (AMU) in livestock production represents a major contributor to the global AMR burden, with food-producing animals accounting for ~73% of the antimicrobial consumption worldwide (Van Boeckel *et al.* 2015; Van Boeckel *et al.* 2019). This highlights the importance of addressing AMR beyond human medicine and reinforces the need for a One Health approach that explicitly includes livestock production systems.

1.3 History of antimicrobial usage in livestock

The discovery of antibiotics represents one of the most important historical advances in human medicine, profoundly transforming the treatment of infectious diseases. However, the impact of antibiotics extended beyond human healthcare and rapidly influenced food production systems. Following their introduction into clinical medicine, antimicrobial drugs were soon adopted in agriculture and veterinary medicine. The first marketed antibacterial drug, prontosil, introduced in Britain in the 1930s, was a synthetic sulphonamide rather than a naturally derived antibiotic, marking the beginning of AMU beyond human medicine (Figure 1). The Second World War catalysed a major shift in the treatment of animal diseases, as the strategic importance of food production, particularly milk, led to early trials of penicillin against bovine mastitis in Britain and Denmark as early as 1943 (Woods 2014). While wartime conditions constrained pharmaceutical production in much of Europe, United States-based companies such as Merck, Pfizer, and American Cyanamid emerged as leading producers of antibiotics, with strong interwar links between pharmaceutical and feed industries facilitating the mass medication of herds and flocks through medicated feed and water (Landecker 2017). Non-therapeutic AMU expanded rapidly after the discovery in the late 1940s that low-dose antibiotics in animal feed could enhance growth and provide prophylactic disease protection, leading to widespread adoption of antibiotic growth promoters in livestock production and a subsequent boom in their commercial use. By the 1960s and 1970s, antibiotic growth promoters were incorporated into a large proportion (up to 80%) of commercially available animal feeds in several European countries, reflecting the scale of AMU in livestock production during this period (Kirchhelle 2018). However, the extensive and routine use of antibiotics in food-producing animals soon began to raise concerns. These included the presence of antibiotic residues in food products, the facilitation of increasingly intensive production systems with potential animal welfare implications, and, most importantly, the selection and spread of antimicrobial-resistant bacteria. While early regulatory responses in many countries focused primarily on controlling residues in food, growing scientific evidence increasingly linked agricultural AMU to the emergence of AMR with potential consequences for human health.

Sweden was among the first countries to respond to these concerns by adopting a precautionary approach. In 1986, Sweden implemented a national ban on the use of antibiotics for growth promotion in livestock, well ahead of most other countries (Beber *et al.* 2025). This policy was accompanied by broader efforts to improve animal health, biosecurity, and management practices to maintain productivity while reducing the reliance on antimicrobial drugs, reflecting the One Health principle that healthy animals require little or no antibiotic treatment. The Swedish experience later served as an important reference point for international policy discussions. Today, Sweden remains among the countries with the lowest AMU in livestock in Europe, with antimicrobial consumption of approximately 6.0 mg/PCU[1] in 2023 compared with an EU average of 45.1 mg/PCU (EMA 2025). Most antimicrobial treatments in Swedish livestock are administered individually and primarily involve first-line agents classified as "prudent use" by the European Medicines Agency, while critically important antimicrobials are rarely used. Despite overall low usage, antimicrobial treatment frequency varies between farms and production stages, and recent regulatory changes, such as the withdrawal of zinc oxide for piglets, may influence AMU patterns (European Commission 2017).

At the European level, increasing scientific evidence and public concern eventually led to a progressive restriction of antibiotic growth promoters, culminating in a complete ban on their use in food-producing animals within the European Union in 2006 (European Commission 2005). This marked a major regulatory shift towards more prudent AMU in livestock production and reflected a growing recognition of the role of agricultural AMU in the development and spread of AMR.

While substantial efforts have been made within Europe to reduce selective pressure from AMU in livestock through the promotion of prudent antibiotic use and improvements in animal health and management, AMR remains a major concern globally. In countries with large-scale and highly intensive livestock production systems, such as the United States and China, AMU in food-producing animals continues at comparatively high levels, driven by production intensity and demand (Mulchandani *et al.* 2023; Zhao 2023). At the same time, in many low- and middle-income countries, the

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[1] Population Correction Unit

challenge is compounded by limited access to veterinary services, diagnostics, and regulatory oversight, as well as restricted availability of appropriate antimicrobial agents (Alhassan 2025). These constraints may lead to inappropriate AMU, including incorrect dosing, suboptimal treatment choices, and reliance on antimicrobials as substitutes for preventive animal health measures. Together, these global disparities highlight that, despite progress in parts of Europe, AMU in livestock remains a complex international issue requiring coordinated, context-specific strategies within a One Health framework.

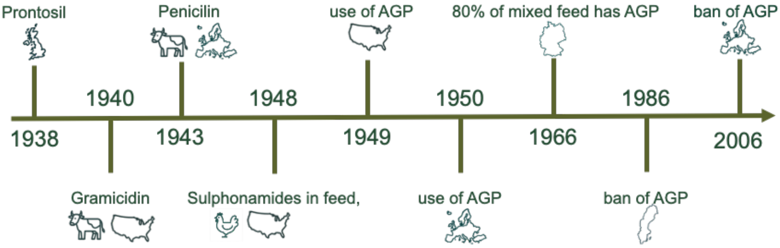

Figure 1. History of antibiotic usage in livestock, based on (Kirchhelle 2018). [2]

1.4 AMR assessment in livestock

A crucial prerequisite for addressing AMR in livestock production is a comprehensive understanding and effective monitoring of the current AMR situation. Routine surveillance of antimicrobial sales, together with monitoring the occurrence of resistant bacteria, provides the basis for analysing temporal trends and assessing fluctuations in response to interventions and policy measures (WHO 2015b). For AMR surveillance, the culture-based isolation and antimicrobial susceptibility testing (AST) of indicator bacteria remain the gold standard and form the foundation of most national and international monitoring programmes (WHO 2015b; WOAH 2023). Traditional phenotypic AST methods, such as broth dilution and agar diffusion, are widely used and are regarded as robust and highly standardised approaches. By broth dilution, the minimum inhibitory concentration (MIC)

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[2] Prontosil – synthetic sulfonamide, gramicidin – peptide antibiotic, penicillin – beta-lactam, AGP – antibiotic growth promoter

of antimicrobial agents can be determined. This method involves culturing indicator or pathogenic bacteria and analysing their MICs against a defined panel of antibiotics, with susceptibility interpreted using established thresholds, such as epidemiological cut-off values (ECOFFs) in the European Union (Kahlmeter *et al.* 2003; European Food Safety *et al.* 2019). Resistance levels are then reported as the proportion of resistant isolates among all representative isolates. Since 2004, the European Food Safety Authority (EFSA) has coordinated harmonised AMR surveillance in food-producing animals based on four bacterial groups: *Salmonella* spp., *Campylobacter* spp., and the indicator organisms *Escherichia coli* and *Enterococcus* spp., which are part of the normal gut microbiota and common carriers of ARGs (EFSA 2012). While this approach provides valuable insight into phenotypic resistance trends and supports long-term comparability, it yields limited information on the broader resistance landscape, as it focuses on a restricted set of bacterial species and does not capture the broader resistome, encompassing the ARG content of all pathogenic, opportunistic and non-pathogenic bacteria (Kim & Cha 2021). Consequently, surveillance based on indicator bacteria may not fully represent the overall occurrence and dynamics of AMR in livestock environments and therefore estimating resistance in such way may mislead conclusions (Andersen *et al.* 2021).

Another widely used approach for AMR surveillance is quantitative real-time polymerase chain reaction (qPCR), which enables sensitive and quantitative detection of a predefined set ARGs in biological or environmental samples (Graesboll *et al.* 2019; Luiken *et al.* 2022; Yang *et al.* 2022). This method is particularly useful for targeted screening and for comparing the abundance of specific resistance determinants across samples or over time. However, qPCR-based approaches are inherently limited by their reliance on prior knowledge of target genes and therefore capture only a fraction of the total resistome. In addition, qPCR provides limited information on the broader bacterial community structure, the genetic context of resistance genes, and their potential for horizontal transfer. Consequently, while qPCR represents a valuable complementary tool for AMR monitoring, it is insufficient on its own to provide a comprehensive assessment of microbial community composition and resistome diversity in complex environments.

DNA microarrays represent another molecular approach for screening bacterial genomes for known ARGs (Call *et al.* 2003). This technology relies on thousands of immobilised probes that hybridise with complementary DNA sequences, generating detectable signals when target genes are present and enabling the simultaneous detection of a broad range of ARGs within a single assay (Frye *et al.* 2010). However, like PCR-based methods, microarrays are inherently limited to the detection of ARGs for which probes have been designed and therefore may fail to identify novel or unexpected ARGs. In addition, microarray analyses are generally qualitative rather than quantitative, providing information on gene presence or absence but offering limited insight into the relative abundance or expression levels of resistance determinants (Jaksik *et al.* 2015).

Recent technological developments in molecular biology have introduced whole genome sequencing (WGS) as a powerful tool for deeper analysis of AMR in individual bacterial isolates, offering high-resolution characterization of ARGs and phylogenetic relationships that enable detailed tracking of AMR transmission and dissemination across clinical, agricultural, and environmental settings (Sorensen *et al.* 2024). The use of WGS for the prediction and characterisation of AMR has been extensively studied, with several bioinformatic tools benchmarked and shown to provide increasingly accurate results (Brouwer *et al.* 2023). WGS enables high-resolution molecular epidemiological analyses, including characterisation of plasmids, insertion sequences, virulence factors, and phylogenetic relationships among isolates. Recognising WGS as a promising alternative to conventional phenotypic testing for AMR monitoring, but acknowledging that only a limited number of EU Member States currently have the capacity to implement it routinely, EU legislation was updated through Commission Implementing Decision (EU) 2020/1729 to permit the voluntary reporting of WGS data – under defined technical conditions to ensure comparability – in place of phenotypic antimicrobial susceptibility testing for extended-spectrum β-lactamase (ESBL-), AmpC-, or carbapenemase-producing *E. coli* (European Commission 2020). Although WGS has been increasingly incorporated into AMR surveillance and offers substantial gains in resolution and epidemiological insight, its isolate-based nature limits its ability to characterise AMR beyond selected target organisms, leaving much of the environmental AMR unexplored.

To overcome the limitations of culture- and isolate-based approaches, whole metagenome sequencing (WMS) has emerged as a powerful, culture-independent method for characterising the AMR content of entire microbial communities directly from environmental samples. This sequence-based method allows analysis of entire microbial communities without the need for isolation and laboratory cultivation, and as such offers more comprehensive and rapid insights into AMR dynamics (Olsen & Riber 2025). Importantly, WMS provides an untargeted view of both taxonomic diversity and ARG diversity, enabling recovery of the full resistome – all ARGs circulating in pathogenic bacteria, antibiotic-producing organisms, and non-pathogenic bacteria – and thereby facilitating a broader understanding of the ecology of AMR at a global scale (Wright 2010; Olsen & Riber 2025). While WMS represents a highly promising tool for comprehensive AMR analysis, its application in routine surveillance requires careful consideration of methodological, analytical, and interpretative challenges.

1.5 Challenges connected to WMS for resistome analysis

As discussed above, WMS represents a highly promising tool for resistome analysis; however, its application in AMR research remains relatively limited, especially in non-clinical and environmental settings. This is largely due to a range of methodological and practical limitations that arise at different stages of the research workflow, from sampling and laboratory processing to data analysis and interpretation.

1.5.1 Sampling strategy for comparable resistome research

When assessing the resistome at the farm level, sampling design is a critical determinant of data quality and interpretability. Considering the resistome as a representation of AMR at the farm level, a central challenge concerns how samples should be collected in a way that accurately reflects on-farm resistance dynamics. Key questions include which sample matrices should be selected, how many samples are required to capture spatial heterogeneity, and how randomisation should be applied to minimise sampling bias. When designing complex and statistically robust resistome studies, it is also essential to consider that, to enable meaningful comparisons between numerous farms, sampling protocols must be both practical and standardised. On the one hand, sampling procedures should be simple and clearly defined to minimise collector bias and ensure consistency across sites. On the other hand, the total number of samples must be carefully balanced against the high costs and substantial time requirements associated with WMS and downstream analyses. These constraints necessitate thoughtful trade-offs between statistical power, feasibility, and resource availability when planning farm-level resistome investigations. Many studies have addressed challenges related to randomisation and pooling strategies for individual animal samples or fresh faecal droppings (Yamamoto *et al.* 2014; Munk *et al.* 2017; Andersen *et al.* 2021). Overall, these studies showed that pooled sampling approaches can provide a representative overview of the farm-level resistome while substantially reducing the number of samples required for analysis. Pooling of randomly selected samples was found to capture dominant ARGs and overall resistome composition with acceptable accuracy compared to individual sampling, especially when the objective was to characterise population-level patterns rather than individual-level variation. However, these studies also highlighted that excessive pooling may reduce sensitivity for detecting low-abundance or rare resistance determinants, indicating that pooling strategies must be carefully optimised depending on study aims and expected resistome heterogeneity (Andersen *et al.* 2021). In addition, resistome composition may vary considerably within farms and over time due to differences in housing, production stage, animal age, and management practices, highlighting the need for adequate spatial and temporal replication. Careful sampling design, combined with standardised protocols and comprehensive metadata collection, is therefore essential to ensure that resistome analyses are robust, comparable, and biologically meaningful.

1.5.2 Effects of sample storage temperature on resistome stability

Another methodological consideration closely related to sampling is the temperature at which samples are stored and the duration of transportation prior to DNA extraction. Suboptimal storage can substantially influence microbial viability, community composition, and DNA integrity, thereby affecting downstream resistome analyses. In particular, delays between sampling and processing combined with inappropriate storage temperatures may lead to microbial growth, cell lysis, or DNA degradation, resulting in shifts in both taxonomic profiles and the apparent abundance of ARGs (Lauber *et al.* 2010). These factors represent a particular challenge in studies conducted on commercial farms, where immediate sample processing and strict temperature control are not always feasible due to logistical constraints. Therefore, sampling strategies and workflows for farm-level resistome studies must be designed to be robust in real-world conditions while minimising potential artefacts introduced during transport and storage. Several studies have demonstrated that storage at ambient temperatures can alter microbial community structure, especially in faecal and environmental samples, due to selective overgrowth of fast-growing taxa and degradation of DNA from more sensitive organisms (Choo *et al.* 2015; Martin de Bustamante *et al.* 2021). Such changes may bias resistome profiles and reduce comparability between samples, especially in longitudinal resistome studies where minimising technical variability is essential for accurate interpretation of temporal trends. Alterations in microbial diversity in equine faecal samples stored at room temperature for extended periods have been reported, whereas human and feline samples showed greater stability during the first 24 hours, indicating that resistome stability may vary across host species and sample types (Martin de Bustamante *et al.* 2021). Consequently, cooling or freezing of samples is generally recommended to preserve microbial DNA and minimise post-sampling alterations. Storage at 4 °C is often considered acceptable for short-term transport, whereas freezing at −20 °C or −80 °C provides better preservation for longer transport times, although freeze-thaw cycles may also introduce bias if not carefully controlled (Knight *et al.* 2018). As a result, the choice of transportation temperature and preservation strategy represents a trade-off between logistical feasibility and data quality.

Overall, temperature control during sample transportation is a crucial factor influencing the reliability and reproducibility of resistome analyses. Careful selection and standardisation of storage conditions are therefore essential to ensure that observed differences in resistome composition reflect true biological variation rather than artefacts introduced during sample handling.

1.5.3 DNA extraction

DNA extraction represents another important pre-analytical step in WMS workflows and has a substantial influence on the observed composition and diversity of microbial communities and ARGs. When using WMS to characterise the resistome, the objective is to recover DNA from the full spectrum of bacteria present in complex livestock environments, which can account for thousands of different species (Scicchitano *et al.* 2024; Yasmin *et al.* 2025). This inherently involves overcoming biological differences between taxa, particularly between Gram-negative and Gram-positive bacteria, which differ markedly in cell wall structure. Gram-positive bacteria possess thick peptidoglycan layers that make them more resistant to lysis, and insufficient disruption can lead to systematic underrepresentation of these organisms and their associated ARGs, thereby biasing resistome profiles (Pollock *et al.* 2018).

Several studies have demonstrated that DNA extraction protocols can strongly influence both taxonomic composition and detected ARG abundance in metagenomic datasets (Bencic *et al.* 2024). Mechanical lysis methods, such as bead-beating, generally improve DNA recovery from resilient bacterial cells, including Gram-positive taxa, and enhance detection of resistance genes (Cullen 2022). However, aggressive lysis introduces a second methodological conflict: while maximising DNA yield is desirable for comprehensive resistome detection, excessive mechanical or chemical disruption can result in substantial DNA fragmentation. This fragmentation may compromise downstream analyses, particularly when long-read sequencing technologies are applied to resolve genomic context, mobile genetic elements, or ARG-host associations (Gand *et al.* 2023).

Conversely, gentler extraction protocols may better preserve high-molecular-weight DNA suitable for long-read sequencing but risk incomplete lysis and selective loss of DNA from more robust bacterial populations. In addition, co-extraction of inhibitory substances such as humic acids, bile salts, or host-derived compounds can further affect DNA quality, library preparation efficiency, and sequencing performance, especially in environmental matrices such as manure, dust, or soil (Knight *et al.* 2018). As a result, DNA extraction methods introduce trade-offs between representativeness, DNA integrity, and analytical resolution.

Together, these challenges highlight that no single DNA extraction protocol is optimal for all resistome studies. Instead, extraction strategies must be carefully selected and optimised based on sample type, study objectives, and downstream sequencing approaches. Standardisation and transparent reporting of DNA extraction methods are therefore essential to ensure robust, comparable, and biologically meaningful resistome analyses.

1.5.4 Sequencing strategy

Another important choice to do in resistome analysis is the choice of sequencing platform. Different technologies offer distinct trade-offs in terms of read length, accuracy, sequencing depth, cost, and suitability for specific analytical objectives. Short-read sequencing platforms such as Illumina, and long-read technologies such as Oxford Nanopore Technologies (ONT) have been applied in taxonomic and resistome studies, each with specific advantages and limitations.

Illumina sequencing remains the most widely used platform for metagenomic studies due to its high throughput, low per-base error rates, and cost-effectiveness, enabling deep sequencing of complex microbial communities. High sequencing depth is particularly advantageous for resistome analysis, as it improves detection of low-abundance ARGs and rare taxa (Zaheer *et al.* 2018; Gweon HS 2019). Several studies have demonstrated that Illumina-based approaches provide robust and reproducible estimates of microbial community composition and ARG abundance, and they are often considered the current standard for characterising complex microbiomes (Abramova *et al.* 2024; Luiken *et al.* 2019; Van Gompel *et al.* 2019; Mencia-Ares *et al.* 2020; Stevens *et al.* 2023).

In contrast, long-read sequencing technologies such as ONT offer substantially longer reads, which facilitate assembly of repetitive regions, reconstruction of complete genes and operons, and improved linkage of ARGs to their bacterial hosts and mobile genetic elements (Weinmaier *et al.* 2023; Sierra *et al.* 2024; Slizovskiy *et al.* 2024). These features are particularly valuable for understanding the genomic context and potential mobility of resistance determinants. However, ONT sequencing is characterised by higher per-base error rates compared with Illumina, which can affect taxonomic classification accuracy and ARG annotation, particularly for closely related gene variants. Although continuous improvements in ONT chemistry and basecalling have markedly increased accuracy in recent years, error rates remain a consideration for resistome studies that rely on precise gene identification.

To balance these trade-offs, hybrid sequencing approaches combining short- and long-read data have increasingly been proposed. Hybrid assemblies leverage the high accuracy and depth of Illumina reads together with the structural resolution provided by long reads, enabling improved assembly quality, ARG detection, and ARG-host association while mitigating platform-specific limitations (Bertrand *et al.* 2019; Brown *et al.* 2021). However, hybrid approaches introduce additional analytical complexity, increased costs, and higher computational demands, which may limit their feasibility in routine surveillance settings.

Depth of sequencing represents an additional critical factor that interacts with platform choice. While long-read sequencing technologies excel at resolving genomic structure, achieving sequencing depths comparable to short-read platforms remains cost-prohibitive for many study designs. As a result, Illumina sequencing often outperforms long-read approaches in terms of total ARG detection and diversity, particularly when the primary aim is quantitative resistome profiling rather than detailed genomic reconstruction (Gweon *et al.*, 2019; Stevens *et al.*, 2023).

Overall, platform selection should be guided by study objectives as well as complexity of the microbiome, expected abundance and diversity of ARGs in the sample, as this determines the sequencing depth required to achieve reliable detection. For comprehensive resistome profiling focused on ARG diversity and abundance across many samples, Illumina sequencing remains the most practical and cost-effective choice. In contrast, long-read or hybrid sequencing approaches are better suited for studies aiming to resolve genomic context, horizontal gene transfer, and ARG mobility, albeit at increased cost and analytical complexity.

1.5.5 Downstream analysis

Bioinformatic analysis represents an important component of the WMS workflow for resistome characterisation, with methodological choices at multiple stages substantially influencing the diversity, abundance, and interpretability of detected ARGs. Key steps include the selection of an assembly strategy, optional binning, and the choice of ARG reference databases. Although several studies have described the advantages and limitations of different approaches, optimal workflows remain highly context-dependent and must be tailored to the specific research question, sample complexity, and intended biological interpretation (Boolchandani *et al.* 2019; Pillay *et al.* 2022; Lee *et al.* 2023).

The most common pipelines for ARG identification from metagenomic data are based either on read-based mapping approaches or on assembly-based strategies, which may be further combined with binning and contextual annotation (Pillay *et al.* 2022). Read-based approaches allow direct quantification of ARG diversity and abundance and are often preferred when the primary objective is comprehensive detection of resistance determinants. However, such approaches are sensitive to false-positive detections, particularly for homologous genes, and provide limited information on genomic context (Abramova *et al.* 2024). In contrast, assembly-based workflows enable localisation of ARGs within longer genomic fragments, facilitating ARG-host association and investigation of mobile genetic elements, but each additional processing step introduces the risk of information loss, particularly for low-abundance ARGs.

Several integrated pipelines have been developed to streamline resistome analysis, including AMRPlusPlus (Doster *et al.* 2020), ARGs-OAP (Xiaole Yin 2023), DeepARG (Arango-Argoty *et al.* 2018), ResFinder/PointFinder (Zankari *et al.* 2017; Bortolaia *et al.* 2020), RGI (CARD) (Alcock *et al.* 2023) and ARGprofiler (Martiny *et al.* 2024), each differing in underlying algorithms, databases, and analytical assumptions. While these pipelines provide standardised workflows, their performance varies depending on sample type, sequencing depth, and ecological complexity, reinforcing that reliance on a single predefined pipeline may not be sufficient for complex environmental resistome studies.

Assembly strategy plays a particularly important role in downstream analysis. Comparative evaluations have shown that for different type of samples different assemblers perform better (Zhang *et al.* 2023; Abramova *et al.* 2024). Hybrid assembly approaches combining short and long reads can further improve contiguity and contextualisation of ARGs but introduce additional computational complexity and may not always outperform optimised single-technology assemblies (Berbers *et al.* 2020; Brown *et al.* 2021). These observations highlight that assembler performance is dataset-specific and that benchmarking remains essential for robust resistome analysis.

Binning represents an optional but influential step in resistome workflows. On the one hand, binning enables reconstruction of metagenome-assembled genomes (MAGs), facilitating investigation of ARG distribution across known and unknown bacterial taxa (Pillay *et al.* 2022). On the other hand, binning is associated with loss of ARG diversity, particularly for genes located on small contigs.

Another major determinant of resistome outcomes is the choice of ARG reference database. A wide range of databases and tools have been developed, differing in scope, curation strategy, and resistance mechanisms covered (de Abreu 2020; Papp & Solymosi 2022). Databases may focus exclusively on acquired ARGs or include chromosomal mutations, biocide resistance, and metal resistance genes, which is particularly relevant for environmental studies. Importantly, ARG detection is inherently constrained by the content and annotation quality of reference databases, meaning that resistance genes absent from or poorly represented in databases cannot be detected, regardless of sequencing depth or analytical sophistication.

To address some of these limitations, machine learning (ML)-based approaches have increasingly been applied to resistome analysis. ML-based tools such as DeepARG and related neural network models can identify putative ARGs based on sequence features rather than strict homology, improving detection of divergent or previously uncharacterised ARGs (Arango-Argoty *et al.* 2018). While these approaches offer promising improvements in sensitivity and discovery potential, they also introduce new challenges related to interpretability, training dataset bias, and validation, and they are not yet routinely applied in regulatory or surveillance contexts (Olatunji *et al.* 2024). Consequently, ML-based methods are currently best viewed as complementary tools that enhance, rather than replace, database-driven ARG detection (Sakagianni *et al.* 2024).

1.6 Drivers of AMR in livestock production

Effective mitigation of AMR in livestock production systems requires a comprehensive understanding of the factors that drive the emergence, persistence, and spread of resistance at the farm level. AMU is widely recognised as the dominant and most direct driver of AMR selection, as exposure to antimicrobial compounds exerts strong selective pressure favouring resistant bacteria and ARGs (Ferri *et al.* 2017; Magnusson 2022; Ye *et al.* 2025). Numerous observational and experimental studies have demonstrated associations between AMU and increased prevalence of resistant bacteria or ARGs in livestock environments, supporting the central role of antimicrobial exposure in resistance development (Munk *et al.* 2018; Van Gompel *et al.* 2019; Mencia-Ares *et al.* 2020; Horie *et al.* 2021; Luiken *et al.* 2022).

However, despite the clear importance of AMU, an increasing number of studies conclude that AMU alone cannot fully explain the substantial variation in resistance patterns and there are other risk factors that can affect farm level AMR (Figure 2). Even in studies where statistically significant associations between AMU and AMR are detected, AMU often explains only a limited proportion of the total variation in resistance outcomes (Luiken *et al.* 2022; Smith *et al.* 2023). This suggests that additional herd-level, management-related, and environmental factors interact with AMU to shape the farm resistome in complex and context-dependent ways (Horie *et al.* 2021).

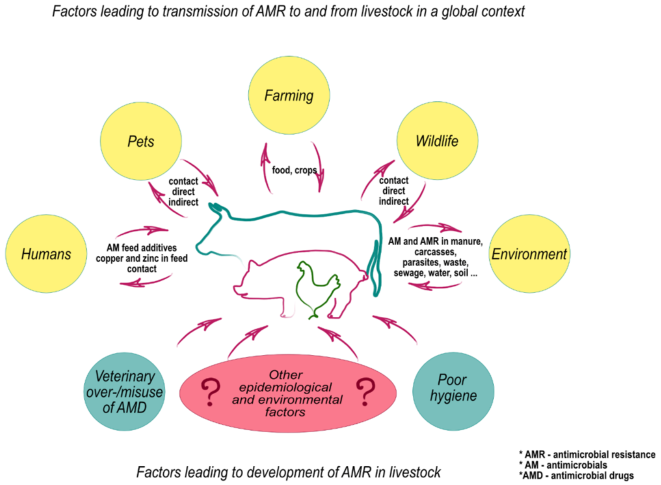

Figure 2. Factors that can be involved in development, spread and persistence of AMR within farm environments.

Biosecurity and hygiene practices are among the most frequently discussed non-AMU drivers of AMR. Several studies have reported associations between internal or external biosecurity measures and resistance levels, although the direction and strength of these associations vary between production systems (van Gompel *et al.* 2019; Luiken *et al.* 2022). High infection pressure, suboptimal hygiene, and increased opportunities for microbial transmission may promote the persistence and dissemination of resistant bacteria even in systems with relatively low AMU (Bosman *et al.* 2014; Dewulf *et al.* 2007). Conversely, improved biosecurity may reduce disease incidence and transmission, thereby lowering the need for antimicrobial treatment and the circulation of resistant bacteria.

Animal-related factors, including species, age, production stage, and stocking density, also appear to influence resistome composition. Young animals, such as piglets and calves, often harbour higher levels of ARGs/resistant bacteria than older animals, potentially reflecting early-life exposure, maternal transmission, or environmental contamination (Burow *et al.* 2019; Kyselková *et al.* 2015). High animal density and housing designs that facilitate close contact have also been associated with increased AMR prevalence, highlighting the importance of animal-to-animal transmission pathways (Chuppava *et al.* 2019).

Environmental factors represent another important category of non-AMU drivers. Seasonal variation and temperature have been associated with changes in resistance prevalence, likely through effects on bacterial survival and transmission dynamics (Schubert *et al.* 2021). In addition, exposure to heavy metals such as zinc and copper, commonly used in feed or present in the farm environment, as well as biocides and disinfectants, has been linked to co-selection of AMR through shared genetic mechanisms (Johanns *et al.* 2019; Maertens *et al.* 2020; Doidge *et al.* 2021). These selective pressures may contribute to the persistence of resistance genes even in the absence of recent antimicrobial treatment.

Farm management practices and indirect exposure pathways further complicate resistance dynamics. Practices such as waste milk feeding, animal purchasing and movement, shared transport vehicles, and environmental contamination with antimicrobial residues have all been associated with increased occurrence or persistence of resistant bacteria (Duse *et al.* 2015a; Duse *et al.* 2015b; Gunn *et al.* 2003). Importantly, several studies report that differences between farms are often larger than differences between treated and untreated animals within the same farm, emphasising the role of farm-specific ecological and management contexts in shaping the resistome (Tams *et al.* 2023; Munk *et al.* 2018).

A critical limitation of the current evidence base is that most studies investigating AMR drivers have been conducted in countries with a long history of relatively high AMU in livestock production. In such settings, elevated background levels of resistance may mask or dilute the effects of additional drivers, making it difficult to disentangle the relative contribution of non-AMU factors. This highlights the importance of studying resistome dynamics in low-AMU systems, where reduced antimicrobial selection pressure may allow the influence of management, biosecurity, and environmental factors to become more apparent. Taken together, these findings indicate that while AMU remains the central driver of AMR in livestock systems, resistance emerges from the interaction of multiple biological, environmental, and management-related factors. A deeper understanding of these interacting drivers is essential for designing effective, context-specific AMR mitigation strategies that extend beyond antimicrobial stewardship alone and align with a One Health approach.

2. Aims

The overall aim of this thesis was to enhance the understanding of the dynamics of AMR in the livestock production indoor environment, using Swedish pig farms as a model.

Specific objectives were:

- Evaluate and optimize key methodological aspects of metagenomics for farm-level resistome analysis.
- Develop a bioinformatic pipeline optimized for environmental resistome analysis in pig farm systems with low AMU.
- Characterize the abundance, diversity, and temporal development of the environmental resistome across a full production cycle in selected Swedish pig farms.
- Characterize phenotypic AMR in indicator bacteria from pig farm environments and evaluate concordance and complementarity with metagenomic resistome profiles.
- Investigate associations between farm-level risk factors, AMU patterns, environmental conditions, and environmental resistome composition.

3. Comments on materials and methods

Materials and methods for each study (I- IV) are described in detail in the papers included at the end of this thesis. A major objective of this project was not only to characterise the AMR load and risk factors affecting its fluctuation within Swedish pig farms, but also to critically evaluate and optimise key methodological steps involved in resistome analysis of complex environmental samples. Consequently, several methodological choices made in each study were informed by, and directly linked to, parallel methodological comparisons performed within the project, including evaluations of sampling strategies, DNA extraction protocols, sequencing technologies, assembly approaches, and ARG databases.

3.1 Farm selection

3.1.1 Methodological Study

The material used in Paper II was collected at the pig farm of the Swedish University of Agricultural Sciences (SLU), situated at the Swedish Livestock Research Centre in Lövsta, outside Uppsala, Sweden. This farm was selected as a controlled and well-characterised production environment suitable for methodological optimisation and benchmarking.

3.1.2 Resistome study

The study farms (Paper I, III-IV) were selected using predefined inclusion criteria to ensure both logistical feasibility and comparability of production systems. Eligible farms were required to be located within a maximum driving distance of approximately 1.5-2 hours from Uppsala, allowing timely sample transport and processing. In addition, farms had to operate a farrow-to-finish pig production system and be able to provide a batch of pigs in which the animals remained on the farm throughout the entire production cycle. Thirteen farms fulfilling these criteria were invited to participate in the study, and ten of them agreed to take part and were subsequently included.

3.2 Sample collection and treatment

3.2.1 Methodological study

Samples were collected in March 2022 from four pig pens, each housing 10-13 three-month-old pigs from the same production batch (Paper II). Environmental sampling was performed using sterile gauze socks placed over clean boots covered with disposable boot covers (Figure 3). One pair of socks was used per pen, and within each pen, 100 steps were taken following a standardized walking pattern designed to cover the entire pen surface. The socks were rotated by 90° after every 25 steps to ensure even contact with the floor.

Figure 3. Example of sock sampling: 20 cm long elastic tubular retention bandage over single use plastic boot coverage.

Following sampling, the socks were immediately transported to the laboratory at SLU. To mimic the effect of different sample transportation conditions, samples were subjected to three storage scenarios upon arrival at the laboratory. To simulate transport under refrigerated conditions (e.g. shipment with ice packs), samples were stored at +4 °C for 48 h. To mimic transportation at ambient temperature (e.g. ordinary postal delivery), samples were stored at +20 °C for 48 h. To represent optimal transport conditions (e.g. shipment on dry ice), samples were processed immediately upon arrival.

For sample processing, each sock was soaked in 50 mL of buffered peptone water and homogenized using a stomacher to extract the sample material. The resulting suspension was concentrated by centrifugation at

3,000 × g for 10 min, after which the pellet was resuspended in 15 mL of supernatant. Aliquots were subsequently transferred to 1.8 mL cryotubes and stored at −80 °C until further analysis.

3.2.2 Resistome study

For the analysis of the environmental resistome in ten Swedish pig farms, samples were collected monthly over a period of approximately six months, starting when the piglets were 1-2 weeks old and continuing until 1-2 weeks prior to slaughter. An exception was Farm 10, where the final sampling was conducted two days after the pigs had been removed from the pens. Sampling took place between February and September 2023. In total, 61 pooled environmental samples were collected; one farm (Farm 4) followed a production cycle approximately one month longer than the others, resulting in seven sampling occasions instead of six.

At each farm visit, up to four environmental sock/boot-swab samples were collected by systematically walking through the pens housing the study batch of pigs (Figure 4). The sampling strategy followed the same general principles as applied in the methodological study; however, in this field study, one pair of socks was used to sample multiple pens housing pigs from the same batch.

Extraction of sample material from the fabric matrices was performed on the day of sampling immediately upon arrival at the laboratory, as described for the methodological study. All sock samples collected on the same date at the same farm were pooled into a single composite sample per farm visit and subsequently stored at −80 °C until DNA extraction.

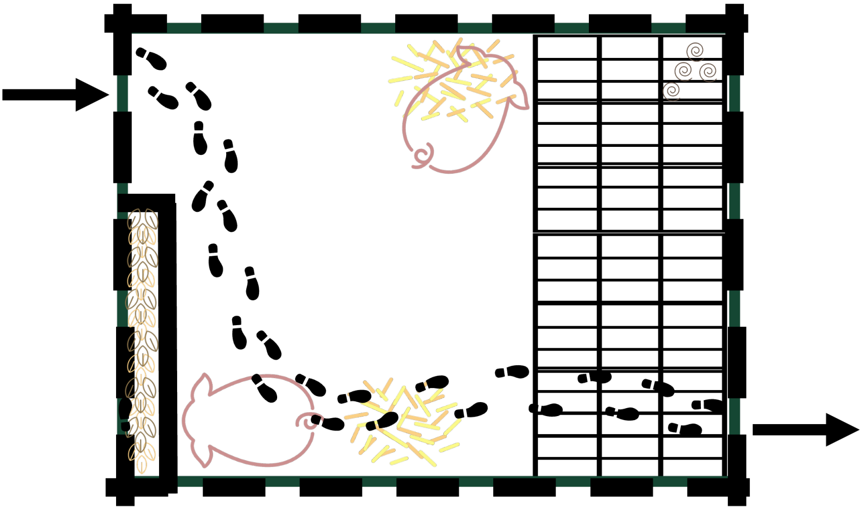

Figure 4. Schematic illustration of the standardised sampling trajectory used for environmental sample collection within pig pens.

In each selected pig pen, which was evenly spatially distributed within the batch of pigs of interest, samples were collected by following a systematic walking trajectory consisting of 25 predefined steps. The trajectory was designed to ensure comprehensive spatial coverage of the pen and included all functionally distinct areas, specifically the lying area, feeding area, and toilet area. This standardised approach was applied consistently across pens to minimize spatial sampling bias and improve comparability between samples.

3.2.3 Phenotypic analysis

For phenotypic analysis of AMR, aliquots of the same pooled environmental samples used for resistome analysis were processed with one modification: prior to storage at −80 °C, the pooled extracts from sock samples were mixed with glycerol to prevent bacterial cell damage during freezing and to preserve bacterial viability for subsequent isolation and phenotypic testing.

3.3 AMU and farm management

Information on farm management practices was collected using a structured questionnaire administered to the farmer and the responsible veterinarian during the first farm visit. The questionnaire captured detailed information on herd management and production characteristics, including housing systems, biosecurity measures, vaccination strategies, cleaning and disinfection routines, and selected environmental parameters relevant to animal health and infection pressure.

To obtain detailed and time-resolved AMU data, farmers were provided with a dedicated journal in which all daily events and antimicrobial treatments administered to the study batch of pigs were recorded throughout the production cycle. Recorded information included the date of treatment, antimicrobial substance, dosage and treatment duration. A summary of the variables derived from this questionnaire is presented in Table 1.

Based on these records, AMU was quantified for each farm and antimicrobial class. Antimicrobial exposure was calculated both for the period preceding each sampling occasion and cumulatively over the entire lifetime of the pigs. AMU was expressed as the total number of pig-treatment days, allowing comparison of treatment intensity across farms and sampling occasions.

Environmental parameters, including temperature and relative humidity, were recorded on site using a combined thermometer-hygrometer (Uni-T UT333 thermometer and hygrometer).

For the analysis of Zn and Cu, material collected from the sampling socks was used. Specifically, material was scraped from complementary socks, defined as one sock from each pair collected for AMR analysis. The scraped material was pooled per sample and submitted for elemental analysis using SS-EN ISO 54321:2021 mod./SS-EN ISO 17294-2:2023 to Eurofins Environment Testing Sweden AB, Sweden. An unused, clean sock was analysed in parallel as a control for potential background metal content associated with the sampling material.

Table 1 Overview of potential farm-level risk factors collected during the longitudinal study of Swedish pig farms. [3]

Risk Factor	Level	Category/Summary	Number (Visits or Farms)
Zn (mg/kg)	Visit	Median 104 (Min 18, Max 754)	60
Cu (mg/kg)	Visit	Median 28.5 Min 8, Max 217)	60
Temperature (°C)	Visit	Median 21.28 (Min 13.05, Max 42.76)	60
Humidity (%)	Visit	Median 72.79 (Min 41.50, Max 97.58)	60
BL	Visit	Median 0 (Min 0, Max 421)	28
AG	Visit	Median 0 (Min 0, Max 14)	4
FOL	Visit	Median 0 (Min 0, Max 5)	7
TET	Visit	Median 0 (Min 0, Max 241)	2
MLS	Visit	Median 0 (Min 0, Max 33)	6
Farm Hygiene	Visit	Clean	45
		Dirty	15
Presence of Flies	Visit	No flies observed	50
		Flies observed	10
Floor Material	Visit	Concrete	47
		Concrete and Plastic/Metal/Straw	13
Farm Size	Farm	Median 2065 (Min 740, Max 3627)	8
Number of Sows	Farm	Median 190 (Min 60, 500)	10
Number of Vaccines Sows	Farm	Median 3 (Min 2, Max 4)	10
Number of Vaccines Piglets	Farm	Median 2 (Min 0, Max 3)	10
Other Animals	Farm	No other animals at the farm	2
		Other animals present at the farm	8
Average Mortality in Nursery (%)	Farm	≤16	5
		>16	5
Average Mortality in Growing Pigs (%)	Farm	Median 1 (Min 1, Max 2,6)	8
Average Mortality in Slaughter Pigs (%)	Farm	Median 1 (Min 0, Max 2)	8
Mixing of Sows	Farm	Sows and gilts not mixed	4
		Sows and gilts mixed	6

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[3] AG (aminoglycosides), BL (beta-lactams), FOL (folic acid synthesis inhibitors), MLS (macrolides-lincosamides-streptogramins), TET (tetracyclines), SPF (specific pathogen free). Antimicrobial use is reported as the number of pig-treatment days preceding each sampling occasion.

Disease Outbreaks	Farm	No outbreak reported	7
		Outbreak reported	3
Nursery Sows	Farm	Nursery sows not used	5
		Nursery sows used	5
Disinfection Practices	Farm	Disinfectants not used	2
		Disinfectants used	8
SPF Farm Status	Farm	No SPF	7
		SPF	3
Other Risk Factors	Farm	No	6
		Work with other animals / hospital	4
Disposable Farm Specific Clothes	Farm	Yes	10
Track of Visitors	Farm	Yes	10
Storage of Wet Manure	Farm	Open	10
Storage of Dry Manure	Farm	Open	9
		No	1
Disinfection of Hands	Farm	Yes	10
Max Age of Sows (years)	Farm	Median 3 (Min 3, Max 4)	10
All-in All-out System	Farm	Yes	10
Cleaning of Water Nipples between Batches	Farm	Yes	10
Cleaning of Food Places between Batches	Farm	Yes	10
Biosecurity	Farm	Basic	5
		Elite	5

The table summarises information on management practices, housing characteristics, biosecurity measures, animal health status, vaccination routines, and AMU collected during repeated farm visits throughout the production cycle. Data were obtained through structured farmer interviews conducted at the first farm visit and through follow-up assessments during subsequent sampling occasions. The column “Level” indicates whether each variable was collected once during the initial interview or repeatedly at each farm visit (six visits per farm). The column “Number (Visits or Farms)” indicates the number of visits or farms in which the respective factor was recorded, present, or assessed. Vaccination practices were recorded separately for sows and piglets. Vaccines administered to sows included those targeting porcine parvovirus, erysipelas (*Erysipelothrix* *rhusiopathiae*), Glässer's disease (*Glaesserella parasuis*), and piglet diarrhoea caused by *Escherichia coli*. Vaccines administered to piglets included porcine circovirus type 2 (PCV2), *Lawsonia intracellularis*, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae* (APP), and Glässer's disease. Information on the occurrence of common clinical conditions was also collected, including arthritis, diarrhoea, joint infections or inflammations, skin wounds, piglet diarrhoea, hoof lesions, mastitis, farrowing fever, and tail biting, when present on the farm. These health indicators were recorded qualitatively based on farmer report; however, no standardised scoring system was applied, limiting their suitability for quantitative comparison across farms. AMU was quantified as the number of recorded pig-treatment days preceding each sampling occasion for the pig batch of interest. Treatments were categorised by antimicrobial class as follows: AG (aminoglycosides), BL (β-lactams), FOL (folic acid synthesis inhibitors), MLS (macrolides-lincosamides-streptogramins), and TET (tetracyclines).

3.4 Measurement of AMR

3.4.1 Phenotypic AMR in indicator bacteria (Paper I)

Thawed sample eluates were inoculated onto selective agar plates for bacterial isolation. MacConkey agar was used for the detection of *E. coli*, and Slanetz and Bartley (SlaBa) agar was used for the detection of *Enterococcus* spp. From each sample, two colonies with typical morphology were selected from each agar medium. Species identification of the selected isolates was confirmed using matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS).

Antimicrobial susceptibility testing was performed using broth microdilution with Sensititre™ panels (ThermoFisher Scientific Inc., Waltham, MA, USA). Minimum inhibitory concentrations (MIC) were determined for twelve antimicrobial substances in *E. coli* and fifteen antimicrobial substances in *Enterococcus* spp. Epidemiological cut-off values (ECOFFs) defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were applied to classify isolates as belonging to the wild-type (WT) or non-wild-type (NWT) populations, with

NWT isolates considered likely to have acquired resistance to the tested antimicrobial agent (EUCAST).

Data analysis and descriptive statistics were performed using Microsoft® Excel, and data visualisation was conducted in R v4.3.1 (R Core Team 2024) using the ggplot2 package v3.4.4 (Wickham 2016).

3.4.2 Genotypic AMR: Metagenomic sequencing of farm resistome

DNA extraction

For method optimisation (Paper II), three commercially available DNA extraction kits incorporating mechanical bead-beating were evaluated on technical replicates of the same sample to assess the quantity and quality of extracted DNA, as well as its suitability for downstream sequencing applications, including more demanding long-read-based sequencing technologies. The DNA extraction kits used in the study were the ZYMOBiomics™ MagBead DNA/RNA Kit (Zymo Research, USA), the E.Z.N.A.® Universal Pathogen Kit (Omega Bio-tek, USA), and the MagPure Stool DNA LQ Kit (Magen Biotechnology, China). All three DNA extraction kits incorporated a mechanical bead-beating step to achieve efficient cell lysis. Bead-beating was performed according to the respective manufacturer protocols using either a Precellys Evolution homogenizer (10,000 rpm; four cycles of 60 s with 60 s pauses between cycles) for the ZYMOBiomics™ MagBead DNA/RNA Kit and the MagPure Stool DNA LQ Kit, or a Vortex-Genie 2 (Scientific Industries, Inc., Bohemia, NY, USA) operated at maximum speed for 5 minutes for the E.Z.N.A.® Universal Pathogen Kit. Two of the evaluated kits (ZYMOBiomics™ MagBead and MagPure Stool DNA LQ) employed magnetic bead-based DNA purification, whereas the E.Z.N.A.® Universal Pathogen Kit relied on column-based purification using a MicroElute column. All extraction protocols were performed in accordance with the manufacturers' instructions. Following the initial comparison of DNA extraction kits, further optimisation of the E.Z.N.A.® Universal Pathogen Kit protocol was performed to increase DNA yield. This optimisation included retaining the entire supernatant after bead-beating, diluting it with a double volume of RBB buffer, and purifying the resulting lysate using a MicroElute column.

Based on the optimisation results, DNA extraction for comparisons of storage temperature effects and sequencing technologies, as well as the applied farm-level resistome analyses was performed using the E.Z.N.A.® Universal Pathogen Kit with minor modifications to the manufacturer's protocol described above.

Sequencing

To evaluate the impact of sequencing technology on downstream resistome analyses, both long- and short-read sequencing approaches were applied in Paper II. Long-read sequencing was performed using a nanopore-based platform ONT. For each sample, one library was prepared using the Ligation Sequencing Kit SQK-LSK109 (Oxford Nanopore Technologies, ONT; Oxford, UK), following the manufacturer's instructions. Sequencing was conducted on MinION flow cells using both R9.4.1 and R10.4.1 chemistries to assess differences in read accuracy and performance. In parallel, short-read sequencing was performed using Illumina technology. Libraries were prepared using the TruSeq DNA PCR-free Library Prep Kit (Illumina Inc., San Diego, CA, USA), with a target insert size of approximately 550 bp, and sequenced on an Illumina NovaSeq 6000 platform using an SP flow cell (250 bp paired-end reads) at the National Genomics Infrastructure (NGI) / SNP&SEQ Technology Platform, Uppsala, Sweden. The comparative performance of sequencing platforms is described in Paper II.

As part of the sequencing method optimisation, the required sequencing depth for short-read sequencing to reliably capture the complexity of the farm-level resistome was also evaluated. To assess the effect of sequencing depth on resistome recovery, *in silico* subsampling was performed on three representative metagenomic samples. Subsampling was applied to quality-controlled reads using the *sample* function of the seqtk package v1.3-r106 with a fixed random seed (100) to ensure reproducibility (*seqtk* 2018). Reads were subsampled to a series of predefined depths (1, 2, 4, 6, 8, 10, 20, 30, 40, 50, 60, 70, 80 and 90 million reads) to simulate increasing sequencing effort.

Based on the results of the methodological comparison, short-read sequencing was used for the investigation of environmental resistomes from pig farms in Paper III and IV. Metagenomic libraries were prepared using the PCR-free NEBNext® Ultra™ II DNA Library Prep Kit (Cat. No. E7645) and sequenced by NOVOGEN UK Company Ltd. on an Illumina NovaSeq X Plus platform (Illumina Inc., San Diego, CA, USA), generating 150 bp paired-end reads. A minimum sequencing depth of 80 million paired-end reads per sample was achieved to ensure adequate coverage of low-abundance ARGs. Raw metagenomic sequencing reads (61 samples from 10 farms) are available in the European Nucleotide Archive under project accession: PRJEB96782.

Bioinformatic analysis

Quality filtering of raw sequencing data for both short- and long-read datasets was performed using fastp v0.19.5 (Chen 2023). Removal of host-derived sequences was conducted by mapping reads to the pig reference genome (*Sus scrofa* 11.1, NCBI). For Illumina short reads, host decontamination was performed using Bowtie2 v2.5.2 (Langmead & Salzberg 2012) followed by filtering with Samtools v1.3.1 (Danecek *et al.* 2021). For ONT long reads, host-read removal was carried out using minimap2 v2.1-r311 (Li 2021), followed by processing with Samtools v1.3.1. Taxonomic annotation was performed on trimmed reads obtained with Illumina and ONT in Kaiju v1.10.1 (Menzel *et al.* 2016) using Kaiju databases Refseq_nr (2023_06_17).

To evaluate the impact of assembly strategy on downstream resistome analysis (Paper II), three types of metagenomic assemblies were generated: short-read assemblies based on Illumina data, long-read assemblies based on ONT data, and hybrid assemblies combining short- and long-read sequencing data. For each assembly type, two assemblers employing different algorithmic approaches were selected and systematically compared. The assemblers and their underlying assembly algorithms are listed in Table 2. In addition, metaplasmidSPAdes was applied specifically to facilitate detection and reconstruction of plasmid-associated sequences. Assembler performance was evaluated using QUAST v5.2.0 (Mikheenko *et al.* 2018) contig statistics and ARG detection results. MetaQUAST (Mikheenko *et al.* 2016) was run in metagenomic mode to obtain genome fraction statistics for the 20 most abundant bacterial species present in the pig farm environment, allowing assessment of assembly completeness and performance across dominant taxa. Based on these evaluations, MEGAHIT (Li *et al.* 2015) was selected for all downstream assemblies in the applied farm-level resistome analyses (Paper II and IV).

Table 2 Tools and assembly algorithms compared in Paper II.

Tool	**Version**	**Assembly**	**Algorithm**	**Reference**
Canu	v2.2	Long reads	Adaptive k-mer weighting and repeat separation	(Koren *et al.* 2017)
metaFlye	v2.9.3	Long reads	Repeat graphs	(Kolmogorov *et al.* 2020)
MEGAHIT	v1.2.9	Short reads	de Bruijn graph	(Li *et al.* 2015)
metaSPAdes	v3.15.5	Short reads	de Bruijn graph	(Nurk *et al.* 2017)
metaplasmidSPAdes	v3.15.5	Short reads	de Bruijn graph	(Antipov *et al.* 2019)
hybridSPAdes	v3.15.5	Hybrid	de Bruijn graph	(Antipov *et al.* 2016)
OPERA-MS	v0.8.3	Hybrid	Scaffold graph	(Bertrand *et al.* 2019)

As part of the bioinformatic pipeline optimisation, the impact of contig binning on resistome analysis was evaluated. Metagenome-assembled genomes (MAGs) were generated from MEGAHIT v1.2.9 contigs by mapping quality-filtered reads back to the assemblies using Bowtie2, followed by binning with MetaBAT v2.12.1 (Kang *et al.* 2019) applied to contigs ≥1500. The quality of the resulting bins was assessed using CheckM v1.0.7 (Parks *et al.* 2015), and bins with estimated completeness below 50% or contamination above 10% were classified as low quality and excluded from further analyses. The usefulness of binning for resistome characterisation was evaluated by comparing ARGs recovery in good-quality bins with ARG detection in unbinned assemblies. Based on the findings, binning was excluded from the final pipeline used for farm-level resistome.

Optimisation of the bioinformatic pipeline included a systematic comparison of five ARG reference databases, as listed in Table 3. Annotation of ARGs against each database was performed using ABRicate v1.0.1 (Seemann). The comparison revealed that individual databases differed in their coverage and resistance classes detected, resulting in complementary rather than overlapping ARG profiles. Based on these findings, all five databases were retained and jointly applied in the final bioinformatic pipeline to maximise the coverage and representativeness of the environmental resistome.

Table 3 ARG databases used in the studies.

Database	Version	Date of download	Number of ARGs	Reference
ARG-ANNOT	v5 (2019 June)	2024-Jun-18	2223	(Gupta *et al.* 2014)
CARD	v3.2.9	2023-Jun-18	4805	(Jia *et al.* 2017)
MEGARes	v2.0	2023-Nov-4	6635	(Doster *et al.* 2020)
NCBI AMRFinderPlus	2024-05-02.2	2024-Jun-18	6863	(Feldgarden *et al.* 2019)
ResFinder	v2.3.2	2024-Jun-18	3194	(Bortolaia *et al.* 2020)

3.5 Risk factor analysis (Paper IV)

Risk factor analysis was performed based on data collected during the first farm visit interview and continuously throughout the entire sampling period (see Table 1). All recorded risk factors were first critically assessed for sufficient variability across farms and sampling occasions, as well as for biological relevance, to ensure statistical interpretability and meaningful inference. Only variables meeting these criteria were retained for further analysis. To improve statistical robustness and avoid sparse category counts, categorical variables were consolidated prior to analysis, such that each variable comprised no more than two categories.

Two AMR outcomes were evaluated separately: resistome alpha diversity and phenotypic resistance score. These two outcomes were chosen to represent complementary genotypic and phenotypic dimensions of AMR while limiting the number of statistical models and preserving interpretability. Resistome diversity was quantified at the level of ARG families using the Shannon diversity index, calculated from relative abundances of ARG families. Phenotypic resistance was summarised using an AMR score derived from MIC data of *E. coli* isolates, based on nine veterinary-relevant antimicrobials, providing a composite measure of resistance per farm visit (Merle *et al.* 2023).

Associations between individual risk factors and each AMR outcome were first explored using univariable linear mixed-effects models using the *lmer* function from the lme4 v 1.1-37 package (Bates D 2015) in R, with farm included as a random intercept to account for repeated measurements. Risk factors with a p-value < 0.20 were considered for inclusion in multivariable modelling.

Multivariable linear mixed-effects models were subsequently constructed to evaluate the combined effects of selected risk factors while minimising collinearity and overfitting. Given the limited number of observations, each model included a maximum of three explanatory variables, consisting of a maximum of one farm-level factor and up to three visit-dependent factors. Farm was retained as a random intercept in all models, and statistical significance was defined as $p < 0.05$.

4. Results and Discussion

This chapter presents the results of four interrelated studies addressing methodological, ecological, and epidemiological aspects of AMR in pig farm environments. Together, these studies evaluate key methodological choices for resistome analysis, compare phenotypic and genotypic indicators of resistance, and examine how AMU and non-antibiotic farm-level risk factors shape both the environmental resistome and expressed resistance.

4.1 Methodological considerations for environmental resistome analysis in pig farm environments (Paper II)

A key outcome of this thesis is the demonstration that methodological choices strongly influence the detection, interpretation, and comparability of resistome data derived from pig farm environments. In contrast to many previous studies that have evaluated isolated methodological steps, this work provides an integrated assessment of sampling strategy, sequencing technology, sequencing depth, and bioinformatic processing, with particular emphasis on their performance in low-AMU production systems that was the focus of the studies.

A central finding is that optimal resistome analysis pipelines are highly context dependent and must be tailored to the complexity of the environmental microbiome, the intensity of AMU, and the specific objectives of the study. Investigations aiming to identify non-antibiotic risk factors for AMR require low-AMU settings, as AMU remains the dominant selective pressure shaping resistome composition. However, such production systems are often characterised by good animal health, high biosecurity standards, and well-managed housing conditions, which are closely interrelated and may collectively limit the overall burden of ARGs. Consequently, low-AMU environments are expected to harbour a comparatively low abundance and diversity of ARGs, making the detection of more subtle, non-antibiotic drivers of resistance particularly challenging.

In this context, methodological sensitivity becomes critical (Bharti & Grimm 2021). Analytical choices that introduce even modest information loss – such as bad assembly outcome, overly aggressive contig binning, restrictive ARG database selection, or insufficient sequencing depth – may disproportionately affect the recovery of low-abundance ARGs and obscure meaningful associations with management practices, environmental exposures, or co-selective agents. Therefore, resistome studies conducted in low-AMU environments must prioritise methodological approaches that maximise information retention while maintaining analytical robustness. This includes careful optimisation of sampling and transportation strategies, sufficient sequencing depth, and bioinformatic pipelines that balance specificity with comprehensive recovery of resistance determinants. Only by preserving as much resistome signal as possible can studies in low-AMU systems reliably detect and interpret the contribution of non-antibiotic drivers to AMR.

4.1.1 Sock sampling as a proxy for the farm-level resistome

Sock sampling proved to be a robust and practical approach for characterising the indoor environmental resistome in pig farms. Previously, sock sampling has been widely applied for surveillance of enteric pathogens such as *Salmonella* and *Lawsonia intracellularis* and shown to reflect herd-level infection status (Buhr *et al.*, 2007; Pedersen *et al.*, 2015; Lillie-Jaschniski *et al.*, 2023). However, its suitability for resistome analysis had not been systematically evaluated.

Our results demonstrate that sock samples yield sufficient quantity and quality of bacterial DNA for shotgun metagenomic sequencing and allow recovery of diverse ARGs. Compared to other common sampling methods, sock sampling offers important advantages for farm-level studies: it is time-efficient and reduces selection bias by integrating microbial material from multiple animals and environmental niches within a pen. In comparison with individual animal sampling, it is also a non-invasive approach, allowing minimising animal stress. In addition, sock sampling is highly suitable for large-scale and surveillance-oriented studies, as it follows a simple and standardisable protocol that can be readily implemented by farmers or responsible veterinarians. The controlled and reproducible nature of the sampling procedure facilitates consistent sample collection across farms and time points, even when performed by different personnel, thereby enhancing feasibility, scalability, and comparability in multi-farm studies.

While previous studies comparing floor manure scraping with individual animal sampling have reported only minor differences in detected resistance profiles (Munk *et al.,* 2017), no direct comparison with individual sampling was performed in the present work. Consequently, the proposed sock sampling approach may have limitations, including potential underestimation of very low-prevalence ARGs or rare bacterial taxa. This limitation is inherent to composite environmental sampling strategies, which prioritise representativeness at group level over sensitivity to rare features.

An additional limitation of sock sampling for longitudinal studies relates to the early production stages, when piglets are housed together with sows. Under these conditions, it is not possible to disentangle the contribution of piglets from that of sows to the environmental microbiome and resistome. This limitation is reflected in the results from Paper III, where principal coordinate analysis (PCoA) showed that samples collected during the first and final farm visits were more similar to each other than to samples collected at intermediate time points. This pattern contrasts with previous faecal microbiome studies, which have shown a pronounced divergence of the piglet microbiome following weaning and a gradual shift toward an adult-like profile resembling that of sows over time (Holman, D. B., 2021). As written earlier, a likely explanation for this discrepancy lies in the nature of the sampled material, which is heavily influenced by sow faeces during period before weaning. As a result, the outcome captured at early time points may reflect a mix of the sow and piglet-associated microbiome as well as resistome rather than that of the piglets themselves. Therefore, for a deeper understanding of how the resistome of young pigs is shaped by maternal and environmental sources, future studies should explicitly compare individual sow resistomes with environmental resistome profiles. Such comparisons would help to disentangle vertical and environmental transmission pathways of ARGs and to clarify the extent to which early-life resistome development is influenced by the sow microbiome versus on-farm environmental reservoirs.

4.1.2 Sequencing strategies and their impact on resistome recovery

WMS represents a critical methodological choice in resistome studies, as sequencing technology and depth directly influence microbiome recovery, assembly quality, and the detection and interpretation of ARGs. In this work, we explicitly compared short-read (Illumina) and long-read (ONT) sequencing approaches with respect to microbiome profiling, metagenome assembly performance, and resistome characterisation in complex pig farm environments.

Short- versus long-read sequencing: microbiome and resistome recovery

A key outcome of this study was that deep Illumina sequencing consistently outperformed ONT sequencing in terms of microbiome resolution and resistome recovery. Illumina data recovered a substantially higher number of bacterial genera, particularly rare and low-abundance taxa, which is most plausibly explained by the marked difference in sequencing depth achieved between platforms. In this study, Illumina sequencing generated tens of millions of paired-end reads per sample, whereas ONT sequencing depth was considerably lower and distributed across a highly diverse microbial community.

High sequencing depth is particularly important in farm environments, where microbial communities are complex and unevenly distributed, and where ARGs often occur at low abundance. Previous studies have shown that deep short-read sequencing improves the detection of rare taxa and stabilises estimates of ARG diversity and abundance (Gweon *et al.,* 2019; Stevens *et al.,* 2023). Consistent with these findings, Illumina sequencing in the present study enabled recovery of a broader microbiome and a higher diversity and number of ARGs compared with ONT sequencing.

Assembly performance and the limited benefit of hybrid assemblies

When comparing assembly strategies, the highest genome fraction was obtained using MEGAHIT, followed by hybridSPAdes and metaSPAdes, while long-read-only assemblies performed substantially worse. Importantly, the incorporation of ONT reads into hybridSPAdes did not result in a meaningful improvement over short-read assembly alone.

This outcome can be explained by several interacting factors. First, the dominance of short-read coverage meant that hybrid assemblies were largely driven by Illumina data, with long reads contributing only marginally to scaffolding or repeat resolution. Hybrid assemblers such as hybridSPAdes are conservative by design and require sufficient per-genome long-read coverage to improve contiguity; in complex environmental metagenomes, ONT reads are distributed across many taxa, resulting in insufficient long-read support for most genomes (Brown *et al.,* 2021; Zhang *et al.,* 2023).

Second, the superior performance of MEGAHIT relative to metaSPAdes is consistent with previous observations that MEGAHIT is particularly well suited for deeply sequenced, highly complex metagenomes, owing to its succinct de Bruijn graph implementation and optimised memory handling (Zhang *et al.,* 2023; Abramova *et al.,* 2024). In such datasets, MEGAHIT may preserve a larger fraction of assembled sequence without excessive fragmentation, even if contig lengths remain moderate.

Together, these findings indicate that, under conditions of high short-read depth and limited long-read coverage, short-read assembly alone represents the most efficient and robust strategy for resistome analysis in farm environments.

Limitations of assembly-based resistome analysis and the role of long reads

Despite the advantages of short-read sequencing and assembly, a fundamental limitation remains: assembly inevitably leads to information loss. Low-abundance ARGs may be missed, fragmented, or excluded during graph simplification, and quantitative interpretation is typically restricted to relative abundance rather than absolute ARG load.

Moreover, assemblies are known to break preferentially around ARGs, which are often located within repetitive regions, transposons, or plasmids (Abramova *et al.,* 2024). This limits the ability to confidently link ARGs to their bacterial hosts or mobile genetic elements when using short reads alone. Long reads have the potential to overcome these limitations by spanning repetitive regions and preserving genomic context, but current ONT sequencing still presents challenges related to per-base error rates and insufficient depth in complex environmental samples.

A promising future solution is the use of PacBio HiFi sequencing, which combines long read lengths with substantially improved accuracy (Han *et al.* 2024). HiFi reads have been shown to enable high-quality metagenome assemblies and improved recovery of plasmids and ARG-host associations in complex microbial communities (Berbers *et al.,* 2020; Zhang *et al.,* 2023). However, there are important limitations of HiFi-based metagenome assembly. Recent work has demonstrated that HiFi-based MAG reconstruction may yield more fragmented assemblies for highly abundant species due to increased strain diversity, which complicates accurate genome reconstruction in complex microbial communities (Feng & Li 2024). These challenges are particularly relevant in livestock environments, where dominant taxa often consist of multiple closely related strains. In addition, the high cost of HiFi sequencing currently limits its application in large-scale farm-level and longitudinal studies.

Implications for resistome studies in low-AMU environments

Taken together, the results of this study demonstrate that deep Illumina sequencing remains the most appropriate choice for resistome analysis in low-antimicrobial-use farm environments, where ARG abundance is low and methodological sensitivity is critical. While long-read and hybrid approaches hold great promise for contextualising resistance genes, their benefits are only realised when sufficient long-read depth and quality can be achieved. Until such approaches become economically and technically feasible at scale, optimised short-read sequencing combined with carefully selected assembly and annotation strategies represents the most reliable compromise between sensitivity, robustness, and cost-effectiveness.

4.1.3 Recovery of ARGs and bioinformatic analysis

Read-based versus assembly-based ARG detection

A central objective of resistome analysis is to recover the maximal possible diversity and abundance of ARGs present in a sample. Two main analytical strategies are commonly used: read-based ARG detection and assembly-based ARG detection, each associated with distinct advantages and limitations (Boolchandani *et al.* 2019).

Read-based approaches rely on direct mapping or classification of raw or quality-filtered sequencing reads against ARG reference databases. This strategy maximises sensitivity, particularly for low-abundance ARGs, as it avoids information loss introduced during assembly and graph simplification. Several studies have shown that read-based methods recover a higher diversity of ARGs compared to assembly-based approaches, especially in complex environmental samples (Boolchandani *et al.,* 2019, Abramova *et al.,* 2024). However, read-based detection is more prone to false-positive annotations, particularly for homologous genes or conserved protein domains, and provides limited information on genomic context.

In addition, short reads are often shorter than full-length ARGs, making it difficult to confidently assign resistance genes to specific bacterial hosts or mobile genetic elements. This limitation complicates ecological interpretation, as the presence of an ARG cannot be unequivocally linked to the organism carrying it (Pillay *et al.,* 2022).

Assembly-based ARG detection and host contextualisation

If the research aim extends beyond describing the resistome to understanding ARG-host associations, assembly-based approaches become essential. However, assembly introduces an inherent trade-off: while it improves specificity and interpretability, it may lead to loss of low-abundance ARGs due to fragmented assemblies, coverage thresholds, and removal of short contigs (Abramova et al 2024). The results demonstrated that, for highly complex pig farm environmental samples, MEGAHIT provided the best compromise between assembly quality and ARG recovery. This is consistent with previous benchmarking studies showing that MEGAHIT performs particularly well for deeply sequenced, high-diversity metagenomes due to its memory-efficient succinct de Bruijn graph implementation (Brown *et al.,* 2021, Zhang *et al.,* 2023).

Importantly, assembler choice remains context dependent. Changes in sample type, microbial complexity, sequencing depth, or dominance of specific taxa may shift optimal performance toward alternative tools, underscoring the need for dataset-specific benchmarking rather than reliance on a single “best” assembler.

ARG databases: scope, bias, and the need for combination

Another critical determinant of ARG recovery is the choice of reference database. ARG databases differ substantially in scope, curation strategy, and historical focus. Many widely used databases were originally developed for clinical surveillance, resulting in underrepresentation of environmentally prevalent or intrinsic resistance genes (Papp & Solymosi, 2022).

Our results confirm that no single database captures the full resistome. Even MEGARes – one of the most comprehensive resources – failed to recover all ARGs detected across combined databases. Consequently, simultaneous querying of multiple databases is required to maximise ARG diversity recovery in environmental studies, as previously recommended.

Nevertheless, all database-driven approaches remain fundamentally limited to known and curated resistance genes. ARGs that are poorly characterised, divergent, or novel remain undetectable using homology-based methods alone. To overcome these limitations, ML-based ARG prediction tools have been developed. Currently the most know tool is DeepARG that uses neural networks trained on curated ARG datasets to identify resistance genes based on sequence features rather than strict homology (Arango-Argoty *et al.* 2018). While AI-based tools can improve detection of divergent ARGs by using ML models rather than strict sequence homology, their performance remains constrained by the quality and scope of curated training datasets, limited ability to identify novel or intrinsic resistance mechanisms, and the need for experimental validation of predicted ARGs (Olatunji *et al.* 2024).

ARG search pipeline

There is a wide range of integrated pipelines currently available for resistome analysis, including AMRPlusPlus, ARGs-OAP, RGI (CARD), ARGProfiler, and DeepARG, each differing in analytical strategy, reference databases, and intended applications. While these pipelines provide valuable, standardised solutions for ARG detection, the present work focused on optimising individual methodological components to address the specific characteristics of pig farm environmental samples under low AMU. As demonstrated throughout this thesis, no single pipeline can be considered universally optimal, and resistome analyses must be tailored to the study objectives, sample complexity, and expected ARG abundance. The pipeline developed here was therefore designed to maximise sensitivity and biological interpretability in low-AMU environments with complex microbiomes, rather than to function as a generic benchmarking or surveillance framework.

4.2 Longitudinal dynamics of farm-level resistome

Paper III provides a longitudinal characterisation of the environmental microbiome and resistome across a full pig production cycle in Swedish farrow-to-finish farms operating under low AMU.

4.2.1 Microbiome vs resistome dynamics

In this study, temporal changes in the environmental microbiome and resistome were analysed in parallel, as shifts in microbial community composition are expected to influence the structure and dynamics of the resistome. Ordination analyses revealed distinct longitudinal patterns for the microbiome and the resistome. PCoA of the microbiome showed greater compositional similarity between samples collected at the first and final sampling occasions compared with intermediate time points. This pattern contrasts with previous faecal-based longitudinal studies, where the microbiome typically diverges after weaning and gradually shifts toward an adult-like profile (Gaire *et al.,* 2019; Munk *et al.,* 2017). In the present study, this pattern is most plausibly explained by the environmental sampling strategy: early samples were strongly influenced by sow-associated microbiota in farrowing areas, while later samples reflected microbiota shed by older pigs, resulting in apparent convergence between early- and late-cycle environmental microbiomes.

In contrast, no such convergence was observed in the resistome. Resistome PCoA revealed that samples from the early production phase were more similar to each other, while greater dispersion was observed during the second half of the production cycle. Across all farms and sampling occasions, the resistome was dominated by ARGs conferring resistance to tetracyclines, aminoglycosides, and MLS antibiotics, consistent with previous porcine resistome studies (Munk *et al.,* 2018; Gweon *et al.,* 2019). Importantly, resistome trajectories did not mirror microbiome patterns, supporting earlier observations that resistome composition may change independently of taxonomic structure (Horie *et al.,* 2021).

Together, these findings suggest that although microbiome fluctuations – particularly those associated with dietary transitions at weaning – may contribute to shaping the resistome, they do not fully explain the observed longitudinal resistome patterns. The divergence between microbiome and resistome dynamics indicates that additional factors, such as environmental exposures, management practices, or historical selective pressures, likely play an important role in structuring the resistome over the production cycle.

4.2.2 Limited effect of AMU

Despite AMU being widely recognised as the primary driver of AMR, no clear temporal association between recorded AMU and resistome composition or diversity was detected. Treatments were infrequent, primarily administered early in life, and no consistent increases in corresponding ARG classes were observed. Risk factor modelling further supported this finding, as AMU was not significantly associated with resistome Shannon diversity. Similar observations have been reported in other low-AMU or farm-level studies, where AMU effects on resistome composition were weak, transient, or masked by other factors (Luiken *et al.,* 2019; Horie *et al.,* 2021).

In this work AMU was quantified using pig-treatment days rather than more granular metrics such as treatment incidence based on defined daily or course doses. While such standardised measures are well suited for larger datasets with frequent antimicrobial exposure, they may be unstable or biologically uninformative in low-AMU settings with sparse treatment events. In addition, calculation of treatment incidence or course-dose-based metrics would have been challenging in the present study due to limited level of detail in the available treatment records, as detailed treatment information was not consistently provided despite being requested.

Pig-treatment days provide a transparent and biologically interpretable measure of exposure at farm. Nevertheless, this simplified metric cannot capture differences in dosing with high precision. Consequently, the absence of a significant association between AMU and resistome diversity in the present study should be interpreted considering both low antimicrobial pressure and limited variability in AMU. These findings do not contradict the established role of AMU as the primary driver of AMR but rather highlight the challenges of detecting AMU effects in production systems characterised by prudent use and high animal health standards.

An additional limitation affecting the detection of AMU-related patterns is the relatively small number of farms included in the study. The longitudinal design and depth of sampling required substantial logistical effort and close collaboration with farmers and veterinarians, which constrained the achievable sample size. While this design provided rich within-farm temporal resolution, the limited number of farms reduced statistical power to detect associations that may only emerge at larger spatial scales or across a broader range of management practices. Consequently, subtle or context-dependent effects of AMU on the resistome may remain undetected and should be further explored in larger multi-farm studies.

Together, these results suggest that in low-AMU production systems, the environmental resistome is relatively stable over time and not strongly driven by contemporary antimicrobial exposure. Instead, ARG persistence likely reflects historical selection, environmental reservoirs, and non-antibiotic drivers such as management and environmental conditions. This highlights both the suitability of low-AMU settings for studying non-antibiotic risk factors and the methodological challenge of detecting subtle resistome changes in environments with low ARG abundance, reinforcing the need for sensitive and carefully optimised metagenomic approaches. In addition, as whole metagenome sequencing provides semi-quantitative information on ARG composition rather than absolute gene abundance, future studies combining metagenomics with quantitative approaches may help clarify between-farm differences in total resistome load.

4.3 Phenotypic versus genotypic AMR

In this thesis, no clear correlation was observed between resistome diversity and phenotypic resistance in *E. coli*, despite both being derived from the same longitudinal samples. This decoupling supports previous observations that high ARG abundance in metagenomic data does not necessarily translate into phenotypic resistance in indicator organisms (Horie *et al.,* 2021; Munk *et al.,* 2018). Phenotypic resistance reflects resistance traits in a limited subset of culturable bacteria, while resistome-based approaches capture resistance determinants across a much broader microbial community, including non-target species and unculturable taxa.

Several mechanisms may explain this discrepancy. First, many ARGs detected in metagenomic data may be intrinsic, poorly expressed, or not associated with the indicator species under investigation (Martínez *et al.,* 2015). Second, resistance genes may be present but not phenotypically expressed due to regulatory constraints, lack of selective pressure, or absence of compatible genetic backgrounds (Andersson & Hughes, 2010). Third, phenotypic testing is inherently limited by the choice of indicator organisms and antimicrobial panels, whereas metagenomics captures resistance potential beyond clinically relevant bacteria (Boolchandani *et al.,* 2019).

In low-antimicrobial-use settings such as Swedish pig production, these differences become particularly pronounced. Phenotypic resistance levels in *E. coli* were low and decreased over time, consistent with limited antimicrobial exposure and national surveillance data (EFSA, 2022). In contrast, the resistome remained relatively stable and dominated by ARG classes commonly reported in pig production systems, such as tetracycline, aminoglycoside, and macrolide resistance genes, even in the absence of corresponding treatments. This finding aligns with previous studies showing that resistomes can persist independently of recent AMU, likely due to historical selection, co-selection, and environmental persistence of ARGs (Munk *et al.,* 2018; Luiken *et al.,* 2019). Together, these results demonstrate that phenotypic and genotypic AMR metrics are not interchangeable but rather provide complementary insights.

To fully understand how metagenomic resistance potential translates into expressed phenotypic resistance, additional experimental layers are required. One important next step would be whole-genome sequencing of the cultured indicator isolates analysed phenotypically in this study. Linking isolate-level genomes to resistance phenotypes would enable direct comparison between detected ARGs, their genomic context, and observed susceptibility profiles, thereby improving interpretation of discrepancies between metagenomic and phenotypic findings.

An additional and largely unexplored dimension in farm-level AMR research is the incorporation of metatranscriptomic analyses. While metagenomics identifies which ARGs are present in a microbial community, it does not indicate whether these genes are transcriptionally active. Transcriptomic data would provide insight into which resistance genes are expressed under prevailing environmental and management conditions, allowing discrimination between dormant resistance potential and functionally active resistance determinants. When combined with phenotypic resistance data, such an integrated framework would enable a three-dimensional view of AMR in livestock environments: genotypic potential (metagenomics), functional activity (transcriptomics), and expressed resistance (phenotype). Although technically demanding and costly, such multi-omics approaches represent a promising avenue for future studies seeking to unravel the complex ecology and drivers of AMR in farm environments.

4.4 Farm-level risk factors and statistical considerations

In this work, a broad range of farm-level characteristics was collected based on their biological plausibility to influence the development, spread, or persistence of AMR. The full set of assessed factors is presented in Table 1 and includes AMU, biosecurity measures, housing characteristics, hygiene practices, environmental parameters, and management routines. While this comprehensive approach allowed a multidimensional description of the farm environments, not all collected variables were suitable for inclusion in statistical analyses. Several factors showed either minimal variability across farms or near-uniform presence or absence, limiting their statistical interpretability and leading to their exclusion from further modelling.

The collection of such detailed longitudinal data – both on potential risk factors and on the environmental resistome – is highly time- and resource-intensive, requiring repeated farm visits, close collaboration with farmers and veterinarians, and extensive laboratory and bioinformatic processing. Consequently, this study should be regarded primarily as an exploratory or pilot investigation, aimed at testing the applicability and sensitivity of the methodological framework rather than providing definitive confirmation of individual risk factor effects. Nevertheless, the results provide valuable empirical guidance for the design of larger, hypothesis-driven studies and help identify which variables merit prioritisation in future investigations.

Some assessed factors, such as pen cleanliness, were based on subjective evaluations at the time of sampling. While such assessments may capture biologically relevant variation, they also introduce observer-dependent uncertainty. For future large-scale studies, these variables would benefit from more objective or semi-quantitative measurements, for example through standardised scoring systems. Such systems could include structured cleanliness scores for drinking water systems to assess biofilm formation within pipes, as well as hygiene scoring of feeding and watering areas to capture differences in organic contamination. In addition, the use of quantitative indicators of organic load would improve reproducibility and reduce observer-related bias when assessing hygiene- and environment-related risk factors. Similarly, to improve statistical robustness and biological interpretability, categorical variables were deliberately simplified. Where appropriate, variables were reduced to a maximum of two biologically meaningful categories – for example, grouping floor materials as "concrete" versus "concrete combined with other materials" (e.g. straw, plastic, peat). This approach reduces sparsity in the data and improves model stability while preserving relevant biological contrasts.

From a statistical perspective, care was taken to minimise data-driven inference and avoid exploratory "significance hunting". Risk factors were first critically screened based on biological relevance and data structure, and univariable mixed-effects models were then used to identify variables showing at least a weak association ($p < 0.20$) with the outcomes of interest. Only these candidates were considered for inclusion in multivariable models, which were deliberately restricted in complexity to reduce overfitting given the limited number of observations. This stepwise strategy ensured that multivariable analyses were guided by prior biological reasoning as well as statistical evidence.

Two complementary AMR outcomes were analysed: resistome diversity, quantified using the Shannon diversity index, and phenotypic resistance, summarised as a composite resistance score for *E. coli*. The use of a single outcome per sampling occasion for each AMR dimension was a deliberate choice to maintain statistical power and interpretability. While analysing resistance patterns separately for individual antibiotic classes could provide additional detail, such an approach would substantially increase the number of models and overlook the well-documented co-occurrence and co-selection of resistance genes across antimicrobial classes. By focusing on integrated outcome measures, the analyses captured broader resistance patterns while avoiding fragmentation of the dataset. Alternative approaches, such as the use of other alpha-diversity indices (e.g. richness-based measures) or beta-diversity analyses to assess between-sample compositional differences, could potentially provide complementary insights and may be explored in future studies. However, these were not prioritised here to limit model complexity and preserve statistical power given the sample size.

In this work the multivariable analyses results showed that resistome diversity was primarily associated with non-antibiotic management and environmental factors, including copper concentration and mixing of sows and gilts, whereas no association was observed with biocide use, suggesting limited evidence for biocide-driven co-selection under the studied conditions. In contrast, phenotypic resistance in *E. coli* was associated with floor material, with lower resistance observed in pens with concrete flooring compared to other materials. A negative association between the phenotypic resistance score and the presence of flies was observed, indicating that higher fly presence was associated with lower levels of phenotypic resistance. However, this unexpected finding may reflect seasonal or production-stage confounding rather than a direct causal effect of flies. AMU did not remain in the final models for either outcome, likely reflecting the low and homogeneous AMU levels across farms and highlighting the relevance of non-antibiotic drivers of AMR in low-AMU production systems.

Overall, this risk-factor analysis highlights both the methodological challenges and the scientific value of studying AMR in low-antimicrobial-use livestock systems. The findings reinforce that disentangling the effects of AMU from other management and environmental drivers requires not only sensitive molecular tools, but also carefully structured epidemiological data and conservative statistical approaches. The framework developed in this study provides a foundation for future, larger-scale investigations aimed at identifying actionable non-antibiotic strategies for AMR mitigation.

4.5 Conclusion

Taken together, the findings of this thesis demonstrate that AMR in pig farm environments emerges from a complex interplay of biological, environmental, and farm management factors. Methodological decisions strongly influence resistome characterisation and must be carefully optimised and transparently reported. In production systems with low AMU, the environmental resistome appears to be shaped less by direct antibiotic selection and more by ecological persistence, management practices, and potential co-selection mechanisms. These insights highlight the importance of integrating environmental resistome analysis into One Health surveillance frameworks to expand AMR mitigation strategies beyond antimicrobial stewardship alone. However, while comprehensive data collection is desirable, effective surveillance requires approaches that remain simple, standardised, and feasible in terms of time and cost, necessitating careful prioritisation of *what* to sample (e.g. pig groups or environmental compartments), *when* to sample (number and timing of sampling points), *which* sequencing strategies to apply, and *which* resistance genes and farm-level risk factors to monitor.

5. Future perspectives

- **Refinement of sampling strategies**
  Future studies should compare sock sampling with individual animal sampling (e.g. faeces, rectal swabs) to quantify potential losses of low-prevalence ARGs and rare taxa. In early production stages, parallel sampling of sows, piglets, and the environment would help disentangle maternal, environmental, and piglet-associated contributions to resistome development.
- **Benchmark the proposed bioinformatic pipeline against established resistome analysis pipelines**
  Evaluate differences in ARG recovery, computational efficiency, and biological interpretability.
- **Integration of multi-omics approaches**
  Combining metagenomics with metatranscriptomics and isolate-level whole-genome sequencing would enable a more complete, multi-layered understanding of AMR, linking resistance gene presence, transcriptional activity, and expressed phenotypic resistance. Such integrated approaches would help clarify which ARGs are biologically relevant under specific farm conditions.
- **Advancement of long-read sequencing applications**
  Continued improvements in long-read sequencing technologies, particularly increases in accuracy and throughput, may enable routine recovery of ARG-host associations and mobile genetic elements. PacBio HiFi sequencing represents a promising future option, although its performance in highly strain-diverse livestock environments requires further evaluation.
- **Improved quantification of resistome burden**
  Development and validation of approaches for estimating absolute ARG abundance – such as the integration of quantitative standards, spike-ins, or complementary qPCR measurements – would strengthen causal inference and improve comparisons across farms, time points, and production systems.
- **Larger-scale longitudinal and comparative studies**
  The exploratory findings of this thesis highlight the need for larger, multi-farm longitudinal studies with sufficient statistical power to robustly assess non-antibiotic drivers of AMR. Comparative studies

across production systems with differing AMU intensities would help clarify context-dependent effects.

- **Standardisation of farm-level data collection**
  Future research would benefit from more standardised and objective measurements of management and hygiene factors (e.g. cleanliness scores, environmental load indicators), reducing subjectivity and improving comparability across studies.

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Popular science summary

Antimicrobial resistance is one of the most serious global health challenges of our time. Bacteria that can resist antibiotics make infections harder to treat in both humans and animals, and resistant bacteria and their resistance genes can spread between people, animals, and the environment. Because of this close connection, antimicrobial resistance is considered a One Health problem that requires solutions across medical, veterinary, and environmental fields.

Farm animals play an important role in the ecology of antimicrobial resistance. Livestock farms host large and diverse microbial communities, and resistance genes can persist in animals, manure, on housing surfaces, and in the wider farm environment. In modern livestock systems, the use of antibiotics is known to be the main driver that accelerates the selection and spread of resistance genes, but recent research suggests that other factors - such as housing conditions, hygiene, and the surrounding environment - may also influence how resistance persists, especially in systems where antibiotic use is low. However, these non-antibiotic factors are difficult to study in systems where antibiotic use is high, because strong antibiotic pressure can mask their effects.

In this thesis, I focused on pig farms in Sweden, a country with a long history of strict and very low antibiotic use in livestock, as well as high standards of animal health and biosecurity. This provided a unique opportunity to study antimicrobial resistance in a setting where antibiotic pressure is minimal. The work first developed and optimised a practical and scalable method to study resistance genes in the farm environment using modern DNA-based techniques. A simple “sock sampling” method was used to collect material from pig pen floors, followed by shotgun metagenomic sequencing, which allows detection of thousands of resistance genes at once.

Using this approach, the environmental resistome - the collection of all resistance genes in the pigs’ immediate environment - was studied in ten pig farms across an entire production cycle. Although the microbial communities in the environment changed over time, particularly around weaning, the resistance gene profiles were relatively stable and showed no clear link to antibiotic use. This reflects the low antibiotic pressure in Swedish pig production. Importantly, resistance genes detected by DNA sequencing did not always match resistance observed in laboratory testing of bacteria, showing that genetic potential for resistance and actual expressed resistance represent different but complementary aspects of the problem.

The study also examined farm-level risk factors and found that non-antibiotic factors influenced resistance genes and phenotypic resistance in different ways. These findings highlight that antimicrobial resistance in farm environments is shaped by a complex combination of biological, environmental, and management factors, not antibiotics alone.

Overall, this thesis provides a methodological foundation for future large-scale studies of antimicrobial resistance in livestock environments. By combining practical sampling, sensitive genomic tools, and careful epidemiological analysis, this work contributes to a better understanding of how resistance persists and spreads in the pig farm environment and supports the development of more effective, sustainable antimicrobial resistance mitigation strategies within a One Health framework.

Populärvetenskaplig sammanfattning

Antimikrobiell resistens är ett av de allvarligaste globala hälsohoten i vår tid. Bakterier som kan motstå antibiotika gör infektioner svårare att behandla hos både människor och djur, och resistenta bakterier och deras resistensgener kan spridas mellan människor, djur och miljön. På grund av dessa nära samband betraktas antimikrobiell resistens som ett One Health-problem som kräver lösningar inom både human- och veterinär-medicin samt miljösektorn.

Lantbruksdjur spelar en viktig roll för antimikrobiell resistens. Djurhållningssystem rymmer stora och diversifierade populationer av mikrober och resistensgener vilka kan finnas i djuren, deras gödsel, stallmiljön och den omgivande gårdsmiljön. I moderna produktionssystem är användningen av antibiotika den främsta faktorn som driver selektion och spridning av resistensgener. Samtidigt tyder ny forskning på att andra faktorer - såsom stallmiljö, hygien och omgivande miljö - också kan påverka hur resistens kvarstår, särskilt i system med låg antibiotikaanvändning. Dessa icke-antibiotikarelaterade faktorer är dock svåra att studera i miljöer med hög antibiotikaanvändning, där starkt selektionstryck kan maskera andra effekter.

I denna avhandling har jag fokuserat på grisgårdar i Sverige, ett land med lång tradition av strikt och mycket låg antibiotikaanvändning inom djurhållningen, samt höga krav på djurhälsa och biosäkerhet. Detta gav en unik möjlighet att studera antimikrobiell resistens i en miljö där antibiotikaselektionen är minimal. Arbetet inleddes med utveckling och optimering av en praktisk och skalbar metod för att studera resistensgener i gårdsmiljön med hjälp av moderna DNA-baserade tekniker. En enkel så kallad ”sockprovtagning” användes för att samla material från stallgolv. Från materialet isolerades DNA som sedan analyserades med metagenomisk sekvensering, vilken möjliggör samtidig detektion av tusentals resistensgener.

Med denna metod studerades resistomet - den samlade uppsättningen resistensgener i grisarnas närmiljö - på tio grisgårdar under en hel produktionscykel. Trots tydliga förändringar i bakteriepopulationerna över tid, särskilt kring avvänjning, var resistomet relativt stabilt och visade inget tydligt samband med antibiotikaanvändning. Detta speglar det låga antibiotika-selektionstrycket i svensk grisproduktion. Det var också tydligt att resistensgener som identifierades genom DNA-sekvensering inte alltid reflekterade den resistens som observerades vid laboratorietester av odlade bakterier från samma prover, vilket visar att de gener som utgör potentialen för resistens och den resistens som uttrycks av de bakterier som odlas fram utgör olika men kompletterande aspekter av problemet.

Studien analyserade även riskfaktorer på gårdsnivå och visade att icke-antibiotikarelaterade faktorer påverkade resistensgener och uttryckt resistens på olika sätt. Dessa resultat understryker att antimikrobiell resistens i gårdsmiljöer formas av ett komplext samspel mellan biologiska, miljömässiga och skötselrelaterade faktorer - inte enbart av antibiotikaanvändning.

Sammanfattningsvis ger denna avhandling en metodologisk grund för framtida storskaliga studier av antimikrobiell resistens i djurmiljöer. Genom att kombinera praktisk tillämpbar provtagning, känsliga genomiska verktyg och noggrann epidemiologisk analys bidrar arbetet till en ökad förståelse för hur resistens kvarstår och sprids i grisgårdsmiljöer, och stödjer utvecklingen av mer effektiva och hållbara strategier för att motverka antimikrobiell resistens inom ramen för One Health.

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BRIEF COMMUNICATION **Open Access**

Antimicrobial resistance among indicator *Enterococcus faecium* and *Escherichia coli* in Swedish pig farms

Valeriia Ladyhina[1*], Susanna Sternberg-Lewerin[1], Linus Andersson[2] and Elisabeth Rajala[1]

Abstract

Monitoring the use of antimicrobials and the emergence of resistance in animals and people is important for the control of antimicrobial resistance, and for establishing sustainable and effective disease management practices. In this study, we used *Enterococcus* spp. and *Escherichia coli* as indicator species to investigate antimicrobial susceptibility patterns and how these change over time, on ten Swedish pig farms. Indoor environmental sock sampling was performed once a month during the entire production cycle of one batch of pigs on each farm, resulting in 60 samples collected in total. Selective culture for *E. coli* and *Enterococcus* spp. resulted in 122 isolates of *E. coli*, 74 isolates of *E. faecium*, but no isolates of *E. faecalis*. Microdilution was used to determine minimum inhibitory concentrations for twelve antimicrobial substances in *E. coli* and fifteen substances in *E. faecium*. The overall prevalence of resistance was low. Among the *E. coli* isolates, the proportions non-wild type (resistant, NWT) isolates were as follows: azithromycin and amikacin 1% (n = 1), trimethoprim and sulfamethoxazole 2% (n = 3), ampicillin 6% (n = 7) and tetracycline 9% (n = 11). Among the *E. faecium* isolates, the NWT proportions were: teicoplanin, linezolid and gentamicin 1% (n = 1), daptomycin 3% (n = 2), erythromycin 26% (n = 19), tetracycline 27% (n = 20), quinupristin/dalfopristin 58% (n = 42). The resistance patterns differed between the farms, likely due to different antimicrobial use, biosecurity measures and source of the animals. The NWT prevalence among *E. coli* decreased over time, whereas no similar trend could be observed in *E. faecium*. The results of the current study illustrate the complex factors affecting the antimicrobial resistance patterns observed on each farm, indicating that specific practices and risk factors have an impact on the prevalence and type of antimicrobial resistance. Further studies of the farm environments in combination with antimicrobial use and other risk factor data are needed to elucidate the multifaceted drivers of antimicrobial resistance development on livestock farms.

Keywords AMR, Environmental sampling, Indicator bacteria, Pig, Surveillance, Swine

Findings

Antimicrobial resistance (AMR) is a serious health threat for animals and humans that requires urgent actions [1]. The consequences of AMR for animals are similar to those for humans e.g., treatment failures leading to suffering and decreased welfare, increased mortality, and reduced productivity with major impact on livelihoods and global food security [2]. Monitoring the use of antimicrobials and the emergence of resistance in animals and people is important for the control of

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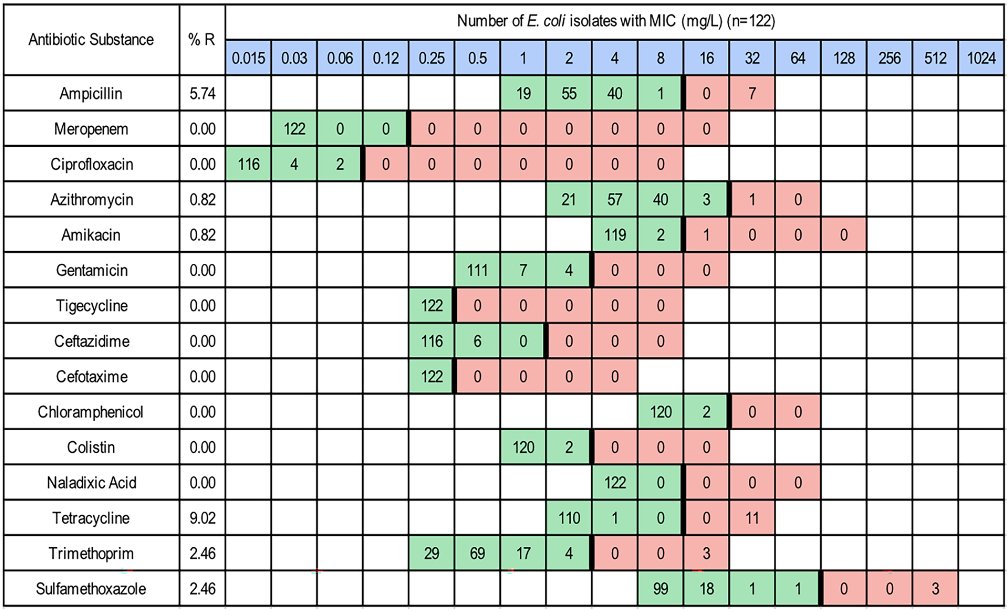
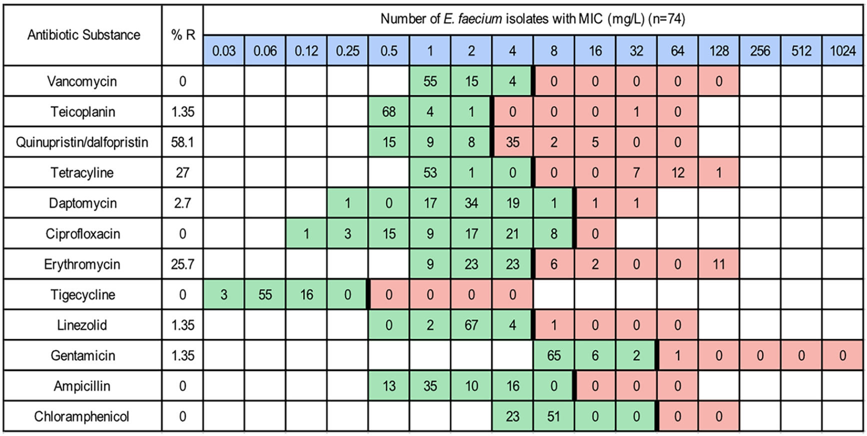
AMR, and for establishing sustainable and effective disease management practices. The overall aim of this study was to investigate the prevalence of AMR in selected Swedish pig farms using two indicator bacteria, *Escherichia coli* and *Enterococcus* spp. Specific objectives were to (i) investigate if AMR differed between the farms, (ii) how AMR changed over time, and (iii) to assess the differences in the temporal dynamics of the resistance patterns between the two indicator species.

The material used in this study originated from environmental sock samples (boot swabs) obtained in 2023 from ten farrow-to-finish pig farms located at a maximum two hours driving distance from Uppsala. Sock samples have proven to be efficient for obtaining a representative picture of the bacteria that are present in a pig herd [3]. One group of pigs from each farm was selected for the 6 month study, with monthly visits throughout the entire production cycle. A total of 60 samples were collected, one pooled sample per herd and sampling occasion. The method is described here in brief, for more details see Additional file 1. For each sampling occasion, samples were kept cold and immediately transported to the laboratory at the Swedish University of Agricultural Sciences (SLU). Upon arrival at the laboratory, material was extracted from the sock samples by immersing them in sterile buffered peptone water. The samples were then processed in a stomacher, followed by centrifugation for sample concentration. Finally, they were preserved in 86% glycerol and stored at −80 °C for long-term storage. The thawed sample eluate was inoculated onto selective agar plates, MacConkey agar for detection of *E. coli*, and Slanetz and Bartley (SlaBa) for detection of *Enterococcus* spp. From each sample, two isolates with typical morphology were selected and Matrix Assisted Laser Desorption/Ionizaton–Time-of-Flight (MALDI-TOF) was used to confirm the identification of the isolates. Microdilution using Sensititre™ (ThermoFisher Scientific Inc., Waltham, MA, USA) panels was used to determine minimum inhibitory concentrations (MIC) for twelve antimicrobial substances in *E. coli* and fifteen substances in *Enterococcus* spp. Epidemiological cut-off values for the MIC, as determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [4] were used to classify isolates as belonging to the wild-type drug-susceptible population or the non-wild type (NWT) population and likely to be resistant to the tested drug. Data analysis and descriptive statistics were done in Microsoft® Excel and data visualization was performed with R (v4.3.1) [5] using package ggplot2 (v3.4.4) [6].



Antibiotic Substance	% R	Number of *E. coli* isolates with MIC (mg/L) (n=122)																
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024
Ampicillin	5.74							19	55	40	1	0	7					
Meropenem	0.00		122	0	0	0	0	0	0	0	0	0						
Ciprofloxacin	0.00	116	4	2	0	0	0	0	0	0	0							
Azithromycin	0.82								21	57	40	3	1	0				
Amikacin	0.82									119	2	1	0	0	0			
Gentamicin	0.00						111	7	4	0	0	0						
Tigecycline	0.00					122	0	0	0	0	0							
Ceftazidime	0.00					116	6	0	0	0	0							
Cefotaxime	0.00					122	0	0	0	0								
Chloramphenicol	0.00										120	2	0	0				
Colistin	0.00							120	2	0	0	0						
Naladixic Acid	0.00									122	0	0	0	0				
Tetracycline	9.02								110	1	0	0	11					
Trimethoprim	2.46					29	69	17	4	0	0	3						
Sulfamethoxazole	2.46										99	18	1	1	0	0	3	

Fig. 1 Distribution of MIC values of *E. coli* isolates (n = 122). Red and green cells indicate the range of tested concentrations. Vertical black lines indicate EUCAST epidemiological cutoffs



Antibiotic Substance	% R	Number of *E. faecium* isolates with MIC (mg/L) (n=74)															
		0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024
Vancomycin	0						55	15	4	0	0	0	0	0			
Teicoplanin	1.35					68	4	1	0	0	0	1	0				
Quinupristin/dalfopristin	58.1					15	9	8	35	2	5	0	0				
Tetracyline	27						53	1	0	0	0	7	12	1			
Daptomycin	2.7				1	0	17	34	19	1	1	1					
Ciprofloxacin	0			1	3	15	9	17	21	8	0						
Erythromycin	25.7						9	23	23	6	2	0	0	11			
Tigecycline	0	3	55	16	0	0	0	0	0								
Linezolid	1.35					0	2	67	4	1	0	0	0				
Gentamicin	1.35									65	6	2	1	0	0	0	0
Ampicillin	0					13	35	10	16	0	0	0	0				
Chloramphenicol	0								23	51	0	0	0	0			

Fig. 2 Distribution of MIC values of *E. faecium* isolates (n = 74). Red and green cells indicate the range of tested concentrations. Vertical black lines indicate EUCAST epidemiological cutoffs

From the 60 samples, 122 isolates of *E. coli*, 74 isolates of *E. faecium*, but no isolates of *E. faecalis,* were identified and further analysed. The proportions of NWT (resistant) *E. coli* were as follows: azithromycin and amikacin 1% (n = 1), trimethoprim and sulfamethoxazole 2% (n = 3), ampicillin 6% (n = 7) and tetracycline 9% (n = 11) (Fig. 1). Among the *E. faecium* isolates, the NWT (resistant) proportions were: teicoplanin, linezolid and gentamicin 1% (n = 1), daptomycin 3% (n = 2), erythromycin 26% (n = 19), tetracycline 27% (n = 20), quinupristin/

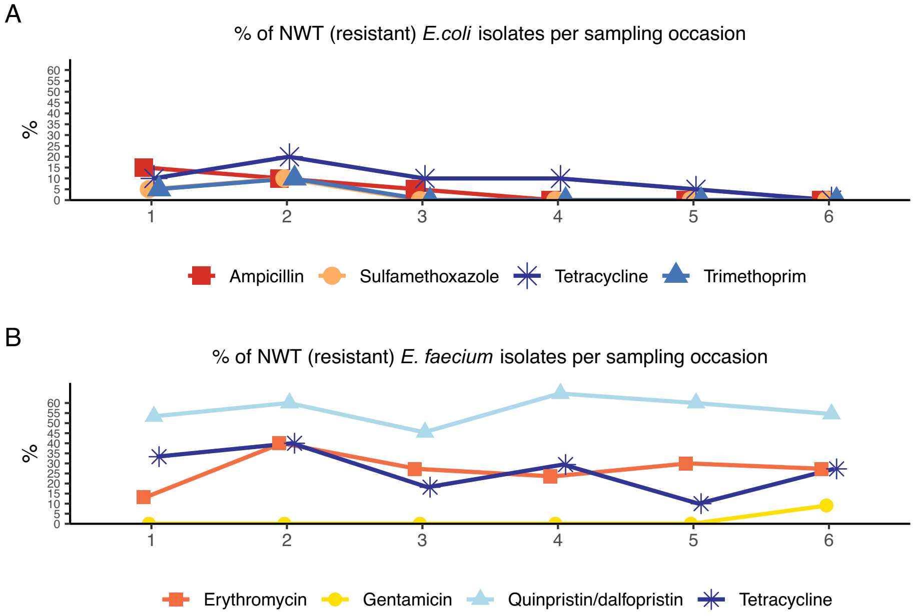


Fig. 3 Proportions of non-wild type isolates from Swedish pig farms over a 6-month period. A. *E. coli* isolates (n = 122), B *E. faecium* (n = 74)

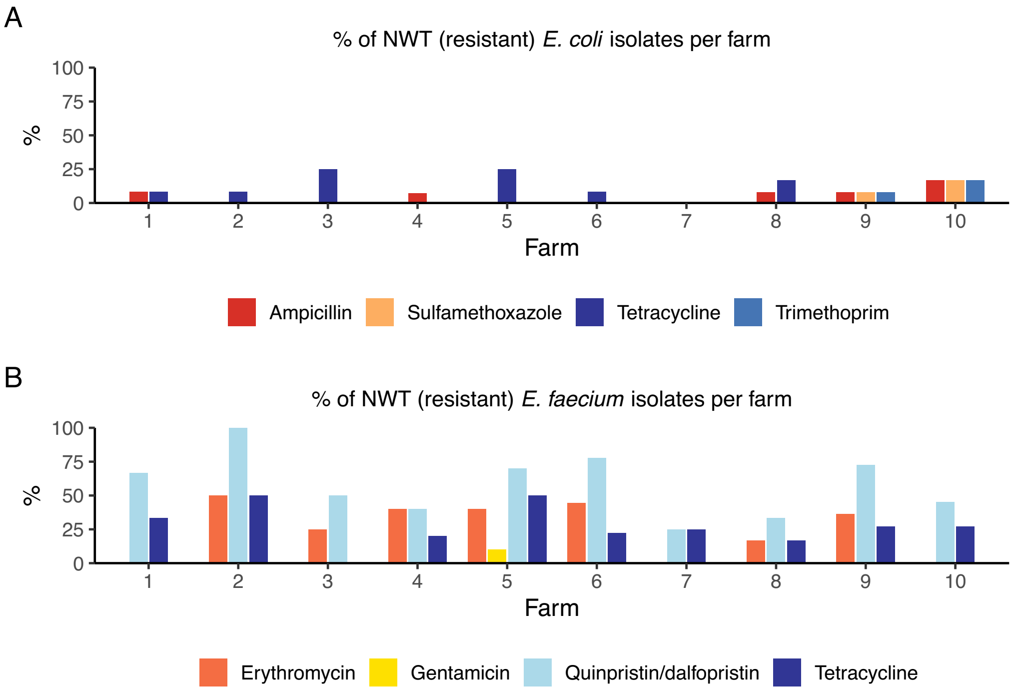


Fig. 4 Proportions of non-wild type isolates from 10 Swedish pig farms. A. *E. coli* isolates (n = 122), B *E. faecium* (n = 74)

dalfopristin 58% (n = 42) (Fig. 2). A majority of the *E. faecium* isolates classified as NWT (resistant) to quinupristin/dalfopristin, tetracycline and erythromycin had MIC values just above the epidemiological cutoff. Among the *E. coli* isolates, AMR decreased over time (Fig. 3A), while no such trend could be observed in the *E. faecium* isolates (Fig. 3B). The AMR patterns for each farm were different for both bacteria (Fig. 4). All farms except farm 7 had *E. coli* isolates that exhibited resistance to either ampicillin or tetracycline, or both, at some point during the production cycle (Fig. 4A). From farms 7, 8, 9 and 10, there were also isolates that were NWT (resistant) to at least one of the following substances: azithromycin, amikacin, trimethoprim or sulfamethoxazole. All farms yielded *E. faecium* isolates NWT (resistant) to quinupristin/dalfopristin. In addition, all farms except farm 3 had isolates with resistance to tetracycline at some point in the production cycle (Fig. 4B).

The results demonstrated differences in AMR patterns between the investigated pig farms, likely due to variation in antimicrobial use and other risk factors between farms, as previously reported [7]. The overall prevalence of AMR in *E. coli* was low, which is in line with previous published research in Sweden [7, 8]. A study from New Zealand presented similar findings as the current study with *E. coli* being susceptible for ciprofloxacin, but NWT (resistant) for ampicillin and tetracycline [9]. In contrast, a study in Spanish pig herds demonstrated much higher proportions of NWT *E. coli*, for most antibiotics tested [10]. The overall prevalence of AMR in *E. faecium* was higher compared to *E. coli*, this was also similar to the New Zealand study [9]. The higher AMR prevalence in *E. faecium* could partly be explained by this species exhibiting intrinsic low susceptibility to certain antimicrobials, e.g., quinupristin [11]. All *E. faecium* isolates in the current study were susceptible to ampicillin, and vancomycin, while resistance to erythromycin was seen on nearly all farms. Similar prevalence of resistance to erythromycin and tetracycline in *E. faecium* have previously been reported in Estonia [12], but higher prevalence has been shown in southern Europe (Portugal [13] and Spain [10, 14]). The current study also showed that AMR in *E. coli* decreased during the lifespan of the pigs, which is in line with previous research [15, 16]. This result reflects common practices of antimicrobial use, where most treatments in pig production in Sweden are administered to piglets [7].

The initial plan was to isolate both *E. faecium* and *E. faecalis*. However, most samples yielded no colonies of *E. faecalis*, despite a repeated attempt to isolate these from the stored frozen samples. This may be due to the sampling methodology, while individual fecal sampling or rectal swabs might have made detection of *E. faecalis* isolates more feasible. However, other European studies have also shown a higher prevalence of *E. faecium* than *E. faecalis* in faecal samples from pig farms [10, 16, 17]. The predominant isolation of *E. faecium* can be explained by its presence in typical fecal microbiota and its tendency to survive longer than other enterococci on dry material [18]. In addition, the microdilution method, with subsequent two-fold dilution steps may yield a one-step deviation in MIC results [4]. This is particularly of note when considering results that are just above or below cutoff, such as the quinupristin/dalfopristin resistance observed in our *E. faecium* isolates. However, this would only have had a small effect on the results of this study, as most MIC results leading to classification of an isolate as NWT (resistant) were above the cutoff by more than one step.

In conclusion, the overall prevalence of AMR in *E. coli* was low among the tested pig herds, while a higher prevalence of AMR was observed in *E. faecium*. Furthermore, the AMR prevalence differed between farms and decreased with age among *E. coli*. The results of the current study emphasize the complex factors leading to the specific AMR pattern observed on a specific farm. Further studies of entire farm environments in combination with data on antimicrobial use and other risk factors are needed to elucidate the multifaceted drivers of AMR development.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13028-024-00756-8.

Supplementary Material 1.

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Author contributions
ER, VL, and SSL designed the study. LA performed the laboratory work, ER, VL, SSL and LA analysed the data, ER, VL and SSL drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The project followed all national and international codes of conduct regarding legal and ethical aspects in research. No ethical approval was required as no personal or sensitive data, and no samples from humans or animals were collected.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Supplementary Information

Methods

The material used in this study originated from environmental sock samples (boot swabs) obtained from ten Swedish farrow-to-finish pig farms. One batch of pigs on each farm was followed by monthly sampling visits during the 6-month production cycle. The sampling was performed from February 2023 until August 2023 and started when the piglets reached an age of ~1 week and ended 1-2 weeks before slaughter. Depending on the number of pens, 3 or 4 pairs of socks samples were collected per farm visit. One hundred steps were taken in each pair of socks and sampling was performed in such way as to equally cover the whole group of pigs spatially. For most of the farms, collection of samples was performed in different pens in the same building. However, starting from the age of 2 months in farm 5 and 6, the pig group of interest was distributed in 2 different buildings. To avoid contamination from the environment around the pens of these pigs, additional plastic boot covers were used.

The samples were kept cold during transport to the laboratory at SLU immediately after sampling. Upon arrival at the lab, 50 ml of sterile buffered peptone water (BPW) was added to each sample and extraction performed by stomacher. After centrifugation at 3000g for ten minutes, the pellet was resuspended in 15 ml of BPW. All samples from each visit were pooled into one, resulting in a total of 60 samples (one pooled sample per farm per visit). In order to preserve the bacteria, glycerol 86 % was added to the samples before storage at -80°C.

For this study, the samples were thawed, re-suspended in BPW to obtain a concentration of 0.4g/ml and vortexed before inoculating onto selective agar plates. For culture of *Escherichia coli*, MacConkey agar plates (one per sample) were incubated for 24 hours at 35°C, and for *Enterococcus* spp. Slanetz and Bartley agar plates (four per sample) were incubated for 48 hours at 44°C. Two typical colonies were picked from each plate and sub-cultured to obtain pure cultures. Bacterial species confirmation was performed by Matrix Assisted Laser Desorption/Ionizaton–Time-of-Flight (MALDI-TOF).

Each confirmed isolate was subjected to antimicrobial susceptibility testing by Sensititre™ (ThermoFisher Scientific Inc., Waltham, MA, USA) panels, according to the manufacturer's instructions. Sensititre EUVENC panels were used for *Enterococcus faecium* and Sensititre EUVSEC3 were used for *E. coli*. Two control strains: *E. coli* (ATCC25922) and *Enterococcus faecalis* (ATCC29212), provided by the manufacturer, were used for quality control.

Epidemiological cut-off values, as determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used to classify isolates as either wild-type drug-susceptible or non-wild type that likely to be resistant to the tested drug. Data collection and analysis was done in Microsoft® Excel (Microsoft Co. Redmond, WA, USA). Data visualisation was performed using Microsoft® Excel (Microsoft Co. Redmond, WA, USA), R (v4.3.1) and Rstudio (v2023.09.0+463). The majority of plots were visualized using the ggplot2 (v3.4.4) package.

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Methodological aspects of investigating the resistome in pig farm environments

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ABSTRACT

A typical One Health issue, antimicrobial resistance (AMR) development and its spread among people, animals, and the environment attracts significant research attention. The animal sector is one of the major contributors to the development and dissemination of AMR and accounts for more than 50 % of global antibiotics usage. The use of antibiotics exerts a selective pressure for resistant bacteria in the exposed microbiome, but many questions about the epidemiology of AMR in farm environments remain unanswered. This is connected to several methodological challenges and limitations, such as inconsistent sampling methods, complexity of farm environment samples and the lack of standardized protocols for sample collection, processing and bioinformatical analysis. In this project, we combined metagenomics and bioinformatics to optimise the methodology for reproducible research on the resistome in complex samples from the indoor farm environment. The work included optimizing sample collection, transportation, and storage, as well as DNA extraction, sequencing, and bioinformatic analysis, such as metagenome assembly and antibiotic resistance gene (ARG) detection. Our studies suggest that the current most optimal and cost-effective pipeline for ARG search should be based on Illumina sequencing of sock sample material at high depth (at least 25 M 250 bp PE for AMR gene families and 43 M for gene variants). We present a computational analysis utilizing MEGAHIT assembly to balance the identification of bacteria carrying ARGs with the potential loss of diversity and abundance of resistance genes. Our findings indicate that searching against multiple ARG databases is essential for detecting the highest diversity of ARGs.

1. Introduction

Antimicrobial resistance (AMR) is a typical One Health problem that imposes a considerable burden on global health and economy (Murray et al., 2022). AMR in pathogenic bacteria poses substantial challenges to effective treatment, leading to increased healthcare costs both in human and animal medicine. Globally, intensive livestock production is among the major consumers of antimicrobials (Van Boeckel et al., 2015). The increasing prevalence of resistant bacteria in livestock raises concerns for animal health. In addition, livestock may serve as a reservoir for resistant zoonotic bacteria and resistance genes that can transfer to human pathogens (Tang et al., 2017). Antimicrobial usage (AMU) is recognized to be one of the main causes for the emergence of AMR in bacteria found in humans, animals, and the environment. Recent studies on livestock have shown that there are additional risk factors such as different biosecurity measures, and co-selection of antibiotic resistance genes (ARGs) in the presence of biocide and metal resistance genes within the farm environment (Horie et al., 2021; Li et al., 2022).

Most surveillance of AMR is based on culturing of indicator bacteria. This approach provides an insight into the phenotypic resistance but may not be representative for the overall occurrence of ARGs in the commensal microbiota as it exludes non-culturable bacteria (Andersen et al., 2017). Another popular screening method is quantitative real-time polymerase chain reaction (qPCR) focused on a limited selection

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of ARGs (Graesboll et al., 2019; Luiken et al., 2022; Yang et al., 2022). However, this method is insufficient to obtain a comprehensive picture of the bacterial community and the resistome. Recent technological developments in molecular biology have increased our ability to investigate and quantify the gene content of microbial communities by whole metagenome sequencing. The main advantage of using metagenomics is the ability to look at the whole microbiome community including bacterial species that cannot be cultured in vitro and the resistome, including both known and novel ARGs in samples, improving our understanding of microbial communities (Noyes et al., 2016; Pillay et al., 2022). Although metagenomics is a very promising technique for resistance analysis, there is still only a limited number of studies that use this technique, escpecially in the area of non-clinical longitudinal research. This can be explained by the complexity of samples as well as a lack of standardized protocols for sample collection and further sample manipulation, including steps of sequencing and bioinformatic analysis. Previous methodology-based studies usually focused on comparison of one specific step of the resistome analysis pipeline. Many studies address the problem of randomisation and pooling of individual animal samples or fresh droppings (Yamamoto et al., 2014; Munk et al., 2017; Andersen et al., 2021). Another question for debate is the selection of sequencing technique: short reads, long read based sequencing or a combination of both methods. Most of the currently published studies on the resistome in farm environments are based on shotgun metagenome sequencing (Illumina) of individual fecal samples, which is still seen as a golden standard in metagenome analysis of resistance due to the possibility to obtain big amounts of good quality sequence data at a low cost (Luiken et al., 2019; Van Gompel et al., 2019; Mencia-Ares et al., 2020; Stevens et al., 2023). However, the length of the short reads is not enough to cover the length of most ARGs and, therefore, a search on raw Illumina reads results in missing genomic context and can lead to appearance of false positives due to spurious mapping (Boolchandani et al., 2019). This problem can be solved in two ways: either perform assembly of short reads or use a sequencing technique based on long reads. There is a list of studies that suggest that long read sequencing (Oxford Nanopore Technologies) is the future for resistome research as it helps to avoid the computationally heavy step of assembly while providing enough of genome context information to not only recover ARGs but also their connection to the microbiome (Weinmaier et al., 2023; Sierra et al., 2024; Slizovskiy et al., 2024). However, long-read sequencing suffers from low-accuracy base calling and the excessive costs of high-depth sequencing needed for high complexity samples (Brown et al., 2021; Zhao et al., 2023; Abramova et al., 2024). Therefore, short-read sequencing is often required to improve genome accuracy, which increases costs and turnaround time (Brown et al., 2021; Zhao et al., 2023; Abramova et al., 2024). As stated earlier, another way to tackle the problem of detection of false positives is to perform assembly, however this is hampered by the tendency of metagenomic assemblies to break around ARGs (Abramova et al., 2024). Another issue is that each additional step of analysis, such as assembly or binning, results in some loss of data. Furthermore, different assemblers employ varying algorithms for constructing contigs, making the choice of assembler a trade-off between the quality of the resulting assemblies, computational requirements, the time required for assembly and the sequencing depth that the assembler can handle. Previous research also showed that sample complexity may affect the choice of the most suitable assembler (Abramova et al., 2024). Therefore, constant development of the technology of long read sequencing as well as new and improved assembly tools requires the re-evaluation of existing pipelines for ARG search for each specific sample type. Another important question that is often addressed in many studies but not thoroughly evaluated is the sequencing depth required to obtain an adequate representation of the resistome. To our knowledge, only one study has specifically addressed this question (Gweon et al., 2019). However, changes in sample material and the anticipated baseline levels of resistance may necessitate recalculating the appropriate sequencing depth. The last but not least step in resistome analysis is the search for ARGs and choice of a suitable database. There is a list of articles that compare advantages and disadvantages of different ARG-databases based on sample and goal for research (de Abreu et al., 2020; Papp and Solymosi, 2022). While metagenomic sequencing has advanced resistome analysis, most previous studies have focused on individual steps of the pipeline, such as sequencing techniques or assembly methods, rather than addressing the pipeline as a whole. We propose that a comprehensive approach, tailored to sample type, complexity, and research goals, is crucial for accurate and reliable ARG detection.

To our knowledge, this study is the first to focus on a comprehensive evaluation of different steps in the environmental metagenomics research pipeline with the aim of advancing farm resistome analysis. This included evaluating various sample transportation temperatures, comparing usage of short and long-read sequencing techniques for microbiome analysis as well as evaluation of tools for assembly and ARG search in the bioinformatical pipeline for investigation of the environmental resistome.

2. Materials and methods

2.1. Sampling and experimental design

The material used in this study originated from environmental sock samples (Fig. 1) collected from the Swedish University of Agricultural Sciences (SLU) pig farm at the Swedish Livestock Research Centre in Lövsta, outside Uppsala, Sweden. Samples were collected in March 2022 from four pens, each housing 10 to 13 three-month-old pigs from the same batch. The pens were located in different parts of the same room. All pigs were managed the same way, except that the pigs in pen number one had recently been treated with penicillin. Sterile gauze socks were put on clean boots covered with clean disposable foot cover. One pair of socks was used in each pen and in every pen, 100 steps were taken, turning the socks 90° after every 25 steps. The study was divided into two parts.

2.1.1. Part 1

Technical aliquots of one sample (Sample 0) were used for comparison of three commercial DNA extraction kits in terms of their influence on the taxonomic composition of the sample as well as compatibility with long-read sequencing (Oxford Nanopore Technologies plc., Oxford, UK (ONT)), which relies on high-quality DNA with long, intact fragments for optimal performance. This sample was handled on the day of collection and kept at −80 °C for long-term storage.

2.1.2. Part 2

Seven samples were used for investigation of the effect of

Fig. 1. Sock sampling: 20 cm long elastic tubular retention bandage over single use plastic boot coverage.

temperature during transportation and sequencing technique on the taxonomical profile and abundance of ARGs, as well as for the development of a bioinformatics pipeline, addressing crucial steps of assembly and ARG search. To simulate different transportation methods, we compared three schemes of storage before extraction of sock samples in the lab. For simulation of transportation of material in the best conditions (on dry ice), sock samples were handled on the day of sampling. To mock sample transportation via ordinary mail, a two-day storage was done at two different temperatures: room temperature (RT, +20 °C) for samples that would be transported without cooling, and + 4 °C to mock sample transportation on ice pack. For handling of the samples, the socks were soaked in buffered peptone water (50 ml per sock) and put in a stomacher to extract sample material. The resulting suspension was concentrated by centrifuging (3000 g for 10 min) and the pellet was resuspended in 15 ml of supernatant. After this, samples were transferred to 1.8 ml cryotubes for storage at −80 °C. A more detailed description of the study procedure can be found in Table 1.

2.2. DNA extraction

DNA was extracted from three technical replicates of sample 0 in order to compare three commercially available kits: (1) ZM = ZYMOBiomics MagBead DNA/RNA (Zymo Research, Irvine, CA, USA), (2) EZ = E.Z.N.A Universal Pathogen Kit (Omega Bio-tek, Norcross, GA, USA) and (3) MP = MagPure Stool DNA LQ Kit (Magen Biotechnology, Guangzhou, Guangdong, China). All three kits employ a bead beating step in order to achieve mechanical lysis which was performed according to the protocol settings either using Precellys Evolution homogenizer (10,000 RPM, 4 cycles × 60 s with a 60 s pause in between each cycle for ZM and MP) or using a Vortex-Genie 2 (Scientific Industries, Inc., Bohemia, NY, USA) at the highest speed for 5 min (EZ). Two out of three kits are based on magnetic bead purification (ZM and MP), while EZ includes a step of DNA purification with a MicroElute Column. The extraction protocols were carried out following the manufacturers' guidelines.

For further comparison of effects of storage temperature and sequencing technique, the EZ kit was used for DNA isolation but with minor adjustments compared with the manufacturers protocol: 1) After bead beating disruption, the entire volume of supernatant in the disruptor tube was transferred to a centrifuge tube and diluted in RBB buffer (in volume equal to double volume of supernatant); 2) the whole volume of the obtained sample was transferred and centrifuged in a MicroElute column. These protocol modifications markedly increased the yield of extracted DNA (2–3 fold) without compromising its quality. This is particularly advantageous for nanopore sequencing, which often requires a DNA cleanup step that can result in the loss of at least half of the initial DNA quantity. We also compared two bead-beating options: via vortexing at the maximum speed for 5 min and using Precellys Evolution homogenizer (10,000 RPM, 4 cycles × 60 s with a 60 s pause) and its compatibility for short and long reads sequencing. A 1 % agarose gel electrophoresis was used for estimation of the length of DNA fragments obtained by using these two bead-beating schemes. Based on the results, aliquots of samples obtained via Precellys Evolution homogenizer were used for further Illumina sequencing, while vortexing was used for DNA extraction for nanopore sequencing. The DNA quality was assessed using a NanoDrop spectrophotometer, and Qubit dsDNA HS and BR Assay Kits were used for quantification of the concentration of extracted DNA.

Table 1
Overview of the procedure comparisons done in the study.

Study part	Sample	Pig pen	Storage prior to handling	DNA extraction kit	Sequencing technique
1	Sample 0	0	0d	EZ	ONT
1	Sample 0	0	0d	ZM	ONT
1	Sample 0	0	0d	MP	ONT
2	Sample 1	1	0d	EZ	ONT, Illumina
2	Sample 2	1	2d at +4 °C	EZ	ONT, Illumina
2	Sample 3	1	2d at RT	EZ	ONT, Illumina
2	Sample 4	2	0d	EZ	ONT, Illumina
2	Sample 5	2	2d at RT	EZ	ONT, Illumina
2	Sample 6	3	2d at +4 °C	EZ	ONT, Illumina
2	Sample 7	3	2d at RT	EZ	ONT, Illumina

RT – room temperature, EZ - E.Z.N.A Universal Pathogen Kit, ZM - ZYMOBiomics MagBead DNA/RNA, MP - MagPure Stool DNA LQ Kit, ONT – Oxford Nanopore Technology.

2.3. DNA sequencing

2.3.1. Nanopore sequencing

Nanopore sequencing was performed on all samples of the study. One nanopore library preparation per sample was constructed using the ligation sequencing kit SQK-LSK109 (Oxford Nanopore Technologies (ONT), Oxford, UK). Sequencing of samples for kit comparison (Part 1) was performed on MinION Flow Cells R9.4.1, while Flow Cells with chemistry type R10.4.1 were used for experiments on sequencing technique comparison (Part 2). Sequencing proceeded for 72 h using MinKNOW software to collect raw sequencing data. Fast5 files were basecalled using Guppy (v.6.1.7) and output DNA sequence reads were saved to fastq files.

2.3.2. Illumina sequencing

Illumina sequencing was used on samples from Part 2 of the study. Following the manufacturer's instruction, we constructed one DNA paired-end (PE) library with an insert size of 550 base pairs per sample with TruSeq DNA PCR-free Library Prep (Illumina Inc., San Diego, CA, USA). Shotgun metagenomic sequencing was carried out on an Illumina NovaSeq 6000 instrument (Illumina Inc.) using an SP flowcell (250 bp PE) with v1.5 sequencing chemistry (Illumina Inc.) at the National Genomic Infrastructure (NGI)/ the SNP&SEQ Technology platform, Uppsala, Sweden.

2.4. Data analysis

2.4.1. Illumina reads quality filtering

Raw reads were filtered to remove adaptor contamination, low-quality reads (<20, base call accuracy ~99 %), reads shorter than 50 bp and host genomic DNA. Pre-processing of raw reads by sequence quality was performed with fastp v0.19.5 (Chen et al., 2018; Danecek et al., 2021; Chen, 2023). The clean Illumina sequences were screened with bowtie2 v.2.5.2 (Langmead and Salzberg, 2012) and Samtools v.1.3.1 (Danecek et al., 2021) against the pig reference genome (*Sus scrofa* 11.1, NCBI) downloaded with pre-built indexes for bowtie2 alignment (https://genome-idx.s3.amazonaws.com/bt/Sscrofa11.1.zip) to remove contamination with host genome sequences.

2.4.2. ONT reads' quality filtering

ONT raw reads were filtered from low quality reads (<15, base call accuracy ~96.8 %). For cleaning of host contamination from long reads we performed alignment against the pig reference genome with minimap2 v2.1-r311 (Li, 2018; Li, 2021) followed by Samtools v.1.3.1.

2.4.3. Taxonomic annotation

Taxonomic annotation was performed on trimmed reads obtained with Illumina and ONT in Kaiju v1.10.1 (Menzel et al., 2016) using Kaiju databases Refseq_nr (2023_06_17).

2.4.4. Assembly of bacterial genomes

Three types of assemblies were performed: assembly of short reads (Illumina), assembly of long reads (ONT) and hybrid assembly (combination of short and long reads). For each type of assembly, two tools were compared. The names of tools and assembly algorithms can be found in Table 2. We also used metaplasmidSPAdes as a tool for plasmid search.

2.4.5. Quality assessment of obtained assemblies

To compare the performance of different assemblers, QUAST v5.2.0. was used to assess contig statistics (Mikheenko et al., 2018). We enabled the MetaQUAST mode to obtain Genome fraction statistics for the 20 most abundant bacterial species presented in pig farm environment (Mikheenko et al., 2016; Chen et al., 2021).

2.4.6. Assembly of metagenome-assembled genomes (MAG)s and quality assessment

MAGs assembly was performed on contigs obtained from MEGAHIT. MAGs were assembled by back-mapping of trimmed reads to obtained contigs using Bowtie2. Binning of contigs was performed using MetaBat v2.12.1 on mapped reads with a minimal length of 1500 bp (Kang et al., 2015). Quality assessment of obtained bins was performed in CheckM v1.0.7 and bins with completeness <50 % and contamination level > 10 % were classified as bad quality bins and excluded from further analysis.

2.4.7. ARG search

The search for ARGs was performed both on contigs obtained with five assembly tools (hybridSPAdes, metaSPAdes, MEGAHIT, metaFlye, metaplasmidSPAdes) and bins obtained from MEGAHIT contigs using ABRicate v1.0.1 (Seemann). The analysis included a search against five ARG databases: ARG-ANNOT (Gupta et al., 2014), CARD (Jia et al., 2017), MEGARes (Doster et al., 2020), NCBI AMRFinderPlus (Feldgarden et al., 2019) and ResFinder (Zankari et al., 2012). Database versions, number of ARGs and date of data retrieval are presented in Table 3. Minimum DNA identity and minimum DNA coverage were set at 80 %. A dictionary of ARGs that includes standard gene name, description of mechanism of work, the resistance pattern (different antibiotics classes, metals and biocides and antibiotic gene family) was manually created to unify the output from 5 different databases as well as to avoid the appearance of duplicates during ARG search.

2.4.8. Sequencing depth subsampling and rarefaction curves

In order to simulate the effect of sequencing depth, subsampling was performed on three samples. For this purpose, the sample function of the seqtk (v. 1.3-r106) package was used with random seed equal to 100. Subsampling was performed on reads that had already passed quality control into the following set of depth intervals: 1 M, 2 M, 4 M, 6 M, 8 M, 10 M, 20 M, 30 M, 40 M, 50 M, 60 M, 70 M, 80 M and 90 M.

Further analyses were performed in R software v4.3.1 (R Core Team, 2013), using the packages: dplyr, tidyverse and ggplot2.

Table 2
Tools and assembly algorithms assessed in the study.

Tool	Version	Assembly	Algorithm	Reference
Canu	v2.2	Long reads	Adaptive k-mer weighting and repeat separation	(Koren et al., 2017)
metaFlye	v2.9.3	Long reads	Repeat graphs	(Kolmogorov et al., 2020)
MEGAHIT	v1.2.9	Short reads	de Bruijn graph	(Li et al., 2015)
metaSPAdes	v3.15.5	Short reads	de Bruijn graph	(Nurk et al., 2017)
metaplasmidSPAdes	v3.15.5	Short reads	de Bruijn graph	(Antipov et al., 2019)
hybridSPAdes	v3.15.5	Hybrid	de Bruijn graph	(Antipov et al., 2016)
OPERA-MS	v0.8.3	Hybrid	Scaffold graph	(Bertrand et al., 2019)

Table 3
Description ARG databases used in the study.

Database	Version	Date of download	Number of ARGs
ARG-ANNOT	V5 (2019 June)	2024-Jun-18	2223
CARD	V3.2.9	2023-Jun-18	4805
MEGARes	V2.0	2023-Nov-4	6635
NCBI AMRFinderPlus	2024-05-02.2	2024-Jun-18	6863
ResFinder	V 2.3.2	2024-Jun-18	3194

3. Results

3.1. Comparison of quality and quantity using different DNA extraction kits

The Nanodrop analysis showed that the best performance was by the EZ kit, with both higher average DNA concentrations and a better A280/A260 ratio compared with the MP and ZM kits. For the other two kits, the absorbance ratio was below 1.7, which is considered as a lower DNA quality that may impact further analysis. Therefore, an additional precleaning step was performed, which reduced the DNA quantity over twofold. Agarose gel electrophoresis didn't show any visible differences among the three kits in length of obtained DNA fragments, which was approximately 20kbp for each kit. Using Precellys Evolution homogenizer for the bead-beating step of the EZ kit yielded twice as much DNA amount compared to vortexing, however, the obtained DNA fragments were smaller in size (1500 to 5000 bp) and therefore not suitable for sequencing using nanopore technology (ONT).

3.2. Effect of DNA extraction kit on detected diversity and abundance of bacteria

The performance of the three kits was further compared by ONT sequencing. Using this technique, we obtained 600 k (ZM), 3 M (MP) and 5 M (EZ) of reads per sample with similar read length distribution between the three different samples, ranging from 50 to 90,000 bases after quality trimming. The mean sequence quality of the reads after trimming remained at 20 (Phred Score/probability of incorrect call 1 in 100 bases). Taxonomic classification of the sequenced reads showed that the ZM kit extraction missed ~37 % of the total diversity on genus level, while the EZ kit resulted in the biggest variety of genera taxonomy covering ~95 % of the total taxonomic diversity (Fig. 2A). There was no clear visible effect of DNA extraction kit on the abundances of the 40 most prevalent genera in the sample (Fig. 2B).

3.3. Effect of sequencing method on bacterial taxonomy

In comparison with the ONT sequencing, Illumina sequencing yielded vastly higher numbers of reads, at least 50 M reads per sample, which were of high quality (>30 Phred score) and had a length of 250 bp. ONT yielded at least 0.8 M of reads with an average read quality (20 Phred score) and a mean length (after quality trimming) of 900-2700 bp. In total, 3813 different bacterial genera were identified across the samples, Illumina sequencing detected approximately 30 % more bacterial genera compared to ONT sequencing. For the majority of the most prevalent bacterial genera there was no difference in relative abundance obtained between the two sequencing techniques, however Illumina sequencing resulted in higher relative abundances of some genera (Fig. 3).

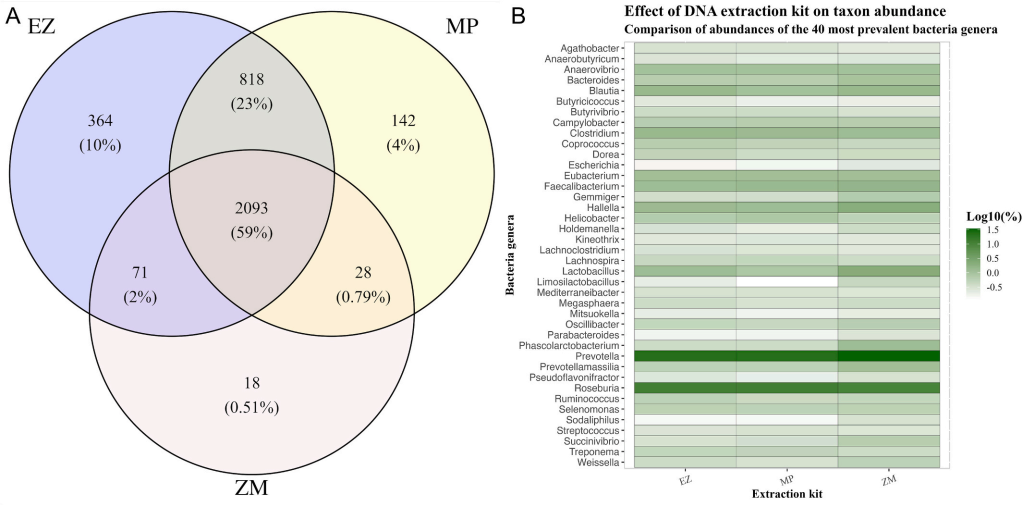


Fig. 2. Comparison of taxonomic profiles from three DNA extraction kits. **A.** Venn diagram showing the number of shared genera, and % of total genera taxonomy covered by sequencing of a sample extracted with each of the kits. EZ - E.Z.N.A Universal Pathogen Kit, ZM - ZYMOBiomics MagBead DNA/RNA, MP - MagPure Stool DNA LQ Kit. **B.** Log10-normalised abundances of the 40 most prevalent bacterial genera: for this analysis the 40 most abundant bacterial genera in the sequencing outcome from three DNA kit extraction were chosen.

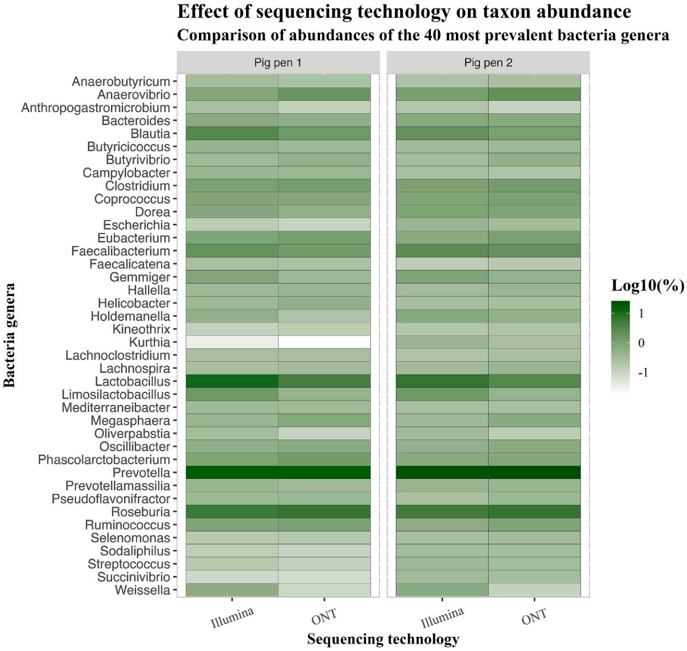


Fig. 3. Effect of sequencing method on bacterial abundance. Log10-normalised relative abundances of the 40 most prevalent bacterial genera in two different samples from pig environment.

3.4. Comparison of different assemblers

The results of assembly quality assessment are shown in Fig. 4. Comparison of assemblies was based on reference-based statistics that included genome fraction, largest alignment and total aligned length, and statistics without reference including largest contig, total length and N50. Overall, assemblers that use Illumina reads performed markedly better than assemblers based only on ONT reads, giving higher values for genome fraction and total aligned length. MEGAHIT showed the best results in genome fraction and total length of assembly.

As the main focus of the method optimisation was analysis of AMR, the assemblers were compared by ARG search. For this analysis, assemblies obtained with hybridSPAdes, metaSPAdes, MEGAHIT, metaFlye and metaplasmidSPAdes were used. There was a very limited number of ARGs (max 6 per sample) found in assemblies with metaplasmidSPAdes. Search of ARGs in metaFlye assemblies resulted in smaller numbers of ARGs within antibiotic classes as well as less varity in antibiotic classes in comparison with metaSPAdes, hybridSPAdes and MEGAHIT (Fig. 5). MetaSPAdes, MEGAHIT and hybridSPAdes resulted in a similar picture of ARGs.

3.5. Comparison of databases used for ARG search

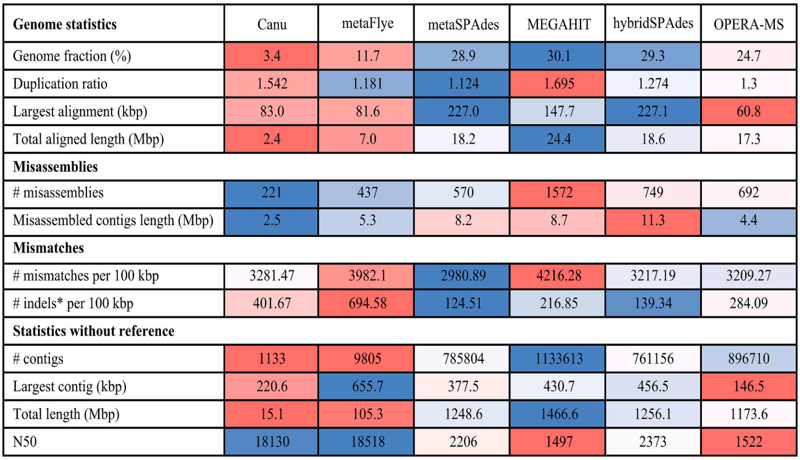
Performing ARG search with ABRicate against 5 databases (Table 3) resulted in a total number of 188 of unique gene names, belonging to 54 families of resistance genes and conveying resistance to 15 antibiotic classes. The largest number of unique ARGs and their sequences were found with MEGARes (Table 4), followed by CARD. Databases such as MEGARes, NCBI AMRFinderPlus and ResFinder contain several sequence variants per ARG. During manual quality control of the ARG-search, it was found that the ResFinder database contains 52 instances where the same accession number is associated with at least two different ARGs, which could potentially lead to errors or misinterpretation of the data. CARD and MEGARes contain ARGs that are involved in resistance against aminocoumarins, fluoroquinolone, mupirocin and peptide antibiotics (Fig. 5). Both these databases also provide information on resistance to biocides, MEGARes additionally gives information on metal resistance genes. The list of these genes can be found in Supplementary Table 1.

3.6. Effect of assembler on ARG identification

The choice of assembler had a visible effect on ARGs detected, which was similar in all tested samples (Fig. 6). MetaplasmidSPAdes gave the worst result with a very small number of ARGs: from 0 to 6 per sample. MetaFlye produced better results than metaplasmidSPAdes (from 5 to 83 ARGs found per sample), however in comparison with search on contigs obtained with hybridSPAdes, metaSPAdes and MEGAHIT, the detected ARGs belonged to a smaller variety of different antibiotic classes as well as a lower number of genes per class. Search of ARGs in hybridSPAdes, MEGAHIT and metaSPAdes assemblies resulted in a similar picture of resistance among the samples: both in the variety of ARG classes and the number of ARGs per antibiotic class. Among the assemblers based on short Illumina reads, ARG searches in metaSPAdes yielded a smaller number of recovered genes (864 in total across 7 samples), whereas hybridSPAdes and MEGAHIT produced nearly identical results, with 883 and 882 recovered genes, respectively.

3.7. Effect of storage temperature on the diversity and abundance of ARGs in the samples

To examine whether different sample storage temperature affects the ARG patterns, we mapped MEGAHIT assemblies against five databases. The analysis did not reveal an observable effect of temperature on the diversity of detected ARGs. Fig. 7 displays the relative abundances (normalised against total length of obtained assembly) of ARGs across seven samples that were collected from three pig pens. In all samples, the largest group of ARGs was associated with the broad-spectrum antibiotic efflux pump class. Other antibiotic classes with a high proportion of associated ARGs included aminoglycosides, beta-lactams, nitroimidazoles, and tetracyclines. Overall, the use of different sample storage temperatures did not have a noticeable effect on the abundance of ARGs within any antibiotic class, with the exception of tetracycline-related genes. For these genes, a slight increase in the number of detected ARGs as the storage temperature increased was observed.



Genome statistics	Canu	metaFlye	metaSPAdes	MEGAHIT	hybridSPAdes	OPERA-MS
Genome fraction (%)	3.4	11.7	28.9	30.1	29.3	24.7
Duplication ratio	1.542	1.181	1.124	1.695	1.274	1.3
Largest alignment (kbp)	83.0	81.6	227.0	147.7	227.1	60.8
Total aligned length (Mbp)	2.4	7.0	18.2	24.4	18.6	17.3
Misassemblies						
# misassemblies	221	437	570	1572	749	692
Misassembled contigs length (Mbp)	2.5	5.3	8.2	8.7	11.3	4.4
Mismatches						
# mismatches per 100 kbp	3281.47	3982.1	2980.89	4216.28	3217.19	3209.27
# indels* per 100 kbp	401.67	694.58	124.51	216.85	139.34	284.09
Statistics without reference						
# contigs	1133	9805	785804	1133613	761156	896710
Largest contig (kbp)	220.6	655.7	377.5	430.7	456.5	146.5
Total length (Mbp)	15.1	105.3	1248.6	1466.6	1256.1	1173.6
N50	18130	18518	2206	1497	2373	1522

Fig. 4. QUAST comparison of assembly quality performed by 6 different tools. Long reads assemblers: Canu, metaFlye. Short read assemblers: MEGAHIT and metaSPAdes. Hybrid-based assemblers hybridSPAdes and OPERA-MS. Colour gradient represents the quality of obtained assemblies, where dark red indicates poorer outcome and dark blue better outcome.*indels = insertions and deletions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

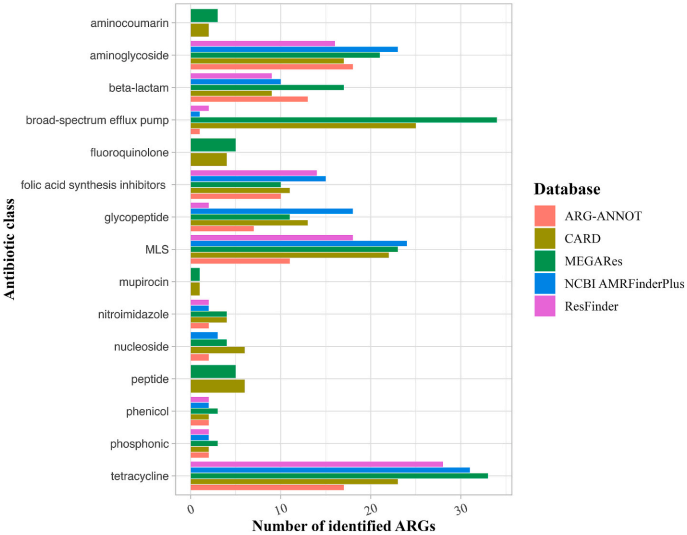


Fig. 5. Comparison of performance of search using five ARG databases. The number of identified ARGs was calculated based on the quantity of unique ARG sequences obtained from in all samples in the study.

Table 4
Identified ARGs in the study samples, based on different databases.

Database	N of unique sequences	N of unique gene names	% of total genes covered
ARG-ANNOT	85	85	45
CARD	147	147	78
MEGARes	177	157	84
NCBI AMRFinderPlus	131	104	55
ResFinder	95	77	41

3.8. Comparison of ARG search results in assemblies and after binning

Fig. 8 shows the results of ARG search after assembly and after binning. It demonstrates that binning prior to ARG search decreases the variety of ARGs in comparison with search without this additional step. On average, binning reduced the total variety of ARGs by 56 %.

3.9. Sequencing depth for ARG search

The results of the simulation of sequencing with different depth followed by ARG search with ABRicate in five ARG databases are presented in Fig. 9. To achieve identification of at least 85 % of all ARG families in the samples, a sequencing depth of 25 M reads was needed (Fig. 9A). To recover 85 % of all gene variants present in the samples, the

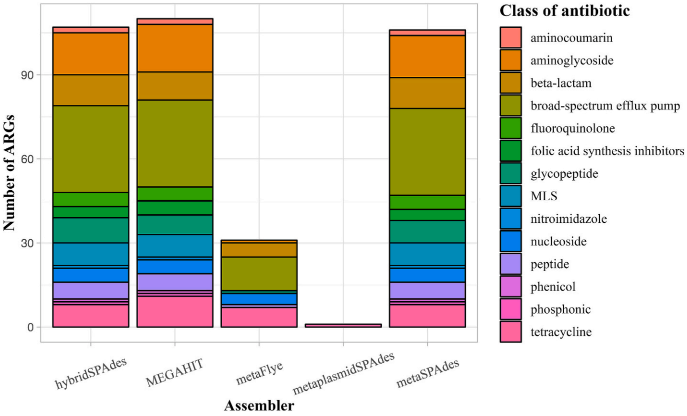


Fig. 6. Effect of assembler on the diversity and abundance of ARGs detected in the sample. The figure shows results from sample 3.

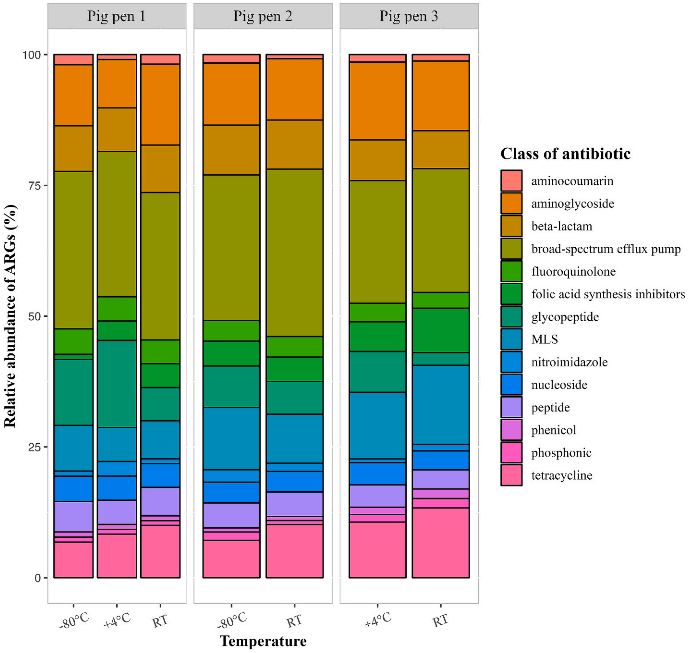


Fig. 7. Effect of sample storage temperature on the diversity of ARGs in different antibiotic classes. Samples were collected from three pig pens and stored at three different temperatures. RT -room temperature.

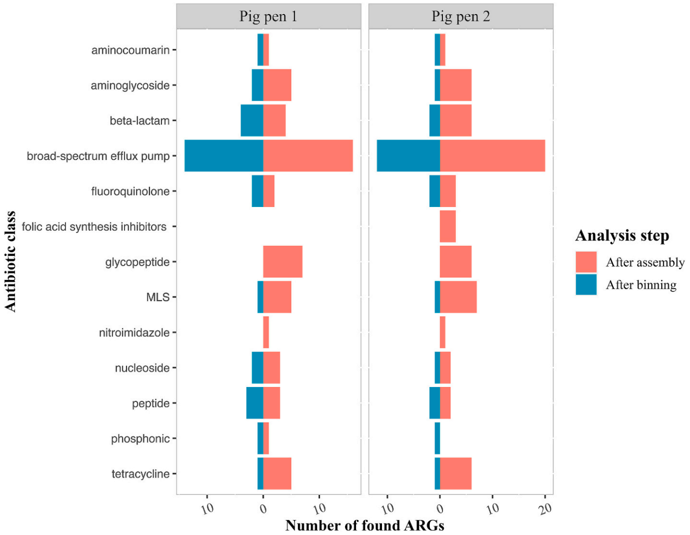


Fig. 8. Detection of ARGs after MEGAHIT based assembly and after MetaBat binning of obtained assemblies. Results presented are based on samples 1 and 4 collected from pen 1 and 2 respectively and stored at −80 °C. The number of genes identified after the assembly step and the binning step is shown on the x-axis.

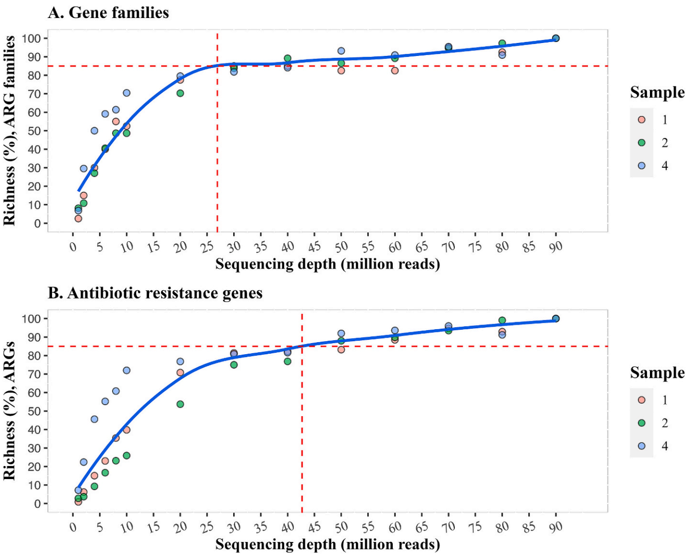


Fig. 9. Rarefaction curves for **A.** ARG gene families, and **B.** unique ARGs. The three samples with lowest concentration of ARGs were chosen for this analysis.

sequencing depth had to be increased to 43 M reads (Fig. 9B).

4. Discussion

There are several critical steps that must be optimised when investigating the farm resistome: sample collection, transportation and storage, DNA extraction and sequencing as well as bioinformatic analysis including metagenome assembly and search for ARGs. Previous studies that evaluated methodology have mainly focused on the comparison of different aspects of one step at a time from the overall pipeline for environmental resistome search. This study aimed to comprehensively evaluate methodologies across several critical steps in the environmental metagenomics research pipeline to advance farm resistome analysis. Specifically, it addressed sample transportation conditions (temperature and duration), comparing short- and long-read sequencing techniques for microbiome analysis, and assessing tools for metagenome assembly and ARG detection within the bioinformatics pipeline to ensure reproducible ARG detection from farm environments.

4.1. Sample collection and storage

We employed and evaluated the performance of sock sampling as a novel method for detecting AMR in the pig farming environment for the first time. Previously this method was concluded to be sufficient to determine the herd status regarding prevalence of bacterial pathogens (Buhr et al., 2007; Pedersen et al., 2015; Lillie-Jaschniski et al., 2023), however it has never been evaluated for suitability for ARG search. Sock sampling is an easy non-invasive method and, based on our results, it yields the needed amount of good quality bacterial material for whole-metagenome sequencing. In comparison with other sampling methods, sock sampling is a cost-effective and not very time-consuming method that helps to avoid the problem of selecting individuals to sample and provides a picture of the entire group of pigs in the pens included in the sample. There is a risk that the sock sampling method might miss low prevalent resistance genes or bacterial genera, however previous studies that compared different sampling strategies showed that a similar strategy based on scraping manure from the floor resulted in only minor differences in resistance in comparison with sampling fresh droppings or individual pig sampling (Munk et al., 2017).

The effect of sample storage temperature on the abundance and diversity of ARGs is a critical area of research, particularly for the purposes of longitudinal studies, where it is important to minimize the role of any factor that might interfere with the stability of the resistome. Previous studies reported that storage of fecal samples at ambient temperatures may exhibit notable changes in their microbiota and resistome profiles over time. For instance, (Martin de Bustamante et al., 2021) reported that fecal samples from equines stored at room temperature for extended periods showed alterations in microbial diversity, contrasting with findings in human and feline samples where no significant changes were observed within 24 h at room temperature. This suggests that the stability of the resistome may vary across species and storage conditions. However, there is a limited number of studies that focus directly on the effect of temperature on the change in resistome. In our study we focused on the effect of short-term storage (48 h) of sock samples at +4 °C and at room temperature (∼ + 25 °C) on the resistome. An increase of temperature did not result in major changes in diversity and abundance of the resistome. Only genes exhibiting resistance against tetracycline showed some slight increase with longer storage at higher temperatures. A similar effect of sample storage temperature increase on tetracycline resistance was shown in the work of Poulsen et al. (2021) in pig fecal samples. However, in his work the total observed abundance of ARGs appeared to be dependent on the storage conditions: cold temperatures (−80 °C and − 20 °C) resulted in a decreased abundance compared to immediate sample processing, while longer storage at RT increased the overall AMR abundance. The difference between our results, and the changes in patterns of resistance in Poulsen's work (Poulsen et al., 2021) could be explained by two factors. Firstly, there is the difference in sample material used: in our study we applied sock sampling, whereas Poulsen's study used pig feces. Secondly, in our study the samples were stored at −80 °C for long-term preservation before DNA extraction and sequencing. This extended storage at low temperatures could reduce any impact that short-term storage at varying temperatures might have on the results. Another possible explanation is that our samples contained lower overall numbers of resistant bacteria with only tetracycline-resistant fast-growing bacteria in sufficient numbers to affect the results. Tetracycline resistance is commonly observed in indicator as well as clinical isolates of *E. coli* from pigs, although the levels in Sweden are much lower than in most countries (SVA, 2024). Regardless, our results support the strategy to transport samples at low temperature.

4.2. Extraction of DNA from complex environmental samples

In our work we didn't reveal any visible difference in bacterial taxonomy of the most abundant bacteria genera in samples processed with different extraction kits. The EZ kit resulted in a larger number of detected bacterial genera, however it still missed ~5 % of the genera that were recovered by ZM and/or MP. Among the tested DNA isolation kits, the EZ kit was the optimal choice because of its easy usage. In contrast with the two other kits, the quality and quantity of DNA obtained with the EZ kit was good enough to avoid the need for further precleaning of samples, which often leads to loss of at least half of total DNA and is especially needed to avoid pore clogging during ONT sequencing. For additional increase in the quantity of obtained DNA some changes can be made in the EZ protocol: 1) usage of the whole available volume of sample after the bead-beating step (as described in materials and methods); 2) exchange the homogenisation step using vortex to Precellys Evolution homogenizer with bead-beating, however this type of lysis leads to a shorter lengths of the obtained DNA fragments and is therefore not recommended for ONT sequencing.

4.3. Illumina vs ONT sequencing

The comparison between Illumina sequencing and ONT sequencing for taxonomy studies and investigations of the resistome revealed distinct advantages and limitations inherent to each platform. Illumina sequencing, known for its high throughput and accuracy, is particularly effective in generating large volumes of data with low error rates, making it suitable for detailed taxonomic studies and microbial diversity, while long reads produced by the ONT platform allows to resolve repetitive regions and complex genomic structures (Boolchandani et al., 2019; Pillay et al., 2022). In this study, the application of both sequencing methods did not reveal any substantial or consistent effect on bacterial taxonomy. However, Illumina sequencing detected 30 % more bacterial genera compared to ONT sequencing, which might be connected to the significantly larger depth of sequencing obtained with short reads. Therefore, deep sequencing with both the NovaSeq 6000 and MinION sequencing platforms appears adequate for assessment of the major part of the microbial community composition, however deep sequencing with the Illumina approach allows for recovery of more rare bacterial genera. Previous research suggests that Illumina is still the current standard for characterizing complex microbial communities (Stevens et al., 2023), however significant improvement in the quality of long read sequencing with ONT has lead to increase in the accuracy of classification and relative estimates and might be preferred for taxonomy studies (Pearman et al., 2020). However, if the focus is on the resistome, the read length obtained with Illumina sequencing can be regarded as sufficient, as shown in our study.

Moreover, regarding ARG abundance, Illumina sequencing showed better results in comparison with the long reads obtained from ONT. More ARGs and antibiotic classes were found in data from Illumina sequencing, which might be connected to the bigger depth of sequencing. Simulation of sequencing with different depth showed that adequate (>80 %) analysis of the resistome based on ARG families needed at least 25 M of 250 bp PE reads, while to reveal the majority of gene variants the sequencing depth has to be increased to 45 M. This corresponds to previous studies where it was shown that to obtain a stabilised number of ARG families in pig caeca, a sequencing depth of ~60 M 150 bp PE reads per sample is needed (Gweon et al., 2019). Achieving similar sequencing depths with ONT sequencing is not cost-effective. Hence, if the focus is on the resistome and the annotation of the ARGs to their bacterial hosts is less important, Illumina would be the method of choice.

4.4. Bioinformatic analysis

There are several crucial steps in bioinformatic analysis to characterise the resistome: choice of assembler, binning and choice of ARG database. Although some authors have described the pro's and con's of different choices (Boolchandani et al., 2019; Pillay et al., 2022; Lee et al., 2023), these must be optimised for the specific purpose of each study, scientific question and complexity of investigated samples. To rely on previously published studies in the choice of methods may seem a robust strategy but, as our study shows, there are many context-specific aspects that must be understood and addressed. The typical pipeline for ARG identification from metagenome data is based either on read-mapping or assembly, which can be further used for binning or an annotation step (Pillay et al., 2022). Every additional step in the pipeline for ARG search brings the risk of additional loss in diversity and abundance of identified ARGs. Therefore, depending on the research question, the assembly and binning step may or may not be performed. A search of ARGs on the basis of raw reads may be recommended if the focus of the research is quantity and variety of different ARGs, but it increases the risk of false-positive detections (Abramova et al., 2024). However, if the focus of the research is the fluctuation of ARGs in regard to specific bacteria, the step of assembly becomes crucial (Abramova et al., 2024). Recent studies suggest that the use of ONT-based long reads, either alone or in combination with Illumina sequencing, can enhance ARG detection and characterization by overcoming the challenge of plasmid reconstruction and the tendency of assemblies to break around ARGs due to surrounding repetitive regions (Berbers et al., 2020; Zhang et al., 2023; Abramova et al., 2024). Taking into account the factors discussed above, we evaluated the performance of three distinct assembly techniques: hybrid, short-read, and long-read assemblies, focusing on their impact on assembly quality. The implications of the resulting assemblies for biological interpretation were assessed by examining the contextualization of ARGs which was the primary focus of this study. We compared seven different assemblers that were seen as most promising for complex environmental metagenome analysis in each type of assembly technique, including two short reads assemblers (metaSPAdes and MEGAHIT) (Brown et al., 2021; Zhang et al., 2023; Abramova et al., 2024), two long reads assemblers (CN and metaFlye) (Latorre-Perez et al., 2020; Brown et al., 2021; Zhang et al., 2023), two tools based on hybrid assembly (hybridSPAdes and OPERA-MS) (Brown et al., 2021; Zhang et al., 2023) and metaplasmidSPAdes to focus on plasmid-located ARGs. In our work, among long-read assemblers metaFlye performed markedly better, while Canu collapsed our data. Similar results with larger size of both total assembly and largest contig were shown in the work of Brown et al. (2021). Previous research showed contradicting results in comparison of MEGAHIT and metaSPAdes. In the work of Abramova et al. (2024) metaSPAdes was suggested as a better option for complex sample scenario, while other studies showed that MEGAHIT outperforms metaSPAdes in generating assemblies from deeply sequenced datasets, which is probably connected to its optimised algorithms to analyze large datasets (Zhang et al., 2023). Similar to the latter, in our work MEGAHIT performed better and preserved the biggest total aligned length among all assemblers. Addition of long reads to short reads increased the length of obtained contigs in hybridSPAdes, however they were still smaller than the ones obtained with metaFlye assemblies, which is expected as short-reads were mainly used to reduce misassemblies rather than fill gaps between contigs (Zhang et al., 2023). Contradictory to previous research (Brown et al., 2021; Zhang et al., 2023), OPERA-MS assembler did not perform well on our data and was excluded from further analysis together with Canu.

Comparison of assemblies based on ARG identification resulted in higher diversity and abundance of ARGs in short and hybrid read assemblies, which is similar to the results of Brown et al. (2021) Overall, among all tested assemblers MEGAHIT showed the best performance in regard of both assembly quality and effectiveness of ARG search.

As stated above, one of the possible steps in ARG search is binning. On one hand, binning may allow to reconstruct genomes of unknown or uncultured bacteria (Pillay et al., 2022), while on the other hand it results in loss of some information. Binning of MEGAHIT contigs reduced the variety of recovered ARGs by 56 %, which is markedly bigger than in the work of Maguire which was performed on a simulated metagenome from 30 chosen genomes and resulted in up to 15 % loss in ARG recovery (Maguire et al., 2020).

Another crucial step in resistome investigation is the choice of ARG database. There are a range of different databases and tools for ARG search that have been described and compared in multiple reviews (Boolchandani et al., 2019; de Abreu et al., 2020; Papp and Solymosi, 2022; Pillay et al., 2022). The databases can be specialised (focused on species specific information) or general, with focus on ARGs from all bacterial hosts, and the tools can be divided into those that work on reads or contigs. In our study we used ABRicate as it allows to standardize and compare outcome of search with several ARG databases. As the focus of the study was the overall resistome of the farm environment, we compared the performance of five general databases: ARG-ANNOT, CARD, MEGARes, NCBI AMRFinderPlus and ResFinder. The choice of databases was based on availability of them in ABRicate and how actively they are curated. Previous research suggested that CARD should be the number one data resource (Papp and Solymosi, 2022), while a lot of environmental and veterinary-based resistome studies are conducted based on the highly cited database ResFinder (Macedo et al., 2021; Ekhlas et al., 2023), however it contains only acquired ARGs (Zankari et al., 2012). In our study, most of the resistance was identified by MEGARes which is based on data from all other databases and had a big number of gene variants (Macedo et al., 2021; Papp and Solymosi, 2022), but it recovered only 84 % of all ARGs found in the study. The ARG-ANNOT database is based on the Lahey Clinic β-lactamase database (Gupta et al., 2014) and therefore had the second best result in detection of ARGs against beta-lactams. Only CARD and MEGARes revealed ARGs conveying resistance to aminocoumarins, mupirocin, fluoroquinolone and peptide antibiotics. The other plus of these two databases is that it is possible to identify biocide and/or metal resistant genes. NCBI AMRFinder Plus recovered the greatest number of ARGs against aminoglygosides, folic acid synthesis inhibitors, glycopeptides, MLS and tetracyclines. One of the minuses of ResFinder identified in this work was that the database has 52 pairs of different ARGs with the same accession number which might lead to misinterpretation of the outcome. Overall, our findings indicate that achieving higher diversity in antimicrobial resistance gene (ARG) detection requires simultaneous searches across multiple databases.

5. Limitations of the study

In this study, we addressed several key factors that impact the reproducibility of ARG identification in complex farm-derived samples. Nonetheless, some limitations remain that future research should address. Firstly, we did not compare the effectiveness of sock sampling to individual fecal samples, leaving unresolved whether the sock sampling method may miss microbial diversity or low-abundance ARGs. Secondly, sequencing data from fresh samples would be valuable to assess potential effects of long-term storage at −80 °C on bacterial and ARG diversity and abundance. Additionaly, this study did not compare ARG detection between assembled and non-assembled data, as quantifying ARGs in non-assembled Illumina short reads presents challenges. Such analysis could clarify the magnitude of potential losses in ARG abundance or diversity connected to the assembly step. The use of samples from several different pens, including the only one where the pigs had been treated with antibiotics, was to obtain some variation and a sufficient number of samples for our purposes. However, as the pigs in Swedish farms are managed batchwise all pigs in the same batch should be regarded as one epidemiological unit.

6. Conclusion

We have developed an approach for reproducible ARG detection in complex samples from the pig farm environment with low levels of antibiotic usage, based on sock sampling as a novel method for detecting AMR. E.Z.N.A Universal Pathogen (Omega Bio-tek, USA) was the optimal choice among the tested kits for metagenome analysis. Choice of sequencing technique didn't drastically affect taxonomy identification; however deep Illumina sequencing allows to recover approximately 30 % more of low-abundance bacterial genera. The optimal sequence depth for ARG detection from farm environment with low levels of antibiotic usage is within 25 to 45 M of 250 bp reads. Storage of sock samples at room temperature up to two days did not affect diversity or abundance of ARGs, however choices made during bioinformatical analysis can markedly change the outcome. In this work, we propose computational analysis based on MEGAHIT assembly as a compromise between need to identify bacteria which carry ARGs and possible loss in diversity and/or abundance of resistance genes. Our research suggested that search against multiple ARG databases is needed for the detection of the highest diversity of ARGs.

Declaration of generative AI and AI-assisted technologies in the writing process

Generative Artificial Intelligence (AI) or AI-assisted technologies were not used in writing or generation of any of the sections of this manuscript.

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Ethics statement

This work did not involve the use of animals or human participants.

CRediT authorship contribution statement

Valeriia Ladyhina: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Elisabeth Rajala:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Susanna Sternberg-Lewerin:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Leila Nasirzadeh:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Erik Bongcam-Rudloff:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Johan Dicksved:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2025.107103.

Data availability

The data supporting this study are available from the corresponding author upon reasonable request. The data will not be published as open access to protect the privacy and confidentiality of the farm.

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Accession number	Gen 1	Gen 2
AB013298	mph(C)	msr(A)
AB016934	fomA	fomB
AB114632	aac(3)-Id	aadA7
AB211959	aac(3)-VIII	aph(3')-Va
AB247327	ant(6)-Ia	aph(3')-IIIa
AB571865	mef(C)	mph(G)
AF015628	vat(C)	vgb(B)
AF024602	aph(3'')-Ib	aph(6)-Id
AF047479	aadA3	catB2
AF079138	erm(30)	erm(31)
AF174129	blaCTX-M-9	dfrA16
AF227520	mef(A)	msr(D)
AF330699	ant(6)-Ia	aph(3')-IIIa
AF337947	aac(6')-Im	aph(2'')-Ib
AJ488494	erm(T)	vat(E)
AJ579365	erm(33)	lsa(B)
AJ584652	aac(6')-30-aac(6')-Ib'	blaIMP-16
AJ746361	dfrA23	sul1
AM040708	aadA8b	dfrA12
AM408573	cfr	fexA
AM850915	blaOKP-A-11	blaOKP-A-12
AY034138	dfrA18	sul2
AY289608	aac(6')-I30	blaOXA-53
AY524415	sul1	sul2
AY553333	blaIMP-15	dfrB2
AY920928	ant(2'')-Ia	blaGES-9
CP013733	aadE-Cc	blaOXA-489
CP091084	TOprJ4	tmexC4
DQ112222	blaOXA-2	fosG
DQ143913	blaVIM-12	sul1
DQ914960	rmtD	sul1
EU370913	OqxA	OqxB
EU855787	aph(3')-Ia	sul1
FJ349556	VanHMX	vanM
FM207631	ARR-3	dfrA1
FN433596	blaZ	tet(M)
FN594949	ant(6)-Ib	tet(44)
FR823292	blaZ	mecC
GQ205627	vat(H)	vga(D)
GQ466184	ant(2'')-Ia	blaOXA-2
HQ652506	tetA(46)	tetB(46)
JN596280	blaGES-19	blaGES-20
KP689347	blaBKC-1	blaBKC

LC633285	TOprJ3	tmexC3
M28829	aph(3'')-Ib	aph(6)-Id
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MN175502	TOprJ2	tmexC2
MN590304	NarA	NarB
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X92946	mdt(A)	str
Y18050	aph(3')-XV	blaVIM-1
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AB016934	fomA	fomB
AB114632	aac(3)-Id	aadA7
AB211959	aac(3)-VIII	aph(3')-Va
AB247327	ant(6)-Ia	aph(3')-IIIa
AB571865	mef(C)	mph(G)
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AM040708	aadA8b	dfrA12
AM408573	cfr	fexA
AM850915	blaOKP-A-11	blaOKP-A-12
AY034138	dfrA18	sul2
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AY524415	sul1	sul2
AY553333	blaIMP-15	dfrB2
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CP013733	aadE-Cc	blaOXA-489
CP091084	TOprJ4	tmexC4
DQ112222	blaOXA-2	fosG
DQ143913	blaVIM-12	sul1
DQ914960	rmtD	sul1
EU370913	OqxA	OqxB
EU855787	aph(3')-Ia	sul1
FJ349556	VanHMX	vanM
FM207631	ARR-3	dfrA1
FN433596	blaZ	tet(M)

FN594949	ant(6)-Ib	tet(44)
FR823292	blaZ	mecC
GQ205627	vat(H)	vga(D)
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HQ652506	tetA(46)	tetB(46)
JN596280	blaGES-19	blaGES-20
KP689347	blaBKC-1	blaBKC
LC633285	TOprJ3	tmexC3
M28829	aph(3'')-Ib	aph(6)-Id
MK347425	TOprJ1	tmexC1
MN175502	TOprJ2	tmexC2
MN590304	NarA	NarB
U13880	aac(6')-Il	catB3
X92946	mdt(A)	str
Y18050	aph(3')-XV	blaVIM-1
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Antimicrobial resistance (AMR) is a major One Health challenge, with livestock serving as reservoirs of resistance genes. While antimicrobial use (AMU) is a key driver, environmental and management factors may also influence AMR. This thesis developed a metagenomic pipeline to investigate the environmental resistome in low-AMU Swedish pig farms. Resistome diversity was not associated with AMU and did not correlate with phenotypic resistance in *Escherichia coli*, highlighting complementary AMR dimensions and the importance of integrated surveillance approaches.

Valeriia Ladyhina completed her doctoral education at the Department of Animal Biosciences, Swedish University of Agricultural Sciences and holds a B.Sc. from V. N. Karazin Kharkiv National University and a M.Sc. from Uppsala University and the University of Groningen.

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