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Metagenome analysis of pig nasal swab samples by Oxford Nanopore and Illumina sequencing

Sertés orrváladék minták metagenomvizsgálata Oxford Nanopore és Illumina szekvenálással

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Abstract

Our study delved into the microbial community dynamics and antibiotic resistance gene profiles within swine samples utilizing Illumina and Oxford Nanopore Technologies sequencing methods. Eukaryota dominated all samples, followed by Bacteria. The detection of 22 bacterial genera, each varying in abundance across different ages, underscores the potential impact of genetic factors, environmental conditions, and antibiotic exposure on microbial diversity.

The detection of 18 resistance genes highlighted the concerning prevalence of antibiotic resistance within swine populations. Aminoglycoside resistance genes, particularly AAC(6')-I-43 and AAC(6')-Il, were prevalent, suggesting a potential role in resistance transmission. The presence of ANT(3'')-IIa and APH(3')-Ia genes underscored the diversity of resistance mechanisms against streptomycin and spectinomycin. Tetracycline resistance genes, including tet(W), were identified. Sulphonamide resistance genes, particularly sul2, were detected by all approach. Fosfomycin resistance gene FosA3 and streptothricin resistance gene SAT-2 were detected, highlighting emerging resistance concerns. Exclusive identification of β-lactamase resistance genes, such as CME-1 and IMP-55, in specific sample groups underscored the diverse nature of antibiotic resistance.

Multidrug resistance genes further emphasized the complex resistance landscape within swine microbiomes. These findings provide valuable insights into swine health and environmental resistance dynamics, calling for comprehensive surveillance and intervention strategies to mitigate antibiotic resistance spread and safeguard public health.

My study elucidates the intricate interplay between microbial composition and antibiotic resistance in swine populations, emphasizing the need for proactive measures to address emerging resistance challenges and ensure sustainable livestock management practices.

Absztrakt

Tanulmányunk sertésminták mikrobiális közösségének dinamikáját és antibiotikum-rezisztencia génprofilját vizsgálta Illumina és Oxford Nanopore Technologies szekvenálási módszerekkel Minden mintában az eukariota dominált, amelyet a baktériumok követtek. A 22 baktériumnemzetség nagy részénél a különböző életkorokban eltéréseket tapasztaltunk, ami aláhúzza a genetikai tényezők, a környezeti feltételek és az antibiotikum-expozíció lehetséges hatását a mikrobiális diverzitásra

A 18 rezisztenciagén kimutatása rávilágított az antibiotikum-rezisztencia sertéspopulációkon belüli elterjedtségére. Az aminoglikozid rezisztenciagének, különösen az AAC(6')-I-43 és az AAC(6')-Il, túlsúlyban voltak. Az ANT(3")-IIa és APH(3')-Ia gének jelenléte a sztreptomicinnel és spektinomicinnel szembeni rezisztencia mechanizmusok sokféleségét hangsúlyozta. Tetraciklin-rezisztenciagéneket, köztük tet(W)-t is azonosítottunk. A szulfonamid-rezisztencia gének közül a sul2-t mindkét technológiával azonosítottuk. A Fosfomycin rezisztenciagén FosA3 és a sztreptotricin rezisztenciagén SAT-2 szintén kimutatható volt. A β-laktamáz rezisztenciagének, mint például a CME-1 és az IMP-55 kizárólagos azonosítása bizonyos mintacsoportokban aláhúzta az antibiotikum-rezisztencia változatos jellegét.

A multidrog-rezisztencia gének tovább hangsúlyozták a sertés mikrobiomokon belüli összetett rezisztenciaprofilt. Ezek az eredmények értékes betekintést nyújtanak a sertésegészségügy és a környezeti rezisztencia dinamikájába és átfogó felügyeleti és beavatkozási stratégiákat tesznek szükségessé az antibiotikum-rezisztencia terjedésének mérséklése és az emberi egészség védelme érdekében.

Dolgozatom adatokkal szolgál a sertéspopulációk mikrobiális összetétele és antibiotikum-rezisztenciája közötti bonyolult kölcsönhatással kapcsolatban, hangsúlyozva a proaktív intézkedések szükségességét a felmerülő rezisztencia-kihívások kezelése és a fenntartható állattartási gyakorlatok biztosítása érdekében.

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List of abbreviations

AG – Aminoglycoside

AMR - Antimicrobial Resistance

ARG - Antibiotic Resistance Gene

CARD - Comprehensive Antibiotic Resistance Database

COVID-19 – Corona Virus Disease 2019

DHPS - Di-Hydro-Pteroate Synthase

DNA - Deoxyribonucleic Acid

HGT – Horizontal Gene Transfer

IAV - Influenza A virus

ISS - International Space Station

MGE - Mobile Genetic Element

NGS - Next Generation Sequencing

ONT - Oxford Nanopore Technologies

ORF - Open Reading Frame

PCR - Polymerase Chain Reaction

RGI - Resistance Gene Identifier

RNA - Ribonucleic Acid

SV - Structural Variant

WGS - Whole-Genome Sequencing

1. Introduction

Continued advancements in nanopore technology, including improvements in base calling accuracy, error rates, and read lengths, hold promise for further expanding the utility of nanopore sequencing in research and clinical settings. As demonstrated by recent studies [1-34], nanopore sequencing platforms like Oxford Nanopore Technologies (ONT) continue to evolve, offering comparable performance to established technologies like Illumina and unlocking new possibilities for genomic analysis and personalized medicine.

The history [1] of nanopore sequencing represents a remarkable journey of innovation, collaboration, and discovery, with profound implications for understanding the complexity of the genome, diagnosing diseases, and improving human and animal health. As nanopore technology continues to mature, its transformative impact on genomics and beyond is poised to accelerate, shaping the future of scientific research and healthcare.

Microbial communities, particularly those found in agricultural settings such as swine farms, play pivotal roles in shaping both environmental and clinical landscapes. Understanding the composition and dynamics of these communities is crucial for assessing factors influencing microbial colonization and the spread of antibiotic resistance genes (ARGs).

The integration of nanopore sequencing into microbial community studies represents a significant advancement, providing researchers with a powerful tool to unravel the intricate interactions within these complex ecosystems. By offering real-time, long-read sequencing capabilities, nanopore technology enables more accurate taxonomic assignment and facilitates the detection of rare or novel microbial species. This enhanced resolution enriches our understanding of microbial community dynamics and their implications for various fields. Thus, the continued refinement and adoption of nanopore sequencing hold immense potential for advancing our knowledge of microbial ecology and informing strategies for mitigating the spread of antibiotic resistance.

In this study, we investigated the microbiome of swine samples using two sequencing methods, Illumina and ONT, to comprehensively characterize bacterial taxa and detect ARGs.

2. Literature review

1.1 History

The original idea for nanopore sequencing design was first envisioned by David Deamer, a research professor at the University of California, Santa Cruz [1]. His suggestion was to create a nanoscopic pore on a membrane through which DNA would be guided by electrophoresis. The pore was supposed to be a transmembrane protein, wide enough for a single strand of DNA to pass through [32]. α-Hemolysin produced by *Staphylococcus aureus* seemed like a possible solution since it's a water-soluble protein monomer that could be bound to the membrane forming a heptameric ion channel [3]. This channel could remain open for extended periods (in optimal conditions [4]) allowing a continuous flow of electrical charge across the membrane, thus enabling DNA or RNA passage through the channel. The first published paper [32] showed that polynucleotide passing through the pore caused current blockade, which was measurable, whereas no blockade was detectable when the pore was open and empty. The duration of the blockade was directly proportional to the length of the polynucleotide. This experiment also showed that the pore made of α-Hemolysin heptamer was too small for the passage of double-stranded polynucleotides. This paper not only tackled the theory of whether polynucleotides can pass through the membrane pores but also proved that it was possible to measure the length of single-stranded polynucleotides, therefore, raising the question of possible application in determining the sequence of a given polynucleotide, if the appropriate conditions were met.

Further study [5] showed that α-Hemolysin pore can be used to distinguish between purine and pyrimidine RNA strands, although it was unclear why each strand provided two distinguishable signals. One of the hypotheses suggested that the orientation of the strain (5'-3' or 3'-5') entering the pore could produce different signals, which was later shown to be true [6].

Identifying purine and pyrimidine polynucleotide DNA strands was not possible because of the speed of transport of DNA strands through the pore. It was suggested [7] that this limitation could be overcome by keeping the strand in the pore for an extended period. This was achieved primarily by forming DNA hairpins [8], and in later years, DNA pseudorotaxane hybrid [9] was formed. All of this showed that there was a possibility for capturing DNA in the pore; allowing enough time for the strands to change the ion current in a detectable manner [7]. Even though

this proved that nanopores could be used for DNA individual nucleobase identification, strands would stay immobilized in the pore, which was inconvenient for real-time, practical usage. To achieve a better resolution, it would also be ideal for a strand to pass through the channel multiple times.

Since there was a need for a strand to stay in the pore for a long enough time and leave it after inducing measurable change, it was suggested the use of the enzymes capable of providing a transient passage of ssDNA through the pore. The enzyme formed an enzyme-substrate complex and as it processed the DNA, DNA passed through the pore more slowly. This led to the retention of the individual bases in the pore, setting the foundation for individual detection of nucleotides.

In the first experiments, the exonuclease I, isolated from *Escherichia Coli*, was used [10]. This enzyme cleaves the nucleobases from one strand of dsDNA in the 3'-5' direction, leaving the strands free to pass through the pore. The speed of ssDNA passage depends on the speed of enzyme cleavage thus the detection rate depends on exonuclease activity. The experiment showed that exonuclease I was able to slow down ssDNA passage through the pore, but the bonds formed between enzyme and DNA were not as strong, so the complex would dissociate very fast. This opened the door for the use of DNA polymerase I, produced by *E. Coli*.

DNA polymerase I has a few roles. It aids in lagging strand synthesis and repairs DNA with its 5'-3' and 3'-5' exonuclease activity. If the enzyme is treated with subtilisin, the so-called "Klenow fragment" [11] will be formed which loses 5'-3' exonuclease activity, retaining the other functions. It was shown that the Klenow fragment forms a complex with DNA halting the DNA passage through the pore [12].

It was discovered that single base discrimination was not possible because the recorded changes in the current were not consequences of the presence of a single nucleotide in the pore, but rather the presence of approximately 12 nucleotides [13]. One of the approaches was adding chemical, peptide tags to separate nucleotides to increase DNA diameter, slow down translocation, and help individual base detection [14]. Experiments demonstrated that adjusting the length, charge, and size of the peptide tags allows for the customization of blockage duration, amplitude, and signature.

The other solution was changing the pore itself. The crystallographic analysis of α-hemolysin's

structure showed that it consists of a mushroom-shaped heptamer with a 5-nm stem in β barrel conformation, and the diameter of its inner channel fluctuates between approximately 1.4 nm to approximately 2.4 nm, depending on the volume of amino acid side chains extending into the pore [3]. One of the conclusions was to change nucleobase recognition sites, since it was discovered that there are three such sites within the channel [15], other was to change the structure of the pore by using MspA, porin from *Mycobacterium smegmatis*, with narrower opening and neutral or positively charged amino acids lining it [16].

The first commercially available nanopore was used in 2014. It was named MinION and was made by Oxford Nanopore Technologies (ONT), which was patented it in 2004 [1]. Since this moment, ONT found its purpose in many areas, and its affordability allowed its wide use in medicine and scientific research.

ONT MinION is small, portable device, rendering it a valuable asset in the field. It enables fast, electronic examination of individual molecules in real-time. Since the device is USB-powered, it can be connected to any computer and can be used for DNA and RNA sequencing [17].

Since its creation, ONT found its application in many areas: clinical research, microorganism detection, infectious disease diagnostics, gene assembly, etc.

3. Applications of Oxford Nanopore Technologies

1.2 ONT in clinical diagnostics

Quick detection of the causative agent of a disease has been imperative in the fast and quick and appropriate treatment of the disease. Diagnosing illnesses accurately can be difficult due to the variety of pathogens that can lead to illness sharing similar clinical symptoms. Existing diagnostic methods, including culturing, nucleic acid amplification tests, and serological assays, typically involve conducting multiple tests in a series in order to pinpoint the specific cause of the illness. Frequently, these techniques still depend on the steps involving the growth and amplification of viable microorganisms in a culture for identification and testing their susceptibility to antimicrobial agents. This process typically requires at least 48 hours for commonly encountered pathogens and even longer for more delicate microorganisms, such as fungi and mycobacteria, which may require weeks for accurate identification and testing.

The power of ONT in the quick detection of the causative agent showed in the *Salmonella* outbreak in the Birmingham hospital. Within 40 minutes from the start of sequencing, it became evident that the probable serovar in question was *Enteritidis*. Once this serovar assignment was made, subsequent analysis focused on a reference tree specifically for *S. Enteritidis* strains. Consequently, it was established within 100 minutes from the beginning of the sequencing that the outbreak strain unequivocally belonged to the primary cluster associated with the hospital outbreak [18].

Traditional sequencing technologies pose challenges for implementation in developing countries due to limited access to reliable power, cold chain infrastructure, laboratory facilities, and adequately trained personnel. Moreover, certain genome sequencing instruments, demand regular calibration, tasks that require the expertise of trained engineers. In 2015, ONT was used in the field setup for genomic monitoring of the Ebola epidemic in West Africa. This analysis also helped with determining the ways of the spread of the virus and monitoring the outbreak [19]. The device was ready for immediate use upon reaching the outbreak area, as it didn't require any specific setup or calibration processes.

Few recent studies [20, 21] showed the superiority of ONT over routine pathogen detection methods, like culturing. The reason for this is that some bacteria are difficult to grow or require a long time, and waiting for the results can lead to antibiotics being prescribed without proper testing.

The recent COVID-19 global pandemic, caused by SARS-CoV-2, had a profound and far-reaching impact across the world. The utilization of nanopore technology for viral genome sequencing unveiled that several patients were presented with viral genomes containing deletions, which can be missed with classical assays [22]. Recombination in coronaviruses is a significant driver of adaptation and identifying recombination events is needed for effective surveillance.

1.3 ONT in cancer

Structural variants (SVs) represent one of the important factors in carcinogenesis. These are somatic DNA mutations that play a role in the initiation and advancement of cancer, contributing to key characteristics associated with genomic instability. The four primary types of SVs are deletions, amplifications, inversions, and translocations of nucleotides in a DNA sequence. Detecting these mutations is crucial for the healthcare industry as it enables the detection, the monitoring of cancer patients, and aids in the early identification of potential relapse. This study [23] showed the ability of ONT to sequence repetitive regions, offering results in minutes, with lower testing costs.

1.4 On-site application

The Ebola outbreak surveillance [19] provided insight into the scope and possibilities of field usage of ONT. The findings from the 2017 research paper [34] showcased the device's capability to detect biomarkers, specifically nucleic acids, in environmental samples, using the MinION device in the Canadian High Arctic. They were able to generate metagenomic and genomic sequences, successfully identifying organisms from all three domains (Bacteria, Archaea, and Eukaryotes), proving that the device can work in harsh and offline conditions.

Space missions can last for many months during which human immune response can be disrupted and microbial pathogenicity may increase. The impact of extended exposure to ionizing radiation and microgravity on microbial populations during these missions is uncertain. This ongoing microbial evolution could affect crew health. The limited ability for intervention from Earth during the mission underscores the importance of on-site analyses and monitoring. Essential in-flight clinical diagnostic capabilities are needed to effectively manage infections, including the administration of targeted antimicrobials. The initial DNA sequencing in space using the MinION platform aboard the International Space Station (ISS), demonstrated that the transport to the ISS and operation in its microgravity environment did not negatively impact sequencing performance. Despite variations in experience, two distinct crew members effectively loaded samples on the ISS, and at the same time, underscoring the MinION sequencing platform’s resilience in space. Interestingly, sequencing performance on the ISS was generally superior, indicating that different operators did not markedly influence the quality of

sequencing, meaning even someone inexperienced with working on nanopore can be able to operate with it and get useful results [25]. This study has shown that flow cells remain stable after six months in orbit, and radiation exposure does not appear to be a significant factor affecting the stability of protein nanopores. Another study confirmed ONT and its reagents' resistance to Mars' level radiations but there were significant levels of degradation in simulated conditions of Jupiter's moon Europa, showing that the device needs additional radiation shielding [26].

1.5 Detection of drug resistant genes

From the start of the commercial use of ONT, scientists explored the possibility of detecting drug resistance genes in microbes. Determining the proper antimicrobial therapy in a timely matter is a priority in the treatment of diseases caused by infectious agents [27]. Unnecessary or inappropriate antibiotic use leads to an increase in healthcare expenses [28] and an increase in antimicrobial resistance [29]. One study [30] showed ONT was able to identify pathogens within 10 minutes of sequencing start and it managed to detect certain resistance genes but not all of them.

Antimicrobial resistance (AMR) is a big concern in veterinary medicine also. Not only it hinders animal treatments but it can lead to AMR gene transfer to pathogens affecting humans. In a comprehensive analysis of 179 studies focusing on interventions to minimize antibiotic usage in farm animals, researchers discovered a connection between these interventions and a decline in the presence of antibiotic-resistant bacteria in animals. The reduction in antibiotic use resulted in a 15% decrease in antibiotic-resistant bacteria and a 24–32% decrease in multidrug-resistant bacteria in animals. Although evidence regarding the impact on humans was less conclusive, a meta-analysis of 13 studies suggested a 24% absolute reduction in the prevalence of antibiotic-resistant bacteria in humans when interventions to reduce antibiotic use in animals were implemented [31].

Nanopore sequencing proves to be vital for monitoring environmental risks linked to pathogens and antimicrobial resistance in veterinary hospitals. One study [32] revealed potential transfers of AMR genes between linked environmental hospital sites, emphasizing waste collection points as notable reservoirs of AMR genes. The research enhanced biosecurity protocols within the hospital and showcased the value of ONT in infection control in veterinary settings.

One research [33] utilized nanopore metagenomic sequencing to analyse Influenza A Virus (IAV) directly from clinical respiratory samples in a UK hospital during the 2018/19 influenza season. The sequencing process had a turnaround time of less than 72 hours, indicating potential for routine clinical applications. ONT sequencing demonstrated 83% sensitivity for IAV, with a need for optimization in samples with lower viral titres. The study emphasized the necessity for improvements in ONT sequencing technology to accurately measure viral loads. Additionally, the research identified a drug resistance mutation in IAV and explored the application of ONT sequencing in mapping outbreaks and transmission. The protocol exhibited over 80% sensitivity for detecting other respiratory viruses, with ongoing efforts to determine limits of detection and enhance sensitivity.

For bacterial genome sequencing utilizing ONT, researchers can choose between ligation-based or rapid library preparation methods. While ligation may provide higher yields, this study [34] emphasizes a significant advantage of rapid preparations - their superior effectiveness in sequencing small plasmids. Since small plasmids may carry AMR genes, excluding them could jeopardize the reliability of conclusions regarding the resistance. Therefore, the study suggests opting for rapid preparations when the recovery of small plasmids is crucial in ONT-only sequencing of bacterial genomes.

4. Illumina

Illumina belongs to the next generation sequencing (NGS) technologies. DNA fragments are applied to a glass flow-cell with specific adapters, undergoing amplification through bridge PCR. The flow-cell, housing amplified DNA clusters, employs a four-color DNA sequencing-by-synthesis technology. Polymerase introduces fluorescence-labelled nucleotides to the strand, with images captured after each addition for base identification. To prevent multiple base incorporations in a cycle, the 3'OH bonds of nucleotides are inactivated. Subsequently, the fluorescent labels are eliminated, and the 3'-end is unblocked for the next incorporation aiding sequence construction [35].

The MiSeq (2011) and HiSeq2500 (2012) sequencers by Illumina offer varying data outputs suitable for gene panels and bacterial genomes. The NextSeq 500 (2014) uses a two-channel system, reducing imaging and cycling, thus lowering sequencing costs. The HiSeq X Ten, HiSeq

3000, and HiSeq 4000 (2015) employ nanowell grids, significantly increasing data output. The NovaSeq (2017), Illumina's latest sequencer, features larger flow-cells, faster imaging, and four flow cell types for diverse sequencing needs. Illumina platforms dominate clinical and research settings due to accuracy, cost-effectiveness, and high throughput [36].

Study from 2014 [37] discusses the genomic surveillance of the Ebola virus during the 2014 outbreak in West Africa. Using massively parallel viral sequencing on Illumina, the researchers obtained 99 Ebola virus genome sequences from 78 patients in Sierra Leone, allowing insights into the virus's origin, transmission dynamics, and evolution. The sequencing revealed a rapid accumulation of genetic variation and identified patterns of viral transmission. The data suggested a single transmission from the natural reservoir, followed by human-to-human transmission. The study highlighted the importance of genomic surveillance for understanding and containing the epidemic.

One study [38] addressed the challenges encountered during the COVID-19 outbreak in South Africa, focusing on the difficulties in obtaining and sequencing SARS-CoV-2 samples. Resource constraints and lockdown measures prompted a shift from Oxford Nanopore to Illumina sequencing methods. The researchers assessed various library preparation kits, highlighting the efficiency and cost-effectiveness of the Illumina kits. Despite obstacles such as varying sample quality and reagent shortages, the study underscores the successful completion of data generation and analysis in a short timeframe.

Illumina can also be used to study pathogenesis of the diseases. One study [39] of Type 1 Diabetes, revealed differences in DNA methylation between diseased and controls before diagnosis. Ten regions exhibited distinct rates of methylation change over time, suggesting that methylation differences precede both the onset of autoimmunity and diabetes diagnosis. The study highlights the potential functional relevance of identified methylation patterns, indicating associations with gene expression in various tissues. While the study contributes valuable insights, further research, replication, and functional validation are needed for clinical applicability.

1.6 Illumina Vs Oxford Nanopore

Illumina utilizes second-generation sequencing technology, employing reversible terminator technology and cluster generation on flow cells for large-scale sequencing. This method creates up to 10 million single-molecule clusters per square centimetre, followed by sequencing using sequencing by synthesis technology with fluorescently labelled nucleotides [40]. In contrast, Oxford Nanopore employs nanopore sequencing technology, utilizing a flow cell with nanopores in an electro-resistant membrane. As DNA or RNA molecules pass through the nanopores, they disrupt the current uniquely, allowing for molecule identification and sequencing [1].

NGS read length, indicating the sequenced base pairs from DNA fragments, is crucial for reconstructing the complete DNA sequence through overlapping regions between reads. Short-read lengths are well suited for sequencing small RNA and gene expression profiling, while long-read lengths are optimal for *de novo* assembly due to increased sequence overlap. Illumina excels in short-read sequencing, while Oxford Nanopore is renowned for long-read sequencing, providing the longest read lengths among NGS technologies [41].

One article [42] compared Illumina and nanopore sequencing platforms in a study focused on a rapid diagnostic assay for unbiased metagenomic detection of DNA-based pathogens in body fluids. The study showed that sensitivities and specificities for bacterial and fungal detection were similar between Illumina and nanopore sequencing. Notably, nanopore sequencing showed lower sensitivity in detecting *Staphylococcus aureus*. This was attributed to higher levels of human host background DNA in nanopore sequencing. Lower read depths and higher error rates in nanopore sequencing were identified as contributors to reduced sensitivity compared to Illumina sequencing. The diagnostic assay focused on identifying pathogen-specific DNA sequences in body fluid supernatant, avoiding host depletion methods like differential lysis that may not suit low-cellularity samples. The protocol excluded mechanical processing steps like bead beating to maintain simplicity and prevent a reduction in detection sensitivity due to the release of human DNA. Clinical scenarios where metagenomic sequencing could be beneficial include identifying culture-negative pathogens, diagnosing rare infections, early testing in critically ill patients, and providing an alternative to extensive send-out tests. The study suggested that both Illumina and nanopore sequencing platforms offer comparable pathogen

detection performance. However, differences in sensitivity for specific organisms and practical considerations in the assay protocol should be taken into account when choosing the appropriate sequencing platform for diagnostic applications.

In this research [43], the sequencing data from ONT (MinION) and Illumina (MiSeq) technologies were compared for three dangerous bacterial species: *Bacillus anthracis*, *Brucella suis*, and *Francisella tularensis*. The study focused on essential tasks for reference laboratories, such as detecting genetic markers and achieving high-resolution genotyping. Illumina produced short, high-quality reads, while ONT produced longer reads with lower per-base quality, but improvements in ONT basecalling accuracy were observed. Both technologies effectively identified species, covered larger portions of reference genomes, and detected chromosomal PCR markers. ONT showcased the capability to assemble closed plasmids, providing an advantage over Illumina. Genotyping methods demonstrated comparable results between Illumina and ONT for *F. tularensis* and *B. anthracis*, with more variability observed for *B. suis*.

One study [44] evaluated the performance of whole-genome sequencing (WGS) for predicting antimicrobial resistance using ONT (MinION) and Illumina platforms. The comparison indicated comparable results between the two platforms, with overall categorical agreement of 88% (ONT) and 90% (Illumina). Despite slightly higher error rates in ONT data, particularly for carbapenem resistance, improvements in chemistry and basecalling models were noted. The study proposed the introduction of areas of technical uncertainty to manage variability and suggested combining AMR genotyping with WGS-antimicrobial susceptibility testing for more precise treatment selection. The ONT platform showed promise, especially for certain antimicrobials, highlighting its potential in clinical laboratories.

5. Materials and Methods

Nasal swab samples were collected from 5-week-old piglets within the same stable as well as from 16 (sample ID: H3, H4) and 19-week-old (sample ID: H1, H2) fattening pigs from two separate pens in the two different stables at a large-scale swine farm, near the town Szekszárd in Hungary. Following sample collection, the nasal swabs were transported on ice and stored at -20 °C until laboratory processing. The swabs were than pooled in nuclease-free molecular biology water following the subsequent procedure: five samples from fattening pigs within the

same stable and pen were combined (sample ID: H1-H4), and two pools of piglet samples, each composed of four piglets, were created (sample ID: M1A, M1B).

1.7 DNA extraction and metagenomics library preparation

DNA extraction was conducted using the QIAamp Fast DNA Stool Mini Kit from Qiagen. The concentrations of the extracted DNA solutions were assessed using Invitrogen Qubit 4 Fluorometer with the Qubit dsDNA HS (High Sensitivity) Assay Kit. However, the concentrations of the two piglet samples were found to be insufficient for library preparation. Consequently, the DNA solutions derived from these two piglet samples were pooled and concentrated using a vacuum concentrator (pooled sample ID: M2).

Library preparation was performed on DNA obtained from two nasal swab samples from fattening pig, named H1 and H3 (16 and 19 weeks old) and one piglet nasal swab sample, M2 (5 weeks old). The remaining two fattening pig samples (H2 and H4) were excluded from the study.

Library preparation steps were executed for short-read sequencing on the Illumina platform and long-read sequencing on the ONT platform. For the metagenomic nanopore sequencing long-read library, the Ligation Sequencing Kit (SQK-LSK110) was employed in conjunction with the PCR-free Native Barcoding Expansion 1-12 (EXP-NBD104) from ONT. Sequencing was carried out using a MinION Mk1C sequencer with an R9.4.1. flow cell from ONT.

1.8 Bioinformatic analysis

The ONT-generated FAST5 files underwent high-accuracy base calling using the Dorado basecaller (v0.5.1, https://github.com/nanoporetech/dorado) with the dna_r9.4.1_e8_sup@v3.6 model. Subsequently, the raw reads were adapter trimmed and demultiplexed using Porechop (v0.2.4, https://github.com/rrwick/Porechop), followed by quality-based filtering with chopper [45] (v0.7.0).

For Illumina short reads, quality-based filtering and trimming were executed with TrimGalore (v.0.6.6, https://github.com/FelixKrueger/TrimGalore), employing a quality threshold of 20 and retaining reads longer than 50 bp.

Additionally, parallel sequencing was conducted using Illumina technology. This method

yielded a substantially higher number of reads compared to ONT sequencing. To ensure comparability, for each sample, a subset of Illumina reads, with a matching number of nucleotides to those generated by ONT sequencing, was randomly sampled. This subset is referred to as the Illumina subset.

The cleaned reads were initially taxonomically classified using Kraken2 [46] on the NCBI non-redundant nucleotide database without applying any confidence limit [47]. Subsequently, employing a 50% confidence limit, taxon classification was conducted using Kraken on a database comprising the complete bacterial reference genomes from NCBI.

For the purpose of constructing longer contigs, de novo assembly was carried out using MEGAHIT (v1.2.9) [48] for the Illumina reads and metaFlye (v2.9-b1779) [49] for the ONT reads.

Open reading frames (ORFs) were extracted from both the contigs and the ONT reads using Prodigal (v2.6.3) [50]. Subsequently, the protein-translated ORFs were aligned to the antibiotic resistance gene sequences from the Comprehensive Antibiotic Resistance Database (CARD, v3.2.8) [51, 52] using the Resistance Gene Identifier (RGI, v6.0.3) with Diamond [53]. ORFs classified as perfect or strict matches were further refined with a threshold of 90% identity and 90% coverage.

All data processing and visualization were conducted within the R environment [54].

6. Results

Figure 1 provides a summary of taxon classification by kingdom using the NCBI NT database. Figure 2 illustrates the results of relative abundance classification of taxa on bacterial genomes at the genus level. Furthermore, Figure 3 depicts the presence of bacterial species with a relative abundance of at least 1% as determined by Illumina sequencing, utilizing both the Illumina subset and nanopore sequences.

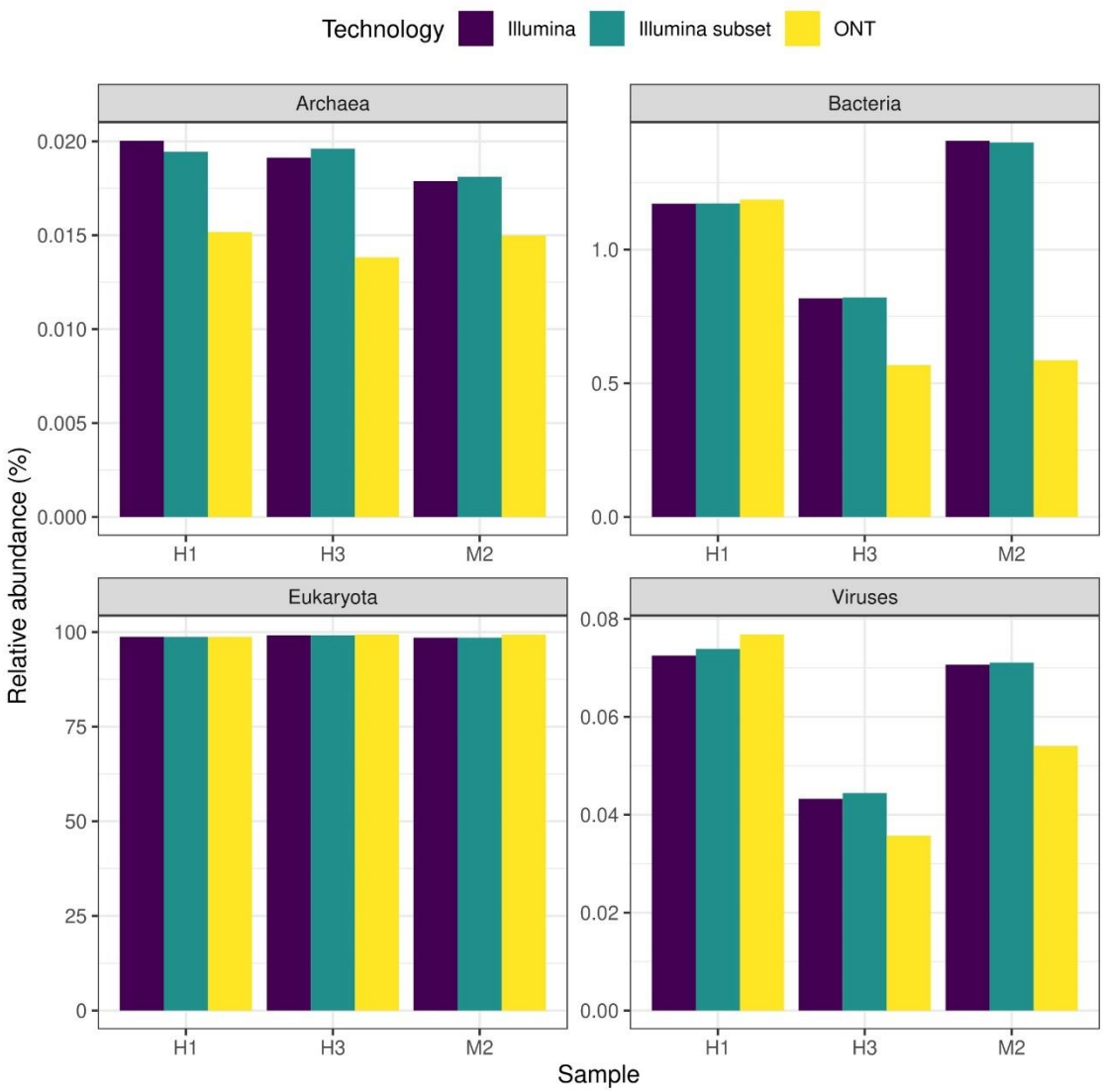

Figure 1. Relative abundance of kingdoms. H1 represents sample from 16 weeks old pigs, H3 from 19 weeks old and M2 from 5 weeks old piglets

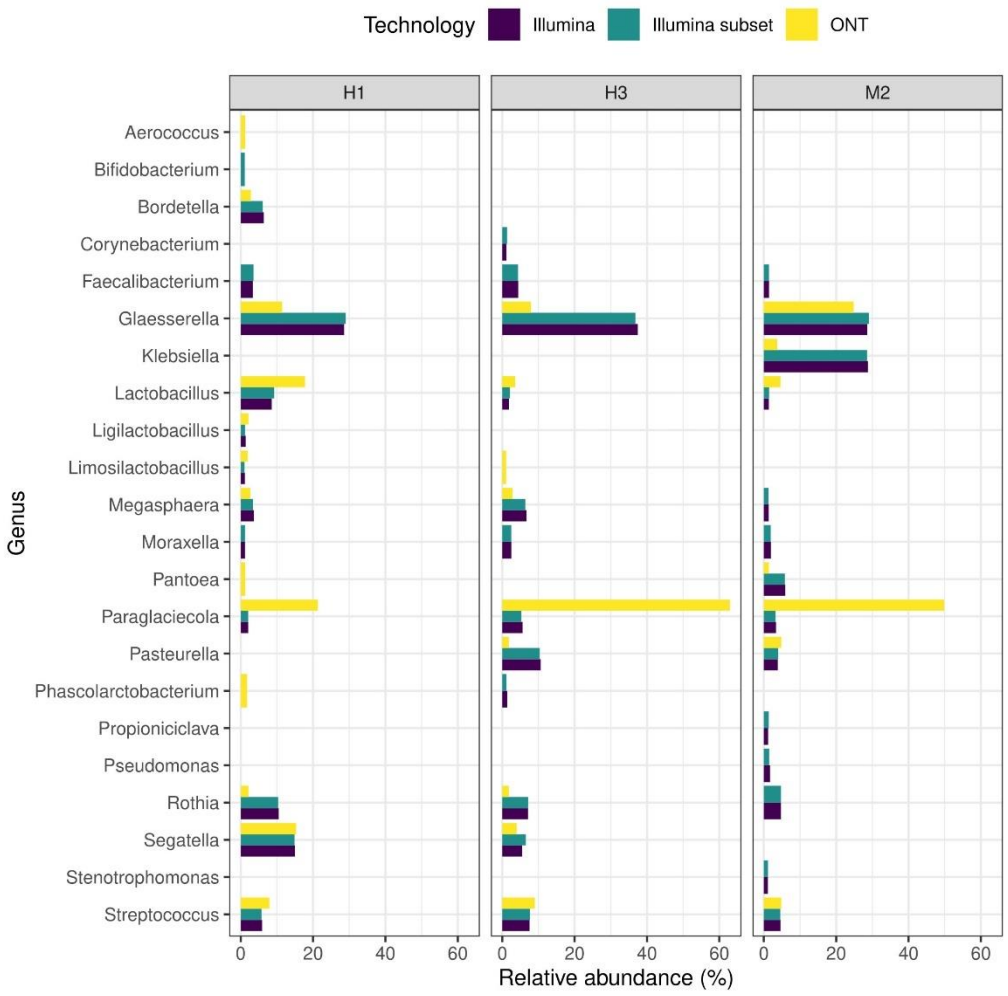

Figure 2 Relative abundance of bacterial genera. H1 represents sample from 16 weeks old pigs, H3 from 19 weeks old and M2 from 5 weeks old piglets

In the ONT long reads extracted from the H1 sample, we detected two ARGs exhibiting a minimum of 60% coverage and 90% sequential identity: CME-1 (coverage: 82.0%, identity: 100%) and IMP-55 (coverage: 66.7%, identity: 100%). Notably, both ARGs share the same antibiotic inactivation mechanism. These genes proved elusive for reconstruction from either the complete or partial Illumina dataset. Furthermore, no other ARGs were discernible in assemblies derived from ONT long reads.

In the contigs originating from Illumina reads, we identified 16 ARGs exhibiting a minimum of 60% coverage and at least 90% sequence identity (Figure 4). These ARGs include AAC(6')-I-43, AAC(6')-Il, ANT(3")-IIa, APH(3")-Ib, APH(3')-Ia, APH(6)-Id, FosA3, MexL, ROB-11, ROB-13, SAT-2, sul2, tet(B), tet(H), tet(W), and tet(Y). Figure 4 illustrates the presence of these genes within the Illumina subset and the ONT long reads.

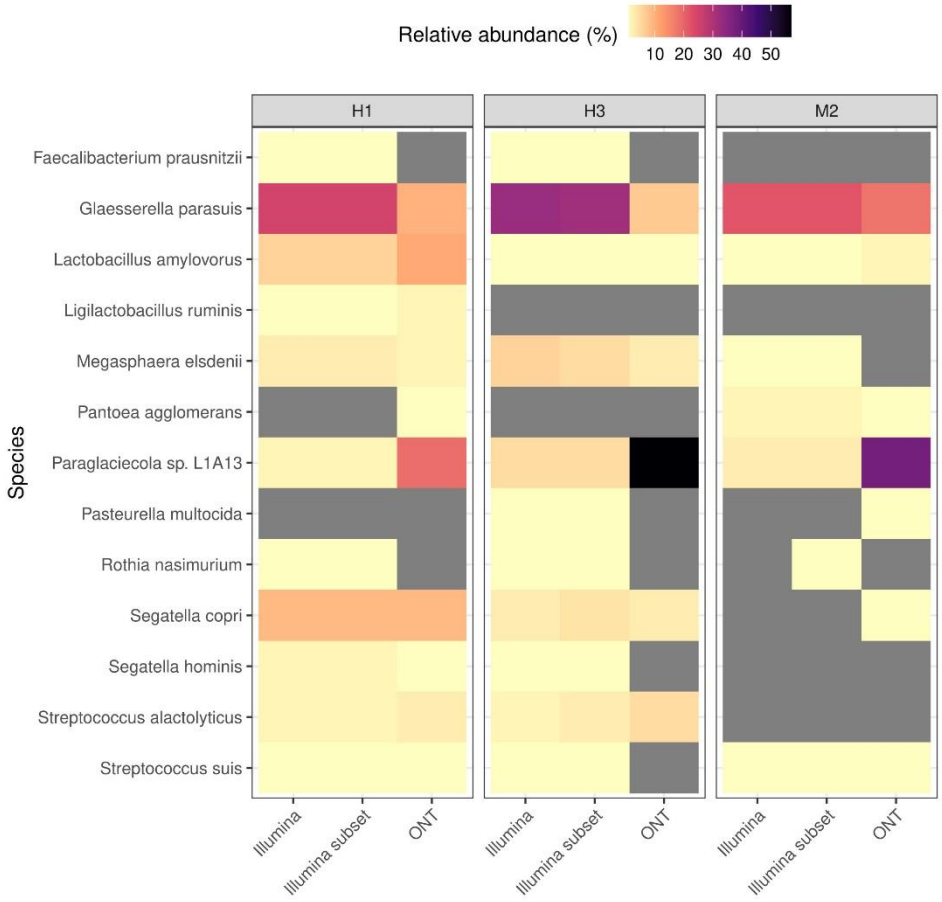

Figure 3. Relative abundance of bacterial species. The grey represents the absence of the species. H1 represents sample from 16 weeks old pigs, H3 from 19 weeks old and M2 from 5 weeks old piglets

Additionally, the resistance mechanism proportions of the identified ARGs were as follows: antibiotic inactivation (62.5%, n=10), antibiotic efflux (25.0%, n=4), antibiotic target protection (6.25%, n=1), and antibiotic target replacement (6.25%, n=1).

Figure 5 illustrates the drug classes impacted by the identified ARGs. Additionally, it provides a summary of the number of ARGs affecting each drug class observed in the samples. Despite the presence of ARGs within the sequences (long reads, contigs), we were unable to ascertain the specific species or genus from which they may have originated.

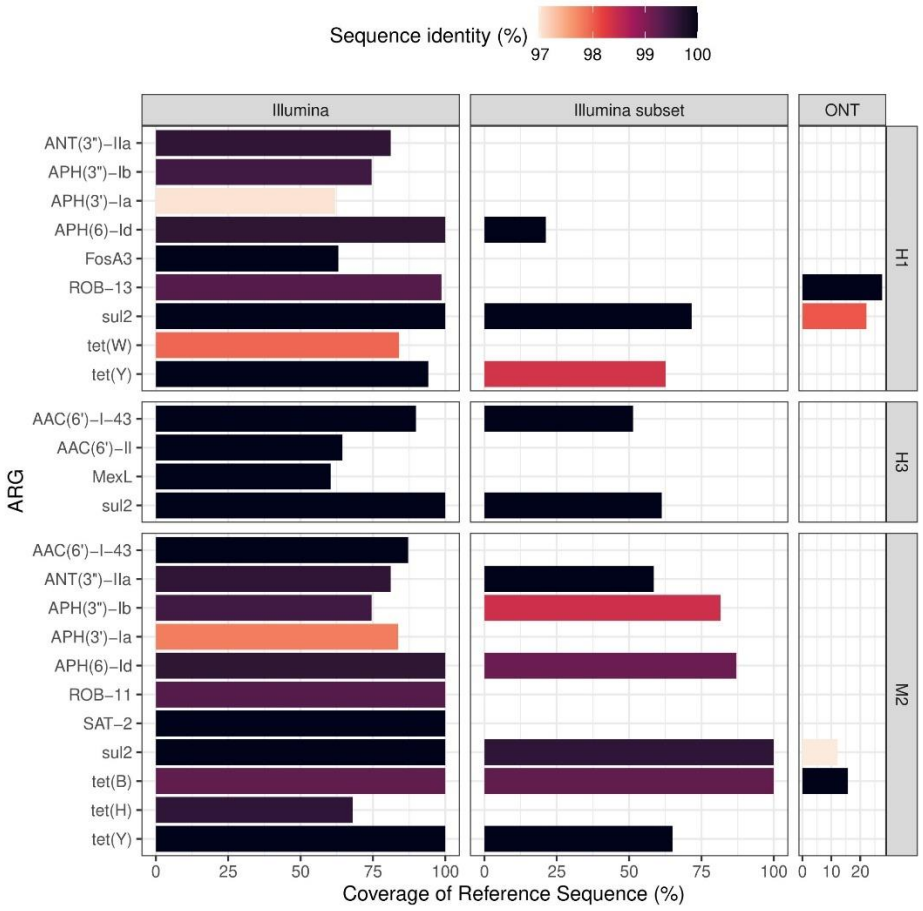

Figure 4. Antimicrobial resistance genes. Illumina sequencing detected ARGs are in the first column. The second and third columns indicate the coverage and sequence identity of ORFs associated with ARGs identified by Illumina subset and ONT, respectively.

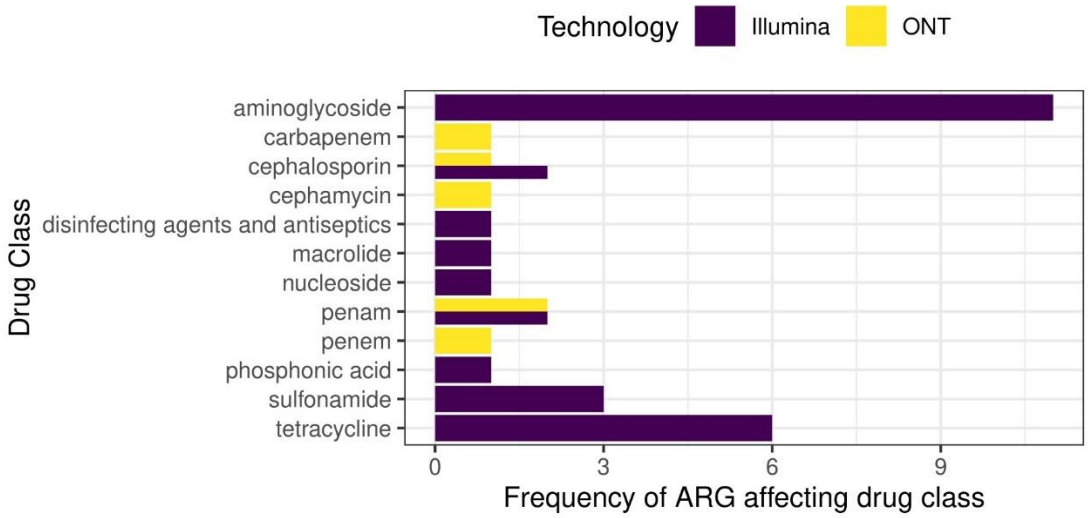

Figure 5. Drug classes affected by detected ARGs. The frequency of ARGs identified might serve as the antimicrobial resistance potential of the bacteriome.

7. Discussion

Our findings revealed that the predominant kingdom present in all three groups is Eukaryota (Figure 1), with nearly equal percentages observed across both methods and in the Illumina subset. These results are expected, as the majority of the detected genes are likely to originate from the host itself.

The second most abundant kingdom identified is Bacteria (Figure 1). Within the M2 sample group, the highest percentage was observed when utilizing both Illumina and the Illumina subset. Across all three sets, the H1 sample exhibited similar levels of bacterial abundance. Notably, this group exhibited the highest bacterial content detected by ONT.

The microbiome development in swine is influenced by different factors, including genetics, environment, diet, antibiotic exposure, and infections. During early stages of life, factors like method of birth, exposure to maternal and environmental microbiomes, and diet play significant roles in shaping microbial colonization and diversity. Environmental conditions and weaning also contribute to microbial exposure and subsequent microbiome composition, with the transition to solid feed being a crucial determinant. Infections and antibiotic use further impact microbial colonization and diversity in growing pigs [55].

In total, we identified 22 bacterial genera (Figure 2). Among these, *Paraglaciecola* was the most

abundant genus detected by ONT across all three samples. However, Illumina sequencing revealed *Glaesserella* as the predominant genus in samples H1 and H3, while in sample M2, *Klebsiella* exhibited a comparable abundance to *Glaesserella*. The Illumina subset produced comparable results to the complete Illumina dataset.

Thirteen bacterial species were chosen based on their abundance exceeding one percent (Figure 3). *Paraglaciecola sp.* L_1A_{13} was the most prevalent species in samples H3 and M2, as indicated by ONT sequencing. *Glaesserella parasuis* was the predominant species in H1 sample, identified by both Illumina and its subset. This bacterium exhibited moderately high abundance across all three sample groups. In total, 18 resistance genes were identified.

1.9 Aminoglycoside resistance genes

Our study revealed a significant abundance of aminoglycoside resistance genes (Figure 5), with the Illumina method being the sole detector of these genes.

Aminoglycoside antibiotics (AGs) are a diverse group of compounds characterized by an aminocyclitol nucleus, connected by glycosidic bonds with amino sugars. These antibiotics are primarily used to treat infections caused by Gram-negative aerobic bacteria, and some Gram-positive bacteria. AGs are typically prescribed alongside other antibiotics like beta-lactams or vancomycin when treating Gram-positive infections, leading to a synergistic effect through enhanced uptake. AGs can be inactivated through various mechanisms [56].

Aminoglycoside modifying enzymes facilitate modifications at the hydroxyl (-OH) or amino (-NH_2) groups of the aminocyclitol nucleus or the amino sugars. These enzymes can belong to categories such as acetyltransferases (AACs), nucleotidyltransferases (ANTs), or phosphotransferases (APHs) [56].

Modifications made by enzymes primarily cause aminoglycoside resistance in Gram-negative bacteria. These enzymes can be found encoded on specific genes, such as N-acetylation on AAC genes, O- nucleotidylation on AAD genes (also known as nucleotidyltransferase genes), and O-phosphorylation on APH genes. These enzymes are further classified based on the specific site of aminoglycoside modification (e.g., 3', 6', and 3'') [57]. In understanding the naming conventions of modifying enzymes, the numbering of carbon centres plays a crucial role. Typically, the aminocyclitol ring is designated without a suffix, while any additional rings are

labelled with primes (') or double primes (") [58]. The resulting resistance phenotype is designated by Roman numerals. Genes within each class are further differentiated using lowercase letters [57]. Acetyltransferases selectively acetylate one of the four amino groups in aminoglycoside antibiotics, decreasing their affinity for the 30S ribosome's tRNA site significantly [59].

The spread of resistance genes through mobile genetic elements is growing due to its potential to rapidly produce multi-drug resistant bacteria. Integron, a genetic element, is notable for its capacity to acquire, express, and remove resistance genes, often found in association with plasmids [57]. In our study, we identified AAC(6')-I-43 (aacA43) and AAC(6')-Il (aaA7) which code 6′-N-aminoglycoside acetyltransferase enzymes and are found on integrons [57, 63].

AAC(6')-I-43 was exclusively identified by Illumina sequencing in the M2 sample, whereas in the H3 sample, it was detected by both Illumina sequencing and in the Illumina subset. This gene was absent in the H1 sample (Figure 4).

AAC(6')-Il was solely identified in the H3 sample and was detected only through Illumina sequencing (Figure 4).

AacA43 carries resistance to kanamycin, amikacin, tobramycin and sisomicin but not gentamicin. In a survey conducted in a Sydney intensive care unit during 2004-2005, aacA43 was found in two *Klebsiella pneumoniae* strains, one *E. coli* strain, and one *Enterobacter cloacae* strain [60].

A significant presence of ARGs was found in soil treated with pigs' manure [61], posing potential risks to human health via the food chain. The exact mechanisms of ARG transmission through the soil-plant-animal food chain remain uncertain. This study showed the important role of ARGs in the food chain, amongst which aacA43 had a significant role. AacA43 was a dominant resistance gene found in lettuce and snail excrement. There was a significant correlation between various mobile genetic elements (MGEs) and AacA43, indicating its potential for dissemination through horizontal gene transfer (HGT). The application of pigs' manure introduced outside ARGs, creating selective pressure that promotes the growth of drug-resistant bacteria indigenous to the soil. Consequently, aacA43, along with other resistance genes, increased in abundance due to the influx of outside ARGs and the proliferation of drug-

resistant bacteria. This highlighted the role of aacA43, MGEs, and manure-induced selective pressures in spreading antibiotic resistance in agricultural ecosystems.

Mycobacteroides abscessus complex belongs to the group of nontuberculous mycobacteria. This complex is highly resistant to many antibiotics and is responsible for causing respiratory, skin, and soft tissue infections. Some of the main targets of resistance genes were AGs, with AacA43 having an important part of these ARGs [62].

The aacA7 gene was discovered as the primary cause of the antibiotic resistance outbreak at Hospital Vargas in Venezuela. It was isolated and sequenced from *Klebsiella aerogenes,* (previously known as *Enterobacter aerogenes*) and *Enterobacter cloacae* [63]. This gene encodes amikacin and tobramycin resistance.

The aacA7 gene frequently co-occurs with various other antibiotic resistance genes within multidrug-resistant bacterial strains like *Pseudomonas Aeruginosa* [64, 65], *Providencia rettgeri* [66], *Klebsiella pneumoniae* [67], etc.

The bacteria carrying the plasmid pEC5207, which harbours resistance genes for aminoglycosides (aacA7), sulphonamides (sul1), and heavy metals, are subjected to selective pressure in the environment where these metals are present. For example, copper is commonly used as a trace element in feed additives on pig farms, while silver is used as a disinfectant in water and on surfaces in the same setting. Under the selective pressure exerted by heavy metals and antimicrobials, bacteria carrying the plasmid pEC5207 have a survival advantage, as they possess genes that confer resistance to these compounds. This selective pressure promotes the persistence of these bacteria in the intestinal environment of pigs. Consequently, there is an increased risk of co-selection, in which bacteria carrying additional resistance genes, are favoured due to their coexistence with the resistant bacteria carrying pEC5207 [68].

Aminoglycoside nucleotidyltransferase uses ATP as a secondary substrate to modify aminoglycoside antibiotics by transferring AMP to their hydroxyl group [69].

In our study, ANT(3")-IIa was detected in the H1 sample through Illumina sequencing, while in M2 sample, it was identified by Illumina and in Illumina subset (Figure 4).

ANT(3")-IIa provides the resistance against streptomycin and spectinomycin [70]. The enzyme [71] alters the 3″-hydroxyl position of streptomycin and the 9-hydroxyl position of

spectinomycin. In a study conducted in 2017 [72], it was demonstrated that ANT(3")-IIa functions as both streptomycin and spectinomycin nucleotidyltransferase in *Acinetobacter baumannii*. Furthermore, the investigation revealed the presence of this gene in *Acinetobacter pittii* and *Acinetobacter gyllenbergii*, indicating its intrinsic presence in these bacterial species. This gene was also identified in *Acinetobacter junii*, and its acquisition was attributed to horizontal gene transfer originating from *A. baumannii*. In the case of *Aeromonas caviae*, there is evidence suggesting the potential acquisition of ANT(3")-IIa genes through gene transfer mechanisms rather than intrinsic means [73].

Clear definition and understanding of ARGs and their hosts have profound implications across various aspects of life and the environment. This study [74] assessed antibiotic resistome pollution in the Henan section of the Yellow River, analysing faecal pollution's contribution to ARGs. The presence of aminoglycoside resistance genes, such as ANT(3")-IIa and APH(3')-Ia, among the detected ARGs in Yellow River sediments underscores their importance. These genes play a significant role in conferring resistance to aminoglycoside antibiotics, which have been extensively used in China for many years. Their prevalence in the sediments suggests that aminoglycoside antibiotics contribute to the overall abundance of ARGs in the environment. Additionally, their ability to be localized on plasmids or integrons facilitates their spread among bacterial populations. The detection of these genes highlights the need for continued monitoring and tighter controls on antibiotic use to mitigate the spread of antibiotic resistance in aquatic environments.

We detected the APH(3')-Ia gene in both the H1 and M2 sample groups, with its presence being solely identified through Illumina sequencing (Figure 4).

APH(3')-Ia carries the resistance against kanamycin, gentamicin B, neomycin, paromomycin, ribostamycin, and lividomycin [58]

The frequent occurrence of APH(3')-Ia gene, implicated in resistance to clinically significant AGs, underscores its role in mediating resistance within clinical contexts. Its identification across bacterial strains such as *E. coli*, *Proteus mirabilis*, and *Morganella morganii* highlights the necessity of vigilant surveillance and an in-depth understanding of both prevalence and mechanisms underlying AG resistance [75]. This extends to antibiotics that are infrequently prescribed, signifying a broader understanding of antimicrobial resistance dynamics.

APH(3')-Ia was identified as a constituent of the gut microbiota in both cattle and swine populations within Korea. The presence of this gene was also detected in various bacterial species such as *Salmonella*, *Escherichia*, *Corynebacterium*, and *Serratia*. This observation suggests a plausible association between the transfer of this genetic element among these bacterial species and their respective animal hosts. Moreover, within the microbiome of Korean cattle, AG resistance genes represented the second most prevalent class of ARGs identified. This finding underscores the significance of AG resistance mechanisms within the microbial communities inhabiting cattle gastrointestinal tracts in Korea [76].

APH(3')-Ia was also found in *E. coli* isolates from wild boars and coyotes, underscoring the role of wild animals in disseminating multidrug-resistant bacteria [77]. This phenomenon has implications not only for human health but also for the well-being of other animal species. To address these risks, it's vital to integrate wildlife into monitoring, surveillance, research, and management efforts to mitigate the spread of multidrug-resistant bacteria and minimize associated dangers.

Aminoglycoside phosphotransferases are commonly encoded by genes located on multidrug resistance elements like R plasmids, transposons, and integrons. These enzymes play a crucial role in altering the antibiotics by phosphorylation, thereby impairing their ability to bind effectively to the A-site of the ribosome, ultimately reducing their efficacy [58]. In our study, we identified the genes APH(3')−Ia, APH(3")-Ib (strA), and APH(6)-Id (strB), all of which belong to this particular group of enzymes. These genes were exclusively detected in the H2 and M2 samples, with all sequences obtained through Illumina sequencing. Additionally, they were identified in the Illumina subset of the M2 sample.

In the UK study [78], *E. coli* isolates from diverse sources including livestock, meat, and humans with serious infections were examined, uncovering the prevalence of antimicrobial resistant genes within each reservoir. Notably, both livestock and human isolates harboured genes such as APH(3')−Ia, APH(3")-Ib, and APH(6)-Id. Although the study did not detect recent gene transfer between animal and human isolates, it did observe a notable relatedness among isolates from distinct farms. On the other hand, a study from 2019 [79] revealed that the source of these ARGs in humans can stem from both swine and bovine sources.

All these phosphotransferases frequently occur together in multidrug resistant bacteria [80, 81, 82] and are amongst the most prevalent environmental and clinical isolates in US hospitals [83].

The significance of APH(3′)-Ia in the spreading of neomycin resistance was shown in clinical *E. coli* in Denmark, following its reinstatement in 2017 for porcine enteritis treatment. This research demonstrates that the APH(3′)-Ia spread is complex, involving multiple procurements of distinct conjugative plasmids of variable sizes, carrying multiple resistance genes. Plasmids with extra resistance genes imply a link between neomycin resistance and the use of other antibiotics. Additionally, genes conferring resistance to heavy metals like mercury and tellurite on these plasmids suggest they could also influence neomycin resistance selection [84].

1.10 Tetracycline resistance genes

Our investigation revealed the presence of four genes associated with tetracycline resistance. Interestingly, these genes were absent in the H3 sample, as illustrated in Figure 4, despite being among the most prevalent after the aminoglycoside resistance genes, as depicted in Figure 5. Among these genes, Tet(Y) stood out as the most predominant tetracycline resistance gene in both the H1 and M2 samples, with detection also in the Illumina subset for both groups. Tet(W) was exclusively identified in the H1 sample, while tet(H) was solely detected in the M2 sample, both exclusively sequenced via Illumina. Additionally, Tet(B) was identified as part of the resistance genes in the M2 sample, being detected by both Illumina and ONT, and it was also represented in the Illumina subset.

Resistance to tetracycline antibiotics can occur through various mechanisms, with efflux pumps and ribosomal protection being key contributors. Efflux pumps, such as those encoded by tet(B), tet(H), and tet(Y), are proteins integrated into the bacterial cell membrane. Their role is to actively remove tetracycline molecules from the cell, reducing the intracellular concentration of the antibiotic and safeguarding the bacterial ribosomes from its inhibitory effects. These efflux mechanisms are prevalent among gram-negative bacterial species. While most efflux proteins confer resistance specifically to tetracycline, they typically do not impact the effectiveness of related antibiotics like minocycline or glycylcyclines. However, tet(B) stands out as an exception, as it codes for an efflux protein that grants resistance to both tetracycline and minocycline, though not to glycylcyclines [85].

The tet(B) gene is widely distributed among gram negative bacteria [85]. However, in a noteworthy discovery in 2019 [86], the expression of tet(B) was identified in one isolate Gram-positive bacteria of the *Streptococcus* genus. So far, confirmation of this tetracycline resistance gene's presence in Gram-positive bacteria was lacking. Prior researches have demonstrated the transfer of antibiotic resistance genes between Gram-positive and Gram-negative bacteria. This finding underscores the potential contribution of *Streptococcus* bacteria to the dissemination of this gene into the environment through horizontal gene transfer mechanisms.

One study revealed that certain antibiotic resistance genes, including strA, strB, and tet(H), have origins predating the modern antibiotic era. These genes were discerned within plasmids isolated from *Psychrobacter psychrophilus* specimens sourced from permafrost sediment, a milieu preexisting the pervasive application of antibiotics [87].

Tet(W) encodes a protein that binds to the ribosome, thereby restricting tetracycline from binding to it [85].

Manure emerges as a significant reservoir of resistance genes, particularly by introducing multiple tetracycline genes into the soil milieu. Notably, tet(W) emerges as a pivotal determinant in the dissemination and persistence of tetracycline resistance within dairy farming environments [88]. Despite variations observed among individual animals, the prevalence of tet(W) remained largely unaffected by intrauterine antibiotic applications in cows. Intriguingly, evidence suggests that calves acquire tet(W) at an early developmental stage, indicating a potential transmission route from mother to foetus. Moreover, the presence of antibiotic residues in milk may transiently elevate the abundance of tetracycline resistance genes in calves. Furthermore, the application of farm-derived manure to agricultural fields results in soil contamination with tetracycline resistance genes, which exhibit sustained presence over prolonged periods. This study underscores the pivotal role of tet(W) in facilitating the dissemination of antibiotic resistance within agricultural contexts.

In the other study [89], an assessment of soil treated with pig manure revealed a notable enrichment of tet(W) abundance, even in the absence of direct manure application. However, the utilization of manure resulted in a discernible augmentation in the levels of specific antibiotic resistance genes within the soil ecosystem.

Both of these studies demonstrated that antibiotic resistance genes could be present in the microbial communities of farm animals, and that farms have a potential role in the spread of antibiotic resistance genes into the environment.

1.11 Sulphonamide resistance genes

Sulphonamide resistance genes emerged as the third most frequent among the resistance genes, as shown in Figure 5, and they were detected through Illumina sequencing. Our findings revealed the presence of the sul-2 resistance gene across all groups, a consistent observation also noted in the Illumina subset. ONT was able to detect the presence of sul2 in the M2 sample, though in lower abundance (Figure 4).

Sulphonamide antibiotics exert their action by inhibiting the di-hydro-pteroate synthase (DHPS) enzyme, thereby disrupting folate synthesis in bacteria. Despite their initial efficacy, resistance to sulphonamides emerged swiftly following their introduction. This resistance can arise through mutations in the chromosomal folP gene, responsible for encoding DHPS, leading to reduced affinity for sulphonamides. Additionally, genes carried on plasmids and integrons, such as sul2, play a role in conferring sulphonamide resistance and are frequently encountered in clinical isolates. This study suggests that the emergence and dissemination of the sul2 gene likely originated from ancient chromosomal mutations in the folP gene within bacterial families *Leptospiraceae* and *Rhodobiaceae*. [90].

The sul2 gene frequently coexists with the strA-strB genes, creating a genetic cluster that imparts resistance commonly seen in a range of bacterial species like *Pasteurella*, *Mannheimia*, *Haemophilus*, and *Actinobacillus*. This gene pairing can manifest in various configurations, with differing gene orientations and occasional insertion sequences between sul2 and strA-strB. Such variations highlight the dynamic nature of genetic arrangements within bacterial genomes. The presence of sul2 with strA-strB genes underscores the pivotal role of horizontal gene transfer in spreading antibiotic resistance throughout bacterial communities [91].

The genes sul2 and tet(W) are proposed markers for evaluating the impact of human and livestock activities on the soil microbiome and the presence of ARGs within it [92].

1.12 Fosfomycin resistance genes

FosA3, a gene associated with resistance to Fosfomycin, was exclusively identified in samples from the H1 sample through Illumina sequencing (Figure 4). However, its presence was noted in relatively lower frequency compared to the previously detected resistance genes (Figure 5).

Recent review delved into the topic of Fosfomycin resistance and its dissemination. Fosfomycin, a broad-spectrum bactericidal antimicrobial agent, is commonly employed in the treatment of lower urinary tract infections. Due to its comparatively lower resistance profile compared to other antibiotics, it is often regarded as a therapeutic option for highly resistant infections. However, despite historically low resistance rates, the emergence of the FosA3 gene has contributed to a gradual increase in Fosfomycin resistance, posing challenges to its continued efficacy. FosA3 is a plasmid-born gene encoding the glutathione transferase enzyme. FosA3 was detected in pet animals despite no prior exposure to Fosfomycin treatment. Additionally, it was shown that pets can transmit this gene to their owners. Until recently, it seemed that this resistance was mainly restricted to Asia, although this observation could be attributed to underreporting. FosA3 stands out as the most widespread gene variant in European countries also [93]. FosA3 frequently co-occurs with other resistance genes [94] like blaCTX-M and blaTEM, indicating a collective dissemination of Fosfomycin resistance alongside other ARGs.

One study investigated how 3-month occupational exposure to swine farm environments affects veterinary students' gut microbiome and resistome. Transferable plasmid-mediated AR genes, including $bla_{CTX\text{-}M}$ and fosA3, increased during farm stays but declined afterward. The simultaneous presence of fosA3 with $bla_{CTX\text{-}M}$ on identical genetic structures suggests the farm environment's role in AR transmission to the human microbiome [95].

1.13 Streptothricin resistance genes

Our investigation isolated the SAT-2 gene solely within the M2 group's samples, utilizing the Illumina sequencing method (Figure 4). The occurrence rate of this gene within this group's samples is comparable to that of Fosfomycin, as illustrated in Figure 5.

The potent antimicrobial activity of streptothricins initially suggested their potential clinical utility, but subsequent studies revealed significant toxicity in mammals, particularly regarding kidney function. Despite early attempts to mitigate toxicity, clinical interest waned. Due to their

toxicity, streptothricins have primarily been utilized in biotechnology, but this led to the rapid emergence of streptothricin-resistant bacteria. Despite the discontinuation of some agricultural practices, the appearance of new streptothricin resistance determinants remains a concern [96].

The SAT-2 gene is part of the group of genes that encode the streptothricin acetyltransferase enzyme, responsible for acetylating the β-lysine amino group of streptothricin. Initial studies on this enzyme revealed its specificity for streptothricin and its reliance on acetyl-CoA as a cofactor. However, despite the identification of multiple streptothricin acetyltransferase genes, the current understanding of these enzymes remains limited, as reflected by their underrepresentation in databases [96].

The SAT-2 is frequently associated with dfrA1 and aadA1 genes within class II integrons. Class II integrons are prevalent in multidrug-resistant bacteria such as *Klebsiella*, *Escherichia*, *Proteus*, and *Acinetobacter*, confirming that the transfer of Sat-2 is linked to the presence of multiple resistance genes [97, 98].

This review emphasizes the importance of not only exploring the medicinal applications of streptothricins but also evaluating strategies for understanding and mitigating the spread of resistance to this compound [96].

1.14 β-lactamase resistance genes

CME-1 stands out as one of the two genes uncovered solely through ONT sequencing, with no detection via Illumina methods in our study. Interestingly, this gene was exclusively found within group H1 samples.

CME-1 belongs to the class A serine β-lactamase. It has the ability to hydrolase the majority of cephalosporins, monobactams, and, penicillins but it does not have the capacity to hydrolyse cephamycins and carbapenems. It is present in bacteria that are classified within the *Elizabethkingia* genus constituting a segment of the bacterial chromosome [99]. CME-1 has been detected in *E. anophelis* [99] and *E. miricola* [100]. One study [101] demonstrated these species were primarily isolated from samples collected from the lower respiratory tract.

The emergence of *E. anophelis* infections has been documented since its initial report in 2011. Subsequent studies have underscored its prevalence as a life-threatening infection across various

regions worldwide. However, the misidentification of this bacterium as *E. meningoseptica* may lead to an underestimation of the true incidence of *E. anophelis* infections. Furthermore, uncertainties persist regarding the routes of infection in some cases. Given the high mortality rates associated with infected patients, these factors are of significant concern [102].

Our investigation identified two ROB genes, known as ROB-11 and ROB-13. ROB-13 was exclusively detected in the H1 sample group and its presence was confirmed through both Illumina and ONT sequencing methods, while ROB-11 was observed solely in the sample group M2 and its identification was accomplished solely through Illumina sequencing.

ROB ARGs are classified under the class A serine β-lactamase group. These enzymes are capable of breaking down penicillins and first-generation cephalosporins through hydrolysis. These genes can be co-located within *Pasteurella multocida*. Notably, one research indicated that the ROB-11 gene exhibits a single amino acid variance from ROB-1, while ROB-13 displays a divergence of three amino acids. Further investigation revealed that these genes are components of an integrative conjugative element, suggesting their potential dissemination through both horizontal transfer and clonal expansion mechanisms [103].

In a study published in 2024, the ROB-11 gene was identified for the first time in *E. coli* isolates obtained from samples collected from pig and cattle farms in Argentina. [104]. This gene was identified within a transposon in the multidrug-resistant genome of *Gallibacterium anatis*, as observed in samples collected from hens in Poland [105].

The IMP-55 gene is the second gene that represents the second gene exclusively sequenced by ONT in our study. This gene was discovered within the H1 sample group. The frequency of this gene was similar to those considered less prevalent, such as Fosfomycin and nucleoside resistance genes (Figure 5).

The IMP-55 gene encodes the enzyme imipenemase, which is classified as a metallo-β-lactamase. These enzymes utilize zinc ions to assist in binding antibiotics, which triggers a nucleophilic attack, resulting in the cleavage of the β-lactam amide bond. This enzyme provides protection against nearly all β-lactam antibiotics, including carbapenems [106].

IMP-55 was initially discovered in clinical isolates of *Acinetobacter baumannii*, contained within a class I integron. Notably, this integron was situated on a plasmid, which played a crucial

role in promoting the dissemination of carbapenem resistance within hospital settings. The rise of multidrug-resistant *A. baumannii* strains in healthcare facilities is a worldwide emergency, severely limiting treatment options to carbapenems due to their high resistance to various antibiotics. These multi resistant strains are believed to function as reservoirs for antibiotic resistance genes linked to integrons, with the potential to disseminate to other bacteria [107].

1.15 Multidrug resistance genes

We observed that the MexL gene was exclusively present in the H3 sample group and was solely detected through Illumina sequencing. This gene significantly contributes to the frequency of tetracycline resistance genes and is associated with the resistance to macrolides and disinfectant agents (Figure 5).

MexL plays an important role in antibiotic resistance by regulating the expression of the MexJK efflux pump, which is a major contributor to multidrug resistance in bacteria like *Pseudomonas aeruginosa*. When MexL is active, it represses the expression of the MexJK pump, limiting its ability to expel antibiotics from the bacterial cell. However, in the absence of MexL repression or under certain conditions, the expression of MexJK is increased, leading to enhanced efflux of antibiotics and decreased susceptibility of the bacteria to these drugs. Therefore, MexL acts as a regulator of antibiotic resistance by controlling the activity of the MexJK efflux pump [108].

This pump is not only responsible for resistance to macrolide antibiotics, such as tetracycline and erythromycin, but also provides protection to bacteria against antiseptics like triclosan, a broad-spectrum biocide. MexJK likely operates as a tripartite antibiotic efflux system, requiring an outer membrane protein channel, a periplasmic membrane fusion protein, and an inner membrane transporter to carry out its function effectively. This study demonstrated that a missense mutation in the MexL gene resulted in the overexpression of MexJK, ultimately leading to high resistance to triclosan. [109].

One study proposed that mutations in MexL might potentially affect *P. aeruginosa*'s resistance to β-lactam antibiotics, highlighting the need for further investigation into this hypothesis [110].

In one study, *P. aeruginosa* had extensive mechanisms to nearly all antibiotics, including mutations in the efflux pump. This particular strain has the potential to develop into a pandrug-resistant bacterium [111].

8. Conclusion

In our study, we utilized both Illumina and Oxford Nanopore Technologies sequencing methods to analyse the microbiome composition and antibiotic resistance gene profiles.

When examining the microbiome composition, both Illumina and ONT sequencing methods demonstrated the prevalence of Eukaryota as the predominant kingdom across all sample groups. This consistency suggests the reliability of both methods in capturing major taxonomic groups. Additionally, the presence of Bacteria as the second most abundant kingdom further underscores the agreement between Illumina and ONT results. Notable differences emerged in the abundance of specific bacterial genera detected by each method. These discrepancies could stem from differences in sequencing depth, error rates, or bioinformatic processing between the two platforms.

Both Illumina and ONT sequencing methods uncovered a diverse array of resistance genes. While Illumina sequencing exclusively identified certain genes, showcasing its sensitivity in detecting specific ARGs, ONT sequencing unveiled others not detected by Illumina, emphasizing its ability to capture unique resistance profiles. The differences in ARG detection between sequencing methods highlight the need for using multiple platforms to fully characterize resistance profiles. While Illumina sequencing offers high sensitivity and accuracy for certain ARGs, ONT sequencing provides unique insights into gene diversity and overall antibiotic resistance mechanisms.

Short-term sequencing prioritizes speed and efficiency to quickly address immediate research needs or applications, while long-term sequencing focuses on comprehensive data generation and analysis over extended periods to achieve in-depth understanding and insights.

Our analysis of Illumina and ONT sequencing highlights their complementary roles in microbiome and ARG profiling. By combining both methods, researchers gain a deeper understanding of microbial communities and antibiotic resistance dynamics, aiding targeted interventions in healthcare and the environment.

9. References

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