MOLECULAR EPIDEMIOLOGY OF ANTIBIOTIC-RESISTANT *ENTEROCOCCUS SPP.* FROM FARM-TO-FORK IN INTENSIVE PIG PRODUCTION IN KWAZULU-NATAL, SOUTH AFRICA

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A dissertation submitted in fulfilment of the requirements for the degree of Master of Pharmacy (Pharmacy) in the School of Health Sciences, University of KwaZulu-Natal.

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This is a dissertation by manuscript with an overall introduction and final summary.

This is to certify that the content of this dissertation is the original research work of Miss Sasha Badul, supervised by;

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DECLARATION

I, Miss **Sasha Badul**, declare that:

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Any omissions and shortcomings that may be identified in this work remain the sole responsibility of the researcher.

S. Badul

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LIST OF ABBREVIATIONS AND ACRONYMS

ABR	Antibiotic resistance
AGISAR	Advisory Group on Integrated Surveillance of Antimicrobial Resistance
AGPs	Antibiotic growth promoters
AMP	Ampicillin
AMR	Antimicrobial resistance
AMU	Antimicrobial use
AS	Aggregation substance
AST	Antimicrobial susceptibility testing
ATLASS	Assessment Tool for Laboratories and AMR Surveillance System
BRICS	Brazil, Russia, India, China and South Africa
CC	Clonal complex
CHL	Chloramphenicol
CIA	Critically Important Antimicrobials
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
Cu	Copper
EMA	European Medicines Agency
ERY	Erythromycin
ESKAPEE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp., and *Escherichia coli*
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EU	European Union

EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agricultural Organization of the United Nations
GAP	Global Action Plan
GEN	Gentamicin
GLASS	Global Antimicrobial Resistance Surveillance System
HLGR	High-level gentamicin resistance
HGT	Horizontal gene transfer
ICE	Integrative and conjugative elements
IPM	Imipenem
IS	Insertion sequence
LEV	Levofloxacin
LZD	Linezolid
MDR	Multidrug-resistant
MLS	Macrolides, Lincosamides and Streptogramins
MLSB	Macrolides, Lincosamides and Streptogramin B
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
NIT	Nitrofurantoin
NTC	No template control
Q-D	Quinupristin-dalfopristin
oriT	Origin of transfer
OIE	World Organization for Animal Health
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PCU	Population correction unit

PVS	Performance of Veterinary Services
REP-PCR	Repetitive element palindromic-polymerase chain reaction
RT-PCR	Real time-polymerase chain reaction
STR	Streptomycin
SXT	Sulfamethoxazole-trimethoprim
TEC	Teicoplanin
TET	Tetracycline
TGC	Tigecycline
VAN	Vancomycin
VARSS	Veterinary Antibiotic Resistance and Sales Surveillance
VCIA	Veterinary Critically Important Antimicrobial agents
VRE	Vancomycin-resistant *Enterococci*
VRE. f*aecium*	Vancomycin-resistant *E. faecium*
WAAW	World Antimicrobial Awareness Week
WAHIS	World Animal Health Information System
WHO	World Health Organization

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ABSTRACT

Background: Substantial antibiotic use and high population densities in intensive farming systems results in the emergence and spread of antibiotic-resistant commensals and pathogens. This study investigated the molecular epidemiology of antibiotic resistance (ABR) and virulence in *Enterococcus spp*. from pigs in an intensive food production continuum from farm-to-fork in the uMgungundlovu district, Kwa-Zulu Natal.

Methods: A total of 174 samples obtained along the pig farm-to-fork continuum (farm, transport, abattoir, and retail meat) were subjected to the quantification and putative identification of *Enterococcus* spp. using the IDEXX Enterolert® method and selective media, respectively. Up to three presumptive enterococcal colonies were picked per sampling point for molecular confirmation by real-time PCR, targeting the genus- and species-specific (*tuf* and *sodA*) genes, respectively. Antibiotic resistance profiles were determined by the Kirby-Bauer disk diffusion method against a panel of antibiotics for *Enterococcus* spp. recommended by the WHO-AGISAR using EUCAST guidelines. Selected antibiotic resistance and virulence genes were detected by real-time PCR. Clonal relatedness between isolates across the continuum was evaluated by REP-PCR.

Results: A total of 284 isolates constituted the final sample. Real-time PCR confirmed 79.2% of the isolates as *E. faecalis*, 6.7% as *E. faecium*, 2.5% as *E. casseliflavus*, 0.4% as *E. gallinarum,* and 11.2% as other *Enterococcus* spp. Antibiotic susceptibility testing revealed resistance to sulfamethoxazole-trimethoprim (78.8%), tetracycline (76.9%), erythromycin (68.1%), streptomycin (62.6%), chloramphenicol (27.0%), ciprofloxacin (8.5%), gentamicin (8.1%), and levofloxacin (5.6%) but no vancomycin, teicoplanin, tigecycline or linezolid resistance was detected. *E. faecium* displayed 44.4% resistance to quinupristin-dalfopristin. A total of 78% of enterococcal isolates were MDR. Phenotypic resistance to tetracycline, aminoglycosides, and macrolides was corroborated by the presence of the *tetM*, *aph(3')-IIIa*, and *ermB* genes in 99.1%, 96.1%, and 88.3% of the isolates, respectively. The most commonly detected virulence genes were: *gelE*, *efaAfs*, and *cpd* in 89.1%, 78.5%, and 77.1% of isolates conferring autolysin and biofilm formation capabilities, cell adhesion, and conjugative plasmid accumulation, respectively. Clonality evaluated by REP-PCR revealed that E. *faecalis* isolates belonged to diverse clones along the continuum with major REP-types, largely consisting of isolates from the same sampling source but different sampling rounds (on the farm). *E. faecium* isolates revealed a less diverse profile. There was minimal evidence of clonal transmission across the continuum.

Conclusion: Multi-drug resistant *Enterococcus* spp. were isolated along the farm-to-fork continuum. Isolates harboured a diversity of antibiotic resistance and virulence genes in different combinations forming reservoirs for the potential transfer of these genes from pigs to occupationally exposed workers

and consumers via direct contact with animals and animal products/food, respectively. The results highlight the need for more robust guidelines for antibiotic use in intensive farming practices and the necessity of including *Enterococcus* spp. as an indicator in antibiotic resistance surveillance systems in food animals.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

"With a lack of development of new antibiotics, and increasing resistance even to last-resort antibiotics, there is a need to conserve the ones available" (von Wintersdorff *et al.,* 2016). Globalization has led to increased interactions between different regions and populations, meaning that all people and places in the world are now interconnected. People and animals are continuously interacting, both with each other and their associated environments, and a complex link exists between the indiscriminate use of antibiotics and the evolution and dissemination of antibiotic resistance in these three sectors. As the population growth rate increases, the demand for food animal products increases, causing a shift to more intensive farming systems where antibiotics are used to not only treat disease but for growth promotion, prophylaxis and metaphylaxis to maintain animal health and productivity (Dewulf *et al.,* 2020). The use of antibiotics, combined with the high population densities of these systems, create favorable conditions for the exchange of bacteria and antibiotic resistance genes (ARGs) by exerting selection pressure (FAO, 2016). The predicted increase in annual global antibiotic consumption in livestock and the increasing emergence of antibiotic-resistant pathogens in animals is a grave public health concern (Van Boeckel *et al*., 2015). Antibiotics used in animal health are often analogues of those used in human health, which can serve as a driving force for disseminating resistance determinants between animals to humans, and vice versa either by direct contact or via the food chain and environment. The degree to which this transmission occurs is of significant interest and has implications for human and animal health (Singer *et al.,* 2016). Therefore, the antimicrobial resistance (AMR) crises cannot be remedied by addressing one sector and necessitates a One Health approach.

An early example of the relationship between antibiotic use in animals and the incidence and subsequent spread of resistance determinants is the use of avoparcin, a glycopeptide structurally related to vancomycin, as a growth promoter in chickens and pigs in many European countries from the 1970s until it was banned in the late 1990s. This led to the frequent isolation of vancomycin-resistant enterococci (VRE) in farm animals and humans alike (Marshall & Levy, 2011). Today, vancomycin is considered a last-line antibiotic for the treatment of severe infections caused by Gram-positive pathogens.

Enterococcus spp. are Gram-positive commensals present in the gut of humans and animals, but they have also emerged as nosocomial pathogens, e.g., VRE, that present a serious challenge to antibiotic therapy (WHO, 2017). In light of this, the World Health Organization (WHO) listed vancomycin-

resistant *Enterococcus faecium* (VRE. *faecium*) as a high-priority pathogen for the development of new antibiotics.

To fully grasp the complexity of enterococci in causing disease, a greater understanding of their ability to survive stresses, their antibiotic resistance, virulence traits, and dissemination pathways are required in all the One Health niches they occupy. While there are some reports of enterococcal infections in human health, there is currently limited information available in South Africa on the molecular characteristics and distribution of antibiotic-resistant enterococci in intensively produced food animals such as pigs. Therefore, it is necessary to investigate antibiotic-resistant *Enterococcus* spp. along the pig production chain, from farm-to-fork, to inform evidence-based measures for its containment.

1.2 Literature review

1.2.1 Antibiotic Resistance

Antimicrobials are medicines used to prevent or cure infections and include antibiotics, antivirals, antifungals, and antiparasitics. AMR develops when pathogens are no longer susceptible to these medicines. Antibiotic resistance (ABR) refers to bacteria that no longer respond to antibiotics that originally killed them and cured the infection (WHO, 2015). This phenomenon may occur naturally but has been intensified by the inappropriate use of antibiotics, poor therapy adherence, excessive use of antibiotics in food-producing animals, and poor hygiene and sanitation (FAO, 2016). A direct consequence of ABR is the failure to successfully treat infections, which leads to increased mortality, prolonged illness, and reduced livelihood and food security. This inadvertently results in higher alternative treatment costs (O'Neill, 2014). It is estimated that unless urgent action is taken, deaths attributable to AMR by 2050 will be 10 million per year, of which approximately 4 million will occur in Africa. (O'Neill, 2014). The contribution of animal food production to the AMR crises may be debated by some on the grounds that we do not see a significant amount of animal associated infections in humans (Robinson *et al.,* 2016). Nevertheless, due to how antibiotics are administered in animal production, metaphylactic, prophylactic, and growth-promoting antimicrobials create an ideal environment for the selection and dissemination of antibiotic-resistant bacteria through the recurrent exposure to low doses of antimicrobial agents (You et al., 2014). These antibiotic resistance genes (ARGs) can subsequently be transmitted to human-adapted pathogens or human gut microbiota via people, contaminated food, or the environment (Robinson *et al.,* 2016).

The rise of ABR in animal food production directly increases the probability of animal mortality, and the failure to treat resistant infections leads to decreased animal performance. Consequently, it decreases the monetary returns in animal food production, resulting in higher food costs for consumers (Dewulf *et al.,* 2020).

1.2.2 Use of antibiotics in food animals

Antibiotics are currently used in food animals for treatment and purposes such as disease prevention and animal growth promotion. Therapeutic use refers to the use of antibiotics to treat clinically infected animals, whereas metaphylaxis involves the administration of antibiotics at therapeutic doses to groups of animals when only some individuals in the group are diseased to prevent further spread of the infection. Prophylactic use is generally defined as the administration of antibiotics to healthy animals to prevent clinical disease (Dewulf *et al.,* 2020). Growth promotion refers to the use of antimicrobial substances at sub-therapeutic concentrations to increase growth rates and/or the efficiency of feed additives in animals. "The term does not apply to the use of antimicrobials for the specific purpose of treating, controlling, or preventing infectious diseases, even when an incidental growth response may be obtained" (You & Silbergeld, 2014).

Singer *et al*., 2016 describes three classes of resistance-driving chemicals, namely antimicrobials (four subclasses: antibiotics, antifungals, antivirals, and antiparasitics); heavy metals; and biocides (i.e., disinfectants and surfactants) and highlights that antibiotics as well as other chemicals can select for resistance genes. Xenobiotics (octanol, hexane, and toluene) are also known to select for resistance (Friedman, 2015; Andleeb *et al.,* 2020). Co-selection of genes that confer resistance to xenobiotics, biocides, antibiotics, and heavy metals is a "potentially ecologically and clinically" significant phenomenon (Singer *et al.*, 2016). Co-selection is achieved in two ways: (1) cross-resistance and (2) co-resistance. Cross-resistance involves mechanisms that provide resistance to multiple resistance-driving chemicals such as antibiotics and heavy metals. For instance, efflux pumps can mediate cross-resistance to multiple antimicrobials by rapid extrusion of the toxins out of the cell (Seiler & Berendonk, 2012). The ability of pathogens to co-select genes that confer resistance highlight a fundamental issue with the use of antibiotics as growth promoters and feed additives in agriculture as it is not just the residual antibiotics in meat but rather the selective pressure exerted by the antibiotics that lead to the emergence of multi-drug resistant (MDR) pathogens (You & Silbergeld, 2014; Molechan *et al.,* 2019).

The use of antibiotics such as avoparcin (a glycopeptide) and tylosin (a macrolide) as growth promoters in European countries were associated with a high prevalence of VRE and macrolide-resistant enterococci in pigs (Boerlin *et al*., 2001). This led to a ban throughout the European Union on the use of avoparcin. The resulting change found a decrease in the prevalence of VRE in livestock; however, this trend was not observed in some European countries (Aarestrup, 2000; Pruksakorn *et al.*, 2016). Nowadays, antibiotics such as vancomycin, a glycopeptide antibiotic, belong to the group of "last resort antibiotics," which refer to the last line of effective antibiotics against resistant bacteria. Resistance to last-resort antibiotics poses a challenge as minimal treatment options remain. (Molechan *et al.,* 2019; Seiler & Berendonk, 2012).

"Co-resistance is defined as two or more genetically linked resistance genes, meaning that genes responsible for resistance to two or more antimicrobials are located next to each other on one mobile genetic element" (Seiler & Berendonk, 2012). As an example, Hasman and Aarestrup (2002) found a genetic link between copper (Cu) resistance encoded by the *tcrB* gene, macrolide resistance encoded by the *erm*(B) gene and glycopeptide resistance encoded by the *vanA* gene in a plasmid originating from E. *faecium* isolated from a pig in Denmark. Here, co-resistance to Cu and antibiotics, all utilized in farm practice was detected. Hasman *et al*. (2006) later showed that in piglets who were fed increased amounts of copper, there was co-selection for erythromycin and vancomycin resistance in enterococcal isolates. This indicates a Cu-induced spread of erythromycin and vancomycin resistance genes to antibiotics relevant in animal and human health sectors. Amachawadi *et al*. (2011) investigated the link between copper intake and the prevalence of Cu resistance (*tcrB)* in enterococci in weaned piglets from America. They collected a total of 180 faecal samples from two groups of pigs, normal Cu diet (control) and elevated Cu diet, at different intervals. The prevalence of *tcrB*-positive enterococci in the elevated Cu group and the control group was 21.1% and 2.8%, respectively. They found a link between the increased levels of copper intake and the prevalence of copper-resistant enterococci in piglets. Using a conjugation assay, they also reported co-transfer of Cu resistance genes (*tcrB)* and erythromycin resistance genes [*erm*(B)] between strains of E. *faecalis* and E. *faecium*, suggesting potential transferability and co-selection. The context in which prophylactic and growth-promoting antimicrobials are administered creates an ideal environment for the emergence and dissemination of antibiotic-resistant bacteria in animals through recurrent exposure to low doses of antimicrobial agents (You & Silbergeld, 2014). For these reasons, the use of antibiotics and heavy metals as growth promoters and feed additives in agriculture should be reassessed.

Wang, *et al.* (2018) aimed to characterize the origin and distribution of the colistin resistance gene *mcr-1* using a data set of 457 *mcr-1*-positive sequenced isolates from humans and farm animals from five continents by sequencing the genomes of 110 bacterial strains and extracting genomic data from publicly available databases. The data was analyzed with novel computational tools that indicated a single emergence of *mcr-1*, dated to the mid-2000s which likely occurred in Chinese pig farms. This highlights a single point of origin for colistin resistance gene mcr-1 followed by its subsequent diversification between multiple genomic backgrounds during global spread, affecting humans and farm animals alike. The authors suggest that possible drivers for the global spread of the ARG are the trade of food animals, and meat, as well as global travel of colonized or infected humans. While the ease of international travel currently creates an avenue for antibiotic-resistant pathogens to spread globally (O'Neill, 2014), the worldwide trade of agricultural products has also contributed to the dissemination of bacteria and the spread of ABR with many countries being pressured to intensify agricultural production supply to meet export demands. International trade requirements may encourage more prudent use of antibiotics in exporting countries; however, importing countries are still at potential risk

of importing resistant bacteria, which may be selected for by the resistance-driving chemicals used in the exporting country (FAO, 2016; Molechan *et al.,* 2019).

Two-thirds of the total antibiotics manufactured each year globally are used in animal husbandry (Singer *et al.,* 2016). It is estimated that >70% of the antimicrobials consumed in the USA, are in food and agriculture production (O'Neill, 2016). In the 2018 European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) report, a total of 31 European countries submitted sales data of veterinary antimicrobials, expressed as milligrams of antibiotic sold per population correction unit (PCU). PCU is a standard unit of measure that considers the number of animals in a country and their estimated weight at the time they are likely to be treated with antibiotics, providing an estimate of the size of the food-producing animal population in a country. Of the overall veterinary antimicrobial sales, the largest amounts were accounted for by tetracyclines (30.7%), penicillins (28.8%), and sulfonamides (8.4%), with these three classes accounting for an overall 67.9% of total sales in the 31 countries (European Medicines Agency, 2020). A similar trend was observed in the UK, with tetracyclines being the most frequently used antibiotic in animals (32%) followed by β-lactams (28%) (Veterinary Medicines Directorate, 2019). Between 2011 and 2018, the overall sales of antibiotics for use in animals in Europe decreased by approximately 34%. It is of importance to note that there was a decreasing trend in the sale of antibiotics considered critically important in human medicine, with third and fourth-generation cephalosporins, polymyxins, and fluoroquinolones recording a 24%, 70%, and 4% decrease in sales between 2011 and 2018, respectively (European Medicines Agency, 2020). Similarly, the UK Veterinary Antibiotic Resistance and Sales Surveillance (UK-VARSS) report recorded a 45% (56.8mg/kg to 25.8 mg/kg) decrease in the sales of veterinary antibiotics for food-producing animals since 2015 in the UK. Overall, in 2019, the total antibiotic sale for use in food-producing animals was 204 tonnes, which accounted for 26% of the total antibiotic use in the UK. This decreased by 12% since 2013. Of interest, the antibiotic consumption in humans also dropped by 6% from 2013 to 2017 in the UK (Veterinary Medicines Directorate, 2019). These are positive outcomes and indicate that the prudent use of antibiotics can be achieved and may in part be due to a One Health approach, which can build communication channels across sectors to work together on research and development activities and inform experts, policymakers, and legislation. However, the above-mentioned reports do not paint a global picture as there can be significant variability in the scale of veterinary antimicrobial use between countries. A challenge presents as many countries currently lack the resources for surveillance and data collection, and the true estimation of the total annual global consumption of antibiotics in agriculture remains elusive (OIE, 2019).

The impact of AMR on the world's GDP is estimated at a cumulated cost of over 100 trillion USD by 2050 (O'Neill, 2016). Van Boeckel *et al*. (2015) estimated global consumption of antibiotics in food animal production at 63,151 (±1,560) tons in 2010. They projected a rise by 67% by 2030, two-thirds of which is anticipated to be due to the increasing number of animals raised for food production with

use in pig and poultry production expected to double and one-third of which was attributed to intensive production systems. China, United States, India, and Brazil account for 50% of total global consumption and this would not change in the next ten years. The authors noted that the highest antibiotic consumption is in countries that have extensive industrial pig, poultry and cattle systems. These projections assume that the way in which antibiotics are used in farms will not change in the future. The application of the WHO's Global Action Plan on Antimicrobial Resistance (WHO, 2015), and the larger overall awareness of ABR, may likely reduce the use of antibiotics in the coming years as it is already being demonstrated in some countries (Dewulf *et al.,* 2020). A responsible approach to antibiotic consumption is necessary to decelerate the rate at which resistance emerges.

1.2.3 One Health approach

Antibiotic-resistant bacteria can develop and move between food-producing animals and humans by direct exposure or through the food chain and the environment irrespective of geographical or ecological borders (FAO, 2016). To better understand the exchange between humans, animals, and the environment, it is vital to consider the interaction of elements in the physical environment (e.g., water, air, and soil) with communal interactions (e.g., between animals within a herd, farmers and animals), in processing (e.g., storage and transport), and human use patterns (e.g., food preparation, meat consumption, and susceptibility to infection) (Landers *et al*. 2012).

Within the animal agricultural sector, resistant bacteria can emerge from the regular use of antibiotics for growth promotion, prophylactic, metaphylactic, and therapeutic purposes in animal production systems. As such, the use of antibiotics in animal production systems may present a risk to human health as livestock, and their associated environment can act as reservoirs for resistant pathogens. In the human health sector, some of the leading drivers of ABR are the use, overuse, misuse, and irrational use of antibiotics, easy and/or illegal access to antibiotics, counterfeit antibiotics and poor hygiene practices (Mitchell *et al.,* 2020; Kelesidis & Falagas, 2015; Schneider & Ho Tu Nam, 2020). The environment can also be contaminated with pharmaceutical waste which is one of the methods by which ARGs can transfer among pathogens in the environment (Mitchell *et al.,* 2020).

"The One Health approach, defined as '...the collaborative effort of multiple disciplines working locally, nationally, and globally – to attain optimal health for people, animals, and our environment…', recognizes that human health is closely associated to animal health and the environment" (Robinson *et al.,* 2016). ABR is clearly evident in each of these three spheres. The key drivers for antibiotic resistance include the scale of antibiotic use in human and animal health sectors; as well as to the relevant pathways in which antibiotics enter the environment, namely municipal and industrial wastewater, greywater, reclaimed and black water, and land application of manure and sludge. The drivers of AMR/ARGs in the environment include soil-borne resistance, animal husbandry, and wastewater and

sludge (Singer *et al.,* 2016). Therefore, AMR is a multisectoral, global problem requiring a One Health approach for its control.

As such, the World Health Organization (WHO), the World Organization for Animal Health (OIE), and the Food and Agriculture Organization (FAO) have formed a tripartite alliance and have collectively, and individually developed action plans to combat AMR effectively (FAO, 2016; OIE, 2016; WHO, 2015). In line with the objective to improve AMR awareness and understanding, the FAO/OIE/WHO alliance have made efforts in increasing overall awareness and understanding of antimicrobial resistance, which include the World Antimicrobial Awareness Week (WAAW) every November and the global campaign "*Antimicrobials: handle with care*" (FAO, 2020; WHO, 2017). Another objective, which is to strengthen knowledge through surveillance and research, saw the development of the FAO Assessment Tool for Laboratories and AMR Surveillance System (FAO-ATLASS) to aid countries in evaluating their national surveillance systems and laboratory diagnostic capacities for AMR detection and characterization (FAO, 2020); the WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS) which aims to build a picture of resistance patterns worldwide (WHO, 2015); and the OIE took the lead in creating a global database on antimicrobial use (AMU) in animals (OIE Annual Reports on Antimicrobial Agents Intended for Use in Animals) that have been published yearly since 2016 (OIE, 2018). The OIE further developed the OIE Performance of Veterinary Services (PVS), a tool for good governance of veterinary services, and the OIE online World Animal Health Information System (WAHIS) Interface which provides free access to world animal health data (OIE, 2019). The latest revisions of the WHO List of Critically Important Antimicrobials (CIA) for Human Medicine and the OIE List of Antimicrobial Agents of Veterinary Importance aim to optimize AMU in human and animal health (OIE, 2018; WHO, 2019). In 2017, the WHO reported that almost 95% of the world's population live in a country that has or is finalizing its national action plan for antimicrobial resistance (WHO, 2017). Countries such as New Zealand, Japan, South Korea, the USA, Canada, Chile, and most countries in Europe publish national reports on veterinary antimicrobial use, indicating that while significant progress has been made, many countries still lack the surveillance capabilities to monitor national antimicrobial usage/ antimicrobial resistance in animals (OIE, 2019).

1.2.4 Animal food production systems

There are three main types of animal production systems: land-based extensive systems, land-based intensive systems, and organic systems (Robinson *et al.,* 2011). Less intensive farming systems (i.e., extensive and organic) are often equated with "sustainable" farming. Land-based extensive farming practices may be locally advantageous to the environment (in terms of biodiversity, water, carbon storage, or environmental health) but naturally also require low inputs and generate lower agricultural yields. These systems may reduce ABR as they require lower levels of antibiotics.

The increasing demand for animal-derived food sources among most of the global population has placed immense pressure on livestock production systems. The key drivers behind the higher food demand are the changes in dietary preferences and population growth, mostly relating to growing affluence and urbanization. To meet the global increased demand, there was a shift in farming practices, from extensive, small-scale livestock production systems to large-scale, commercially orientated intensive farming systems (Robinson *et al.,* 2011). Intensive systems are known to be "land sparing" and farm intensively over a small area (Benton *et al.,* 2011) and involve large numbers of animals housed at high densities (Robinson *et al.,* 2011). This results in higher agricultural yields however the drawback is that the high population density of intensive farming systems results in sharing of both commensal flora and pathogens, which can be favorable to the dissemination of infectious bacteria. Intensive environments thus necessitate robust infection management strategies that often include antibiotics to keep animals healthy and maintain productivity (You & Silbergeld, 2014; Landers *et al*., 2012). Furthermore, intensive farming systems lead to a lack of animal genetic diversity. Considering these points, it is likely for populations of antibiotic-resistant bacteria to displace susceptible ones. Hence, it is possible that intensive production systems that rely on antibiotics for a lack of biosecurity and good hygiene and husbandry practices are faced with the risk of being colonized with resistant pathogens (Molechan *et al.,* 2019).

Brazil, Russia, India, China, and South Africa (BRICS) have adopted intensive production systems, and this rapid intensification occurred in order to meet the massive increase in demand. It is estimated that over 50% of pigs are raised under intensive systems globally. China accounts for 64% of the intensively raised pigs, making China the leading producer of pig meat globally, while high-income countries account for 24% of global intensive production (Robinson *et al*., 2016). Intensive production environments generally rely on antibiotics to maintain animal health and productivity. Therefore, the BRICS countries are contributing to the rise of ABR (Van Boeckel *et al.,* 2015).

1.2.5 Biosecurity

Biosecurity and vaccination are necessary to maintain animal health and thus reduce AMU. External biosecurity aims to keep communicable pathogens out of the herd, while internal biosecurity prevents the spread of disease within the herd (Backhans *et al*., 2015). It is critical to prevent and manage animal diseases in modern livestock production. Productivity and output on the farm can be decreased by outbreaks of disease. This in turn increases treatment costs for the animals. "In modern livestock production, the emphasis should be on disease prevention, and only if this fails should antibiotics be used" (Dewulf *et al.,* 2020). In a study done by Postma *et al.* (2015), 111 veterinarian experts ranked the most effective alternatives for AMU in pig production. The results showed the top five alternatives to AMU to be: "improved biosecurity, increased vaccination, use of zinc/metals, improved feed quality, and improved diagnostics" (Dewulf *et al.,* 2020).

Improving biosecurity may result in decreasing AMU without threatening productivity. A cross-sectional study was conducted in 227 pig herds in four European countries between 2012 and 2013, where associations between biosecurity, AMU, and production parameters were evaluated. The results showed that a higher weaning age, a farrowing rhythm of five weeks or more, and a higher external biosecurity level were significantly associated ($p < 0.05$) with lower AMU (Postma *et al.,* 2016; Dewulf *et al.,* 2020). In another study, the biosecurity status of breeder-finisher pig herds (n=95) in Belgium was quantified using a risk-weighted scoring system. The average external biosecurity score was 65 (range, 45-89), while the average internal biosecurity score was 52 (range, 18-87). The results showed that decreases in disease treatment frequency were associated with higher internal biosecurity scores, implying that biosecurity may help in decreasing AMU (Laanen *et al.,* 2013).

A French study put biosecurity measures into practice in 77 breeder-finisher herds. The results showed that there was a difference in profit margins of around €200 per sow per year between high biosecurity farms and those with low biosecurity (Corrégé *et al.,* 2012; Dewulf *et al.,* 2020). This shows that higher levels of biosecurity also have a cost-benefit. Therefore, improving the biosecurity level should be the foundation of any effort to decrease AMU (Dewulf *et al.,* 2020).

1.2.6 *Enterococcus* spp.

Enterococci are Gram-positive, facultatively anaerobic bacteria and commensals in the gut of animals and humans. They may, as such, provide information on the flow of Gram-positive resistance traits in the food chain (Fisher and Phillips, 2009; WHO, 2017). They are opportunistic pathogens and serve as reservoirs of resistance genes that can be transferred to human pathogens transiting the intestinal tract (Kaye *et al.*, 2004; WHO, 2017). *Enterococcus faecalis*, *E. faecium, E. hirae*, and *E. durans*, are the most prevalent enterococcal species in the microbiota of humans and other mammals. *E. casseliflavus*, *E. gallinarum*, *E. avium*, and *E. cecorum* have also been reported in the microbiota of pigs, although to a lesser proportion (Torres *et al*., 2018). *E. faecalis* and *E. faecium* are two of the most clinically important species. (Quiloan *et al*., 2012). VRE are part of the "ESKAPEE" pathogens, an acronym for *E. faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp., and *Escherichia coli,* so named for their drug resistance mechanisms and ability to evade antibiotics. These bacteria are a common cause of life-threatening nosocomial infections amongst critically ill and immunocompromised patients (Santajit and Indrawattana, 2016).

1.2.7 Critically important "shared" class antimicrobials

Following the recommendations from two FAO/OIE/WHO expert meetings, The WHO List of CIA for Human Medicine and the OIE List of Antimicrobial Agents of Veterinary Importance were initially developed. The purpose of the meetings was to address the human health-related consequences associated with AMU in food-producing animals. The first meeting convened to discuss this matter, recognized that AMR was a global concern for human and animal health and has been impacted by

AMU in these sectors, and noted that the classes of antimicrobials used in animals and humans were often the same, or structurally related. They further concluded that adverse human health consequences resulting from AMU in animals included an increased frequency and/or severity of infections (OIE, 2018; WHO, 2019). For example, quinolone-resistant *Salmonella* spp. infections in humans were linked to animal sources in Denmark (Hald *et al.,* 2007; WHO, 2019). The consequences of ABR are the most severe when pathogens are resistant to CIA for human health. Hence, the overlap of the WHO CIA List and the OIE List of Antimicrobials of Veterinary Importance should be considered for risk management plans in the human sector, the food animal sector, and in agriculture "through a multisectoral One Health approach allowing an appropriate balance between animal health and welfare, and public health" (WHO, 2019).

The criteria used by the OIE to determine the degree of importance for veterinary antibiotic classes considered the fact that many different species have to be treated in veterinary medicine. They firstly considered the response rate to a questionnaire identifying the importance of the antibiotic class. Secondly, antibiotics that treated specific infections and lacked therapeutic alternatives were considered. Antibiotics that met both these criteria were categorized as Veterinary Critically Important Antimicrobials (VCIA). The eight VCIA classes include aminoglycosides, third and fourth generation cephalosporins, macrolides, penicillins, amphenicols, fluoroquinolones, sulfonamides, and tetracyclines, all of which are indicated for use in pigs (OIE, 2018). The WHO CIA List categorized antibiotics used in human medicine as critically important, highly important, and important. Antibiotics which were deemed of critical importance in human health include five of the VCIA classes excluding amphenicols, sulfonamides, and tetracycline. Antibiotics categorized as critically important in human health were further prioritized, and those of greatest significance classified as "highest priority CIA". These include quinolones, third and higher generation cephalosporins, macrolides and ketolides, glycopeptides, and polymyxins (WHO, 2019). Therefore, the highest priority critically important "shared class" antibiotics used in animal and human health include fluoroquinolones and third and fourth generation of cephalosporins. In light of this, the OIE recommended that these two classes and colistin should not be used as growth promoters, prophylactically or as first-line treatment unless justified (OIE, 2018).

1.2.8 Antibiotic resistance in *Enterococcus* spp.

Enterococci have developed resistance to a vast majority of the critically important antibiotics for use in humans and animals - these antibiotics include penicillin's, glycopeptides, aminoglycosides, quinolones, macrolides, and tetracyclines (OIE, 2018; WHO, 2019). **Table 1** lists the antibiotic mechanisms of action and resistance in enterococci.

Table 1: Antibiotic resistance mechanisms in Enterococci to critically important antibiotics

Antibiotic	Mechanism of action	Mechanism of resistance	Genes	Reference
Penicillins (Ampicillin, Imipenem)	Inhibits cell wall synthesis	Low binding affinity for ampicillin due to the presence of the chromosomal gene, *pbp*5, which allows peptidoglycan synthesis in the presence of β-lactams. Drug inactivation mediated by β-lactamase that inactivates ampicillin through hydrolysis of β-lactam ring.	*pbp*5 *blaZ*	(Arias and Murray, 2012) (Miller *et al*., 2014) (Torres *et al*., 2018)
Glycopeptides (Vancomycin+ Teicoplanin)	Inhibits cell wall synthesis	PBPs (VanA-E) have lower affinities for vancomycin. Rare AcrF efflux-mediated resistance has also been known to cause resistance, albeit rarely.	*van*A-E AcrF	(Miller et al., 2014) (Fair & Tor, 2014) (Torres *et al*., 2018)
Aminoglycosides (Gentamycin Streptomycin)	Inhibits protein synthesis	Intrinsic tolerance due to low uptake and decreased binding to ribosomal target Chromosomally encoded *aac(6')-li* and *aph(3')-IIIa* result in intrinsic resistance to aminoglycosides except for gentamycin and streptomycin	 *aac(6')-li* *aph(3')-IIIa*	(Van Hoek *et al.,* 2011) (Hollenbeck *et al.,* 2012)

		Acquired resistance in *E. faecium* can be conferred due to modification of ribosomal target via *efm*M (16S ribosomal RNA methyltransferase enzyme)	*efm*M	(Galimand *et al.*, 2011)
		High-level gentamycin resistance is due to modifying enzyme *aac(6')-le/aph(2")-la* conferring resistance to all aminoglycosides except streptomycin	*aac(6')-le/aph(2")-la*	(Miller et al., 2014)
Macrolide-Lincosamide-Streptogramin B (MLS)	Inhibits protein synthesis by binding to the 50S ribosome	Resistance arises from modification of 23SrRNA target by a variety of methylase genes, most commonly *erm*B	*erm*B *erm*A, *erm*F, *erm*T	(Arias and Murray, 2012)
		Efflux pump	*Isa*A, *mef*A, *mef*E *msr*A, *msr*C, *msr*D *vga*B, *vga*C	
		Inactivating enzymes	*vat*B, *vat*D, *vat*E, *vat*G, *vgb*A	

Tetracyclines	Exert antibacterial effect by binding to the ribosome and interfering with the docking of aminoacyl-tRNA	Efflux pump Ribosomal protection	*tet*K, *tet*L *tet*M, *tet*O	(Torres *et al.,* 2018) (Emaneini *et al*., 2013)
Quinolones (Ciprofloxacin, Levofloxacin)	Interfere with nucleic acid replication, transcription, and synthesis	Capable of acquiring high-level resistance through several mechanisms: Mutation: (*gyr*A and *par*C) alter drug binding Efflux pumps (*Nor*A) has been described in *E. faecium.* Target protection by the formation of the quinolone-gyrase complex, which decreases DNA binding of the quinolone, is described in *E. faecalis.*	 *gyr*A, *par*C *Nor*A *qnr*	(Lopez *et al.*, 2011) (Miller *et al*., 2014) (Kaye et al., 2004) (Tran, Jacoby and Hooper, 2005) (Arsène and Leclercq, 2007)
Amphenicols (Chloramphenicol)	Inhibits protein synthesis	Inactivating enzymes	*cat*, *cat*C, *cat*-TC	(Van Hoek *et al*., 2011)
Linezolid (oxazolidinone)	Inhibits protein synthesis	Mutations of the 23S rRNA binding site	*optr*A, *poxt*A,	(Egan *et al*., 2020)

1.2.9 Virulence factors

There are a variety of genes encoding virulence factors in *Enterococcus spp.* which include aggregation substance (*asa*1), cell adhesins (*efaAfs, efaAfm*), enterococcal surface protein (*esp*), hyaluronidase (*hyl*), sex pheromones (*cpd, cob, ccf,* and *cad*) and secreted factors such as gelatinase (*gel*E) and cytolysin (cylA). Cytolysin (*cyl*A *and cyl*B) is encoded on pheromone-responsive plasmids and has haemolytic and bactericidal activity, while gelatinase (*gel*E) mediates virulence by activating autolysin and biofilm formation. Cell surface determinants such as aggregation substance (*asa1*) contribute to virulence by facilitating aggregation of donor and recipient bacteria for high-frequency transfer of plasmid DNA. Another cell surface determinant, the enterococcal surface protein encoded by the *esp* gene, affects biofilm formation. Hyaluronidase *(hyl)* is a degradative enzyme that acts on hyaluronic acid and is associated with tissue damage (Upadhyaya *et al.,* 2011; Fisher and Phillips, 2009; Arias and Murray, 2012; Biswas e*t al.,* 2016; Kayaoglu and Ørstavik, 2004).

1.2.10 Dissemination of antibiotic resistance and virulence genes

Bacterial genomes comprise chromosomal DNA and accessory genetic elements such as gene cassettes, integrons, insertion sequence common regions, integrative and conjugative elements (ICEs), plasmids, and transposons. While chromosomal DNA encodes genetic information to control the life cycle of the bacterium, accessory genetic elements confer survival advantages, such as antibiotic resistance (Molechan *et al.,* 2019). ABR can be inherited intrinsically or via acquired resistance. Intrinsic resistance is shared by all bacteria of the same genus or species as it is located within the genome of the bacterial species. Mutations of intrinsic genes, or vertical transmission, occur in the chromosomal genes which are then inherited by the bacterial progeny and can often alter gene expression. Acquired resistance allows bacteria to acquire new genetic material via horizontal gene transfer (HGT) or through sporadic mutations of intrinsic genes (Singer *et al.,* 2016).

A primary mechanism for acquired resistance is via HGT and involves mobile genetic elements (MGEs), which "refer to elements that promote intracellular DNA mobility (e.g., from the chromosome to a plasmid or between plasmids) as well as those that enable intercellular DNA mobility" (Partridge *et al.,* 2018). Intracellular MGEs include transposons, insertion sequences (ISs), and integrons. Transposons and ISs are short portions of DNA "that are able to move themselves (and associated resistance genes) to new locations" within a genome. Integrons use "site-specific recombination to move resistance genes between defined sites" (Partridge *et al.,* 2018). The mechanisms that enable intercellular DNA mobility include (1) transformation, (2) transduction, and (3) conjugation. Transformation involves the uptake of naked DNA from the environment. Transduction is where a bacteriophage acts as a vector transporting DNA from one bacterial cell to another (Van Hoek *et al.,* 2011). Conjugation involves the transfer of DNA through direct contact between two bacteria (mediated by plasmids and ICEs) (Wozniak and Waldor, 2010). Conjugative transmission is uninhibited in that they can transfer to other species or genera (von Wintersdorff *et al.,* 2016). It is the numerous

interactions between various types of MGEs that allow antibiotic resistance and virulence determinants to mobilize and transfer between cells and that promote the rapid evolution of diverse multidrug-resistant pathogens (Partridge *et al.,* 2018; Singer *et al.,* 2016).

1.2.11 Mobile genetic elements

ARGs are often located on a conjugative or mobilizable element that is most often a plasmid or transposon. In *Enterococcus spp.*, antibiotic resistance has disseminated due to their ability to transfer resistant and virulent genes via conjugative plasmids and conjugative transposons (von Wintersdorff *et al.,* 2016). Plasmids can be found in almost all bacterial genera and are described as extrachromosomal DNA elements which contain an origin of replication and genes encoding replication functions making them capable of autonomous replication. However, plasmids can also contain genes that encode functions to allow them to transfer via conjugation. "Plasmids that harbor conjugation genes are called conjugative and plasmids that only contain an origin of transfer (oriT), but no conjugation genes are called mobilizable as they can make use of the conjugation functions of conjugative plasmids to transfer to a new host" (Van Hoek *et al.,* 2011). Additionally, plasmids encode antibiotic resistance genes resistance which can be located on a conjugative or mobilizable plasmid giving them the capability to transfer to new hosts. Plasmids may be grouped as "narrow" or "broad host range" where a narrow host range is confined to one bacterial species and a broad host range can transfer between different species (Van Hoek *et al.,* 2011).

Of the "narrow host range" plasmids, pheromone-responsive plasmids represent a unique group of conjugative plasmids. pAD1, pAM373, and pCF10 are the best characterized pheromone-responsive plasmids in enterococci. The conjugative mechanism of these plasmids is based on sex pheromones (extracellular peptides), which are produced by pheromone-responsive donor cells, thereby producing cell aggregation substances (AS) on the cell surface, which facilitates conjugation and DNA transfer (Hegstad *et al.,* 2010). Pheromone-responsive plasmids have been mostly associated with *E. faecalis* (Wardal *et al.,* 2010). Antibiotic resistance determinants are also located on these plasmids. Vancomycin resistance genes (*vanA* and *vanB*) have been located on pheromone-responsive plasmids, with *vanA* being described in *E. faecalis* and *E. faecium* while *vanB* was described in *E. faecalis* only (Wardal *et al*., 2010). Virulence factors, such a AS and cytolysin, which contribute to rapid DNA transfer and haemolytic activity respectively, are also located on these plasmids. Hence, pheromone-responsive plasmids often contain both antibiotic resistance and virulence determinants.

Another class of enterococcal plasmids that play a role in the spread of ARGs include the "broad host range plasmids", also known as incompatibility plasmids (Inc18). One of the best characterized conjugative Inc18 plasmids in *Streptococcus* and *Enterococcus* spp. is pIP501. Inc18 plasmids can encode resistance to a variety of antibiotics, including chloramphenicol, vancomycin, and MLS groups of antibiotics, which can be transferred to other species. These plasmids were found to be responsible

for the transfer of vancomycin resistance (encoded by *vanA* gene) from VRE to methicillin-resistant *Staphylococcus aureus* (MRSA) (Kohler *et al.,* 2018). Recently, the presence of oxazolidinone resistance gene *poxtA* located on Inc18 plasmids (pC25-1 and pC27-2) from CC17 *E. faecium* of pig origin was described in China (Huang *et al.,* 2019). Therefore, HGT has allowed antibiotic resistance and virulence traits to disseminate, and this presents a challenge to treatment options when bacterial strains possess acquired resistance to last-line antibiotics.

A variety of transposons have been described in enterococci as they can encode antimicrobial resistance determinants, virulence factors and facilitate ease of transfer. There are three main categories in which enterococcal transposons can be grouped, namely, composite transposons, Tn*3* family transposons, and conjugative transposons (Hegstad *et al.,* 2010). Composite transposons have been associated with high-level gentamicin resistance (HLGR) and glycopeptide resistance (*vanB1*-type) in enterococci. They are mobilized by flanking copies of ISs, which are identical sequences that move the DNA. The Tn*3* family of transposons move amid different replicons via a replicative mechanism. They have been known to mediate glycopeptide (*vanA*-type) and macrolide-lincosamide-streptogramin B (MLS_B) resistance in enterococci. Conjugative transposons, also known as ICE, encode all the information necessary for their own excision, conjugation, and integration into a new host and have been known to mediate resistance to tetracyclines, MLS-antibiotics, and glycopeptide resistance (*vanB2*- type) in enterococci**.** Conjugative transposons can transfer between a broad host range, possibly carrying clinically relevant resistance determinants between a more extensive bacterial diversity. Members of the Tn*916/*Tn*1545*-family are the most relevant (Hegstad *et al.,* 2010).

1.2.12 Prevalence of ABR in *Enterococcus spp.*

Animals in food production have frequently been reported as reservoirs for the dissemination of resistance genes in humans. (Vignaroli *et al.,* 2011; Hammerum, 2012). Enterococci are commensals of the intestinal tract in animals and humans. In practice, AMU in animals is alleged to be the main driver for ABR development in animal bacteria. However, there is also evidence for transmission of resistance- determinants from animals to humans and vice versa (Crombé *et al.,* 2013; Madec *et al.,* 2017; Dewulf *et al.,* 2019). While the risk of zoonotic disease is rare, more awareness needs to be drawn to the fact that ABR strains may act as a reservoir of resistance determinants for bacteria in the intestinal tract. Of a review of 139 academic research articles that address the issue of antibiotic use in agriculture, 72% (n=100) found evidence of a link between antibiotic consumption in animals and resistance in humans. In contrast, only 5% (n=7) argued that there was no link and 23% (n=32) did not take explicitly take a stance (O'Neill, 2016). Antibiotic resistance determinants have the uninhibited potential to be transferred to other species or genera, and the use of CIA in food animals has implications for human health, as well as animal health and food security since this can create the setting for the emergence of new MDR pathogenic strains.

The following study shows some evidence for a spillover of ABR enterococci from animals to humans and vice versa: Freitas *et al*. (2011) compared the clonal relatedness of VRE in pigs and humans. They compared VRE isolates from pigs (n=29) and healthy humans (n=12) collected from Portugal, Denmark, Spain, Switzerland, and the United States (1995 to 2008) against clinical VRE isolates (140 *E. faecium* and 50 *E. faecalis*) recovered from 23 counties, during the last 3 decades. Clonal relatedness was determined using pulse-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) typing methods. The authors found thirty clonally related *E. faecium* clonal complex 5 (CC5) isolates which were obtained from the faeces of pigs and humans. *E. faecium* CC17 isolates from pig manure (n=1), and healthy human faecal samples (n=5) showed identical PFGE patterns while one E. *faecalis* CC2 isolate obtained from pigs corresponded to MDR clones widely disseminated in hospitals in Italy, Portugal, and Spain. Human-adapted clonal complexes included *E. faecium* CC17 and *E. faecalis* CC2, both of which were found in pigs in this study while animal-adapted strains include *E. faecium* CC5 was also found in humans. Hence, the authors concluded that enterococcal clones belonging to host-adapted clonal complexes of *E. faecium* and *E. faecalis* could be shared by pigs and humans.

In the United States, Donabedian *et al.* (2010) aimed to characterize VRE. *faecium* isolated from human and animal origin. A total of 360 faecal specimens were obtained from humans and their animals (55 samples from pigs) being raised for an exhibit at three county fairs in Michigan in 2008. VRE. *faecium* was isolated in 6 pigs but was not present in humans or other animals. The PFGE patterns and MLST of the six isolates were found to be similar with sequence types belonging to CC5, which is an animal-adapted CC. This could indicate the dissemination of VRE. *faecium* strains among pigs. This highlights the possibility of dissemination of ABR enterococci between animals.

A study by Metiner *et al.* (2013) obtained 47 enterococci isolates from 69 faecal samples on three pig farms in Turkey in 2003 and determined their antibiotic susceptibilities. A large majority of the isolates were identified as *E. faecium* (68%), followed by *E. faecalis* (21.7%). Erythromycin resistance in *E. faecium* and *E. faecalis* was 93.6% and 100%, respectively. Vancomycin resistance and intermediate resistance were observed in eight isolates for *E. faecium* and nine isolates for *E. faecalis.*

A study conducted in Thailand aimed to isolate, identify, and characterize antibiotic susceptibilities of VRE from pig farms. A total of 179 faecal samples were collected from four pig farms in 2011. There was an overall prevalence of 24% (n=43) of VRE in pigs. Of 71 presumptive VRE isolates detected, *E. gallinarum* isolates (62%, n=44) were the most prominent, followed by *E. casseliflavus* (35%, n=25). All isolates were susceptible to teicoplanin, whereas a large number of isolates showed resistance to tetracyclines (86.5%), erythromycin (61.5%), and penicillins (53.8%) (Pruksakorn *et al.*, 2016). This highlights the presence of antibiotic-resistant enterococci among pigs in food production.

Furthermore, in a study conducted by Braga and Lopez (2013), environmental dust from pig breeding facilities (n=171) in Portugal was screened for enterococci and VRE in 2008. *Enterococcus* spp. was

present in 150 facilities (88%) of which, was a 15% prevalence of VRE. MLST typing showed that four VRE. *faecium* isolates carrying the *vanA* genotype were closely related to pig and human isolates from European countries and Brazil. Generally, the species found in dust were similarly found in other environments related to pigs and pig facilities such as faeces, manure, meat, soil, and wastewater. This shows that there are incidences of ABR enterococci that have been isolated from the associated environments of pigs in food production.

In South Africa, a study to determine the prevalence of species distribution, antibiotic resistance, and virulence of *Enterococcus* spp. isolated from pigs. 320 *Enterococcus spp.* isolates were collected from two pig farms in the Eastern Cape. Molecular screening confirmed 37.5% of the isolates as *E. faecium*, 31.25% E. *hirae,* 18.75% followed by 12.5% E. *faecalis*. The results indicated a high prevalence of multi-drug resistant isolates (93.8%) as well as high levels of vancomycin-resistance (100%). The most common virulence factors that were detected included: ace (96.88%), gelE (93.13%), and esp (67.8%) (Iweriebor *et al.,* 2015). The high prevalence of VRE and MDR enterococci in this study agreed with the understanding that to fully appreciate the complexity of *Enterococcus* species in causing disease, a greater understanding of the ability of *Enterococcus* species to survive stresses, its virulence traits, and antibiotic resistance, is needed as *Enterococcus spp*. from pigs could be reservoirs of antibiotic resistance and virulence genes.

1.3 Aim

To investigate the molecular epidemiology of antibiotic-resistant *Enterococcus spp*., from farm-to-fork, in an intensive pig production system in uMgungundlovu district, Kwa-Zulu Natal.

1.4 Objectives

- To enumerate and culture *Enterococcus spp*., using Enterolert® and selective media, across the food production chain from the following sources: faecal and wastewater samples at the farm (bi-monthly), trucks, caecal samples, carcass swabs, and carcass rinsate collected post-slaughter as well as retail meat products.
- To genotypically confirm and speciate *Enterococcus spp*. using real-time polymerase chain reaction (RT-PCR) for the detection of the genus and species-specific genes.
- To determine the antibiotic susceptibility of isolates against a panel of antibiotics, recommended by WHO-AGISAR, using Kirby-Bauer disk diffusion according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.
- To detect the presence of selected antibiotic resistance genes in isolates displaying phenotypic antibiotic resistance via RT-PCR, viz., *tetK*, *tetM*, *ermB*, *aac(6')-le-aph(2'')-la* and *aph(3'-llla*.
- To detect the presence of selected virulence genes, viz., *gelE, cpd, cylA, cylB, efaAfs, efaAfm* in isolated enterococci via RT-PCR

- To elucidate the clonal relatedness of isolates by repetitive element palindromic PCR (REP-PCR)

1.5 Summary of methodology

The WHO-AGISAR guidelines (WHO, 2017) were implemented for the sampling strategy. The sampling points that were included as part of the farm-to-fork continuum are as follows: growth period on the farm (fresh pig faeces and wastewater/slurry), transport (truck), abattoir (carcass swabs, carcass rinsate, and caeca), retail meat (body, head, and thigh). Molecular confirmation by RT-PCR, targeting the genus- (*tuf*) and species-specific (*sodA*) genes, was undertaken. Susceptibility profiles were assessed by Kirby-Bauer disk diffusion using the WHO-AGISAR recommended panel of antibiotics for *Enterococcus* spp. Antibiotic resistance and virulence genes were detected using RT-PCR. Genetic relatedness between isolates across the continuum was evaluated by REP-PCR.

1.6 Study outline

The research findings are presented in three chapters as follows:

Chapter 1 provides the background, literature review, rationale for the study as well as the aims and objectives.

Chapter 2 provides information about the investigations undertaken and the findings and is presented in the form of a manuscript prepared for Science of the Total Environment

Chapter 3 presents the conclusions, limitations, and recommendations for this study.

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CHAPTER TWO

This dissertation is in a manuscript format according to the guidelines of the College of Health Sciences at the University of KwaZulu-Natal) as follows

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Molecular epidemiology of antibiotic-resistant *Enterococcus spp.* from farm-to-fork in intensive pig production in KwaZulu-Natal, South Africa. To be submitted to Science of the Total Environment.

Contributions

- Ms Sasha Badul, as the investigator, worked on the study design with the assistance of supervisors, developed the protocols for the study, executed the laboratory work and wrote the manuscript.
- Mr Daniel Gyamfi Amoako and Dr. Akebe Luther King Abia assisted with laboratory protocols, facilitated data acquisition and analysis of the study.
- Dr. Linda Bester, as co-supervisor, co-designed the study, facilitated data acquisition, laboratory work and data analysis, and contributed to the writing and critical revision of the manuscript.
- Sabiha Y. Essack, as the principal supervisor, co-conceptualized the study, guided the literature review and ethical clearance application, facilitated data collection and analysis and undertook critical revision of the manuscript.

Molecular epidemiology of antibiotic-resistant *Enterococcus spp.* from farm-to-fork in intensive pig production in KwaZulu-Natal, South Africa

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Abstract

Background: Substantial antibiotic use and high population densities in intensive farming systems results in the emergence and spread of antibiotic-resistant commensals and pathogens. This study investigated the molecular epidemiology of antibiotic resistance (ABR) and virulence in *Enterococcus spp*. from pigs in an intensive food production continuum from farm-to-fork in the uMgungundlovu district, Kwa-Zulu Natal.

Methods: A total of 174 samples obtained along the pig farm-to-fork continuum (farm, transport, abattoir, and retail meat) were subjected to the quantification and putative identification of *Enterococcus* spp. using the IDEXX Enterolert® method and selective media, respectively. Up to three presumptive enterococcal colonies were picked per sampling point for molecular confirmation by real-time PCR, targeting the genus- and species-specific (*tuf* and *sodA*) genes, respectively. Antibiotic resistance profiles were determined by the Kirby-Bauer disk diffusion method against a panel of antibiotics for *Enterococcus* spp. recommended by the WHO-AGISAR using EUCAST guidelines. Selected antibiotic resistance and virulence genes were detected by real-time PCR. Clonal relatedness between isolates across the continuum was evaluated by REP-PCR.

Results: A total of 284 isolates constituted the final sample. Real-time PCR confirmed 79.2% of the isolates as *E. faecalis*, 6.7% as *E. faecium*, 2.5% as *E. casseliflavus*, 0.4% as *E. gallinarum,* and 11.2% as other *Enterococcus* spp. Antibiotic susceptibility testing revealed resistance to sulfamethoxazole-trimethoprim (78.8%), tetracycline (76.9%), erythromycin (68.1%), streptomycin (62.6%), chloramphenicol (27.0%), ciprofloxacin (8.5%), gentamicin (8.1%), and levofloxacin (5.6%) but no vancomycin, teicoplanin, tigecycline or linezolid resistance was detected. *E. faecium* displayed 44.4% resistance to quinupristin-dalfopristin. A total of 78% of enterococcal isolates were MDR. Phenotypic resistance to tetracycline, aminoglycosides, and macrolides was corroborated by the presence of the *tetM*, *aph(3')-IIIa*, and *ermB* genes in 99.1%, 96.1%, and 88.3% of the isolates, respectively. The most commonly detected virulence genes were: *gelE*, *efaAfs*, and *cpd* in 89.1%, 78.5%, and 77.1% of isolates conferring autolysin and biofilm formation capabilities, cell adhesion, and conjugative plasmid accumulation, respectively. Clonality evaluated by REP-PCR revealed that E. *faecalis* isolates belonged to diverse clones along the continuum with major REP-types, largely consisting of isolates from the same sampling source but different sampling rounds (on the farm). *E. faecium* isolates revealed a less diverse profile. There was minimal evidence of clonal transmission across the continuum.

Conclusion: Multi-drug resistant *Enterococcus* spp. were isolated in this study. Isolates harboured a diversity of antibiotic resistance and virulence genes in different combinations forming reservoirs for the potential transfer of these genes from pigs to occupationally exposed workers and consumers via

direct contact with animals and animal products/food, respectively. The results highlight the need for more robust guidelines for antibiotic use in intensive farming practices and the necessity of including *Enterococcus* spp. as an indicator in antibiotic resistance monitoring systems in food animals.

1. Introduction

Antibiotic resistance (ABR) occurs naturally but has been exacerbated by the inappropriate and excessive use of antibiotics, poor therapy adherence, over-use of antibiotics in food-producing animals, and poor hygiene and sanitation (FAO, 2016). A direct consequence of ABR is the failure to successfully treat infections, which leads to increased mortality, prolonged illness, and reduced livelihood and food security. With the rise in ABR and a decline in new antibiotic discovery and development, it is imperative to monitor the emergence and spread of ABR in humans and (food) animals.

Antibiotic resistant bacteria can develop and move between food-producing animals and humans by direct exposure or through the food chain and the environment irrespective of geographical or ecological borders (FAO, 2016). There is a shift in farming practices, with a larger proportion of animals projected to be raised in cost-effective intensive farming systems where the high population densities and sub-optimal vaccination, biosecurity and animal husbandry practices results in the over-reliance on the use of antibiotics for the prophylactic and metaphylactic management of infections that subsequently results in the emergence and spread of antibiotic-resistant commensals and pathogens (You & Silbergeld, 2014; Landers *et al.,* 2012).Global consumption of antibiotics in food animal production is projected to rise by 67% by 2030, two-thirds of which is expected to be used in intensive food animal production with use in pig and poultry production expected to double. (Van Boeckel *et al*., 2015).

Enterococci are Gram-positive bacteria and commensals in the gut of animals and humans, and as such, they provide information on the flow of Gram-positive resistance traits in the food chain (Fisher and Phillips, 2009; WHO, 2017). They are opportunistic pathogens and serve as reservoirs of resistance genes that can be transferred to human pathogens transiting the intestinal tract (Kaye *et al.*, 2004; WHO, 2017). *E. faecalis*, *E. faecium, E. hirae*, and *E. durans*, are the most prevalent enterococcal species in the microbiota of humans and other mammals. *E. casseliflavus*, *E. gallinarum*, *E. avium*, and *E. cecorum* have also been reported in the microbiota of pigs, although in a lesser proportion (Torres *et al*., 2018). *E. faecalis* and *E. faecium* are two of the most clinically important species (Quiloan *et al*., 2012).

The pathogenicity of *Enterococcus spp*. has been enhanced by the expression of various virulence and antibiotic resistance genes that have been mobilized on diverse mobile genetic elements and are transferred by horizontal gene transfer (HGT). There is a deficit of data on the molecular characteristics of antibiotic-resistant enterococci in pigs along the farm-to-fork continuum in South Africa. This study investigated the molecular epidemiology of antibiotic-resistant *Enterococcus* spp. from pigs in the food production continuum from farms to retail meat products in the uMgungundlovu district, Kwa-Zulu Natal.

2. Methodology

2.1 Ethical Clearance

Ethical approval was obtained from the Animal Research Ethics Committee (Reference: AREC/007/018) and the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of KwaZulu-Natal (**Appendix 1 & 2**, respectively). This study also received permission to undertake this research in terms of Section 20A of the Animal Diseases Act, 1984 (Act no. 35 of 1984) from The South African National Department of Agriculture, Forestry and Fisheries (Reference: 12/11/1/5; **Appendix 3**).

2.2 Study population and sampling strategy

The study was conducted over 4 months (September 2018- January 2019) at an intensive pig farm and its associated abattoir in the uMgungundlovu District of Kwa-Zulu Natal, South Africa. The implementation of the sampling strategy was done as recommended by the WHO-AGISAR guidelines (WHO, 2017) The sampling points that were included as part of the farm-to-fork continuum are as follows: growth period on the farm (fresh pig faeces and wastewater), transport (truck), abattoir (carcass swabs, carcass rinsate, and caeca), retail meat (body, head, and thigh).

Random sampling was implemented, and a single litter of piglets was selected as the sample population. Sampling was conducted twice weekly over a 4-month period on the farm, which resulted in 9 sampling points. Fresh faecal samples were collected using the block sampling method to ensure that they were representative of the entire herd. Wastewater was collected from two associated drainage pipes. After the growth phase on the farm, the pigs were transported via trucks to the abattoir for slaughter, during which time, swabs of the truck before and after the pigs were loaded were collected. At the abattoir, carcass swabs, caecal content, and carcass rinsates were collected post-slaughter. Swabs of retail meat from the body, head, and thigh portions were obtained from the abattoir the next day. The sampling framework is summarized in **Table 1.**

Table 1: Sampling framework from farm-to-fork

Sampling points:	Source	No. of samples per visit		Total no. of samples	No. of isolates recovered
Farm **(9 sampling points over 4 months)**	Fresh pig faeces	10		90	97
	Wastewater/Slurry	6 (1ml each)		54	48
Transport	Truck floor swabs	60	Pooled into sets of 10	6	44
Abattoir	Carcass swabs	48	Pooled into sets of 12	4	15
	Carcass rinsates	48 (1ml each)		4	7
	Caecal swabs	48		4	17

Retail meat	Head swabs Body swabs Thigh swabs	20 20 20	Pooled into sets of 5	4 4 4	20 28 8
				174 samples	284 isolates

Samples were transported to the laboratory on ice and processed within 6 hours after collection.

2.3 Isolation and identification of *Enterococcus spp*.

The collected samples were diluted 1:10 in sterilized distilled water and vortexed. The samples were then processed using the IDEXX Enterolert® (IDEXX Laboratories, Inc., Westbrook, Maine, U.S.A) method according to manufacturer. The following quantities of each sample were used for the Enterolert® method: 100µL of faecal samples, 10µL of wastewater samples, 1ml of truck samples, 1ml of carcass rinsate, 20µL of caecal content, 1ml of carcass swabs, and 1ml of body, head and thigh samples, all of which were mixed with 100ml of sterilized water, respectively. The Enterolert® reagent was added to each 100ml bottle and poured into a Quanti-Tray®/2000 and sealed with a Quanti-Tray ®sealer PLUS. Each Quanti-Tray® was incubated for 24h at 41°C, to enrich for enterococci. After 24 h, each Quanti-Tray® was viewed under ultraviolet light, and fluorescent wells were presumed positive for enterococci. The samples from fluorescence-positive wells were then inoculated onto selective Chromocult® enterococci agar (Merck, Darmstadt, Germany) and incubated for 24 h at 37°C. Red colonies were positive for *Enterococcus spp.* while non-enterococci produced colourless, blue or turquoise colonies. Individual colonies were then picked and inoculated onto bile-esculin agar (Lab M, Lancashire, UK), and incubated for 24h at 37°C. Observation of dark brown colonies were deemed positive for enterococci. Single colonies were inoculated onto tryptone soya agar (TSA) (Oxoid, Hampshire, England) and further incubated for 24 h at 37 °C. Colonies that were presumed to be enterococci were stored in a 20% glycerol stock solution at -80°C for future use. *Enterococcus faecalis* ATCC 29212 was used as the control strain (EUCAST, 2017).

2.4 Molecular confirmation and speciation of *Enterococcus*

The heat lysis method was used to extract DNA as previously described (Molechan *et al*. 2019). Briefly, after an initial resuscitation on nutrient agar, 3-6 colonies were picked and mixed into nuclease-free water in sterile Eppendorf tubes. They were then vortexed to produce a turbid suspension and boiled in a water-bath for 10 min followed by centrifuging. The supernatant was extracted and stored at -20° C for further use.

The extracted DNA (3 µL) was used as the template in a 10-µL reaction volume made up of 5 µL PowerUp™ SYBR™ Green master mix (ThermoFisher Scientific, Waltham, MA, USA), 0.5 µL of

each forward and reverse primers (final concentration, 0.5 µM), and 1 µl of nuclease-free water. The positive controls, oligonucleotide primer pairs, thermal cycling conditions and melt-curve analysis were as previously described by Molechan et al. (2019), with minor modifications on the initial activation in the cycling conditions for the detection of the Enterococcus genus. Here, the initial activation consisted of an initial incubation at 50° C for 2 min, followed by a second one at 95° C for 2 min.

Similarly, real-time PCR was used for speciation of *E. faecalis, E. faecium, E. gallinarum, E. casseliflavus*. The reactions were carried out in a total volume of 10 µl with Luna® Universal qPCR master mix (New England Biolabs, Ipswich, MA, USA) replacing the PowerUp™ SYBR™ Green master mix. The positive controls, oligonucleotide primer pairs, thermal cycling conditions and melt-curve analysis were as previously described by Molechan et al. (2019), with a minor increase in the number of cycles from 30 to 35. A QuantStudio™ 5 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA) was used to carry out all reactions. A positive control and a No Template Control (NTC) -consisting of the PCR mix and nuclease-free water instead of template DNA was included in each assay. The positive controls, oligonucleotide primer pairs, and amplicon sizes are shown in **Table S1.**

2.6 Antimicrobial susceptibility testing

The Kirby-Bauer disc diffusion method was used to test antibiotic susceptibility. The antibiotic panel recommended by WHO-AGISAR (WHO, 2017) using European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017) guidelines were used. The Clinical and Laboratory Standards Institute (CLSI, 2017) recommendations were used for those antibiotic breakpoints absent from the EUCAST guidelines. The following antibiotics were used: ampicillin (10ug), imipenem (10ug), ciprofloxacin (15ug), levofloxacin (15ug), gentamicin (120ug), streptomycin (300ug), teicoplanin (30ug), vancomycin (30ug), quinupristin-dalfopristin (15ug) *(E. faecium* only), tigecycline (15ug), linezolid (30ug), nitrofurantoin (300ug), sulfamethoxazole-trimethoprim (25ug), erythromycin (15ug), tetracycline (30ug) and chloramphenicol (30ug). The antibiotics were purchased from Oxoid (Basingstoke, United Kingdom). Isolates resistant to one or more antibiotics in at least three distinct classes of antibiotics were defined as multidrug-resistant (MDR). MDR isolates were selected for further analysis based on their source and resistance profiles.

2.7 Molecular detection of antibiotic resistance and virulence genes

A real-time PCR assay was used to detect antibiotic resistance and virulence genes. The reaction concentrations, reagents, total volume, and PCR assay parameters followed were previously described by Molechan *et al.* (2019). All genes were amplified in separate assays and the positive controls, oligonucleotide primer pairs, and amplicon sizes that were used are shown in **Table S2** and **Table S3.**

2.8 Clonality

The clonal distribution among selected MDR E. *faecalis* isolates (n=99) and all E. *faecium* isolates (n=19) were characterized with repetitive extragenic palindromic-PCR (REP-PCR) using the (GTG)5 primer as described by Molechan *et al.* (2019). Briefly, GeneJET Genomic DNA purification kit (ThermoFisher Scientific, Waltham, MA, USA) was used to extract DNA according to the manufacturer's guidelines. Thereafter, amplification was carried out in a 25 µl reaction volume. Amplicons were then electrophoresed in agarose gel and the gels were visualized using the Gel Doc™ XR+ imaging system (Bio-Rad, Hercules, California, USA) while the Bionumerics software version 6.6 (Applied Maths NV, Belgium) was used to analyze the images **(Appendix 4)**. Clusters were obtained at a ≥70.0% similarity cut-off.

Results

1. Prevalence of *Enterococcus spp.*

A total of 284 isolates were obtained along the farm-to-fork continuum of which, 145 (51.1%), 44 (15.5%), 39 (13.7%), and 56 (19.7%) were obtained from the farm, transport, abattoir, and retail sampling points, respectively. Of the 284, 225 (79.2%) were *E. faecalis*, 19 (6.7%) were *E. faecium*, 7 (2.5%) were *E. casseliflavus*, 1 (0.4%) were *E. gallinarum,* while 32 (11.2%) were classified as "other *Enterococcus spp."* **(Figure 1).** The *E. faecalis* isolates were most prevalent throughout the continuum, while *E. faecium* isolates were obtained mainly at the farm level (Round 1, 2, and 6). No *E. faecium* isolates were recovered from transport or retail sampling points. There was a low prevalence of *E. casseliflavus* and *E. gallinarum,* with a few isolates being identified from the farm (Round 1 and 9) and transport vehicles.

2. Antibiotic resistance

AST results showed 100% susceptibility to ampicillin, imipenem, teicoplanin, vancomycin and linezolid while resistance to sulfamethoxazole-trimethoprim (78.8%), tetracycline (76.9%), erythromycin (68.1%), streptomycin (62.6%), chloramphenicol (27.0%), ciprofloxacin (8.5%), gentamicin (8.1%), and levofloxacin (5,6%) was observed. Furthermore, 44.4% of *E. faecium* isolates exhibited resistance to quinupristin-dalfopristin (Quinupristin-dalfopristin is only clinically relevant for *E. faecium* hence antibiotic susceptibility is only reported for this species) **(Table 2)**.

Figure 2 depicts the resistance profiles of the *Enterococcus* isolates along the farm-to-fork continuum. Streptomycin, erythromycin, and tetracycline (>75%) displayed the highest rates of resistance in isolates from the farm level. *E. faecalis* displayed the highest resistance to tetracycline (80%), followed by sulfamethoxazole-trimethoprim (78%), erythromycin (72%), streptomycin (70%), chloramphenicol (25%), gentamicin (15%), ciprofloxacin (9%), levofloxacin (4%), and nitrofurantoin (3%).

Among all the isolates, 222 (78%) were multidrug-resistant (MDR) with a total of 47 antibiograms being identified. Of the MDR isolates, 176 (79.3%) were *E. faecalis*, 13 (5.9%) were *E. faecium*, 5 (2.3%) were *E. casseliflavus*, 1 (0.5%) was *E. gallinarum*, and 27 (12.1%) were 'other' *Enterococcus spp*. *E. faecalis* showed 38 antibiograms while *E. faecium*, *E. casseliflavus*, *E. gallinarum,* and the other *Enterococcus* spp. showed eight, four, one, and nine antibiograms, respectively **(Table 3)**.

3. Prevalence of antibiotic resistance genes

Phenotypic resistance to tetracycline, aminoglycosides, and macrolides was corroborated by the presence of the *tetM*, *aph(3')-IIIa*, and *ermB* genes in 99.1%, 96.1%, and 88.3% of the isolates, respectively. *E. faecalis* displayed the highest occurrence of resistance genes compared to the other enterococcal species with the following distribution: *tetM* (77.6%), *aph(3')-IIIa* (76.0%), *ermB* (67.8%), *tetK* (15.4%), and *aac(6')-Ie-aph(2")-Ia* (5.1%). The *ermB* gene was detected in each species with the prevalence in descending order as follows: *E. faecalis* (67.8%), *Enterococcus spp.* (12.7%), *E. faecium* (6.3%), *E. casseliflavus* (1.0%), and *E. gallinarum* (0.5%). Tetracycline resistance was associated to a larger extent with the presence of *tetM* (99.1%) than with *tetK* (17.1%). Gentamicin resistance, associated with the gene *aac(6')-Ie-aph(2'')-Ia,* was only detected in two *E. faecalis* isolates. The *aph(3')-IIIa* gene was present in 96.1% of isolates with high-level resistance to streptomycin and was more commonly detected in *E. faecalis* isolates (76.0%), followed by *Enterococcus spp.* (12.7%), *E. faecium* (5.4%), and *E. casseliflavus* (2.0%). The one *E. gallinarum* isolate showed the presence of *ermB* and *tetM* **(Table 4)**.

4. Detection of virulence of factors

In all the enterococcal isolates, the frequency of virulence genes was as follo**ws:** *gelE* (89.1%), *efaAfs* (78.5%), *cpd* (77.1%), *cylB* (31.3%), *cylA* (18.3%), and *efaAfm* (3.5%). *E. faecalis* displayed the highest occurrence of virulence genes compared to the other enterococcal species with the allocation as follows: *gelE* (92.4%), *efaAfs* (89.3%), *cpd* (82.7%), *cylB* (35.6%), and *cylA* (21.8%). *gelE* was the most dominant gene detected among *E. faecium*, *E. casseliflavus* and *Enterococcus* spp. isolates at 68.4%, 71.4%, and 84.4%, respectively.

The prevalence of virulence factors detected in all enterococcal isolates were as follows: *gelE* (89.1%), *efaAfs* (78.5%), *cpd* (77.1%), *cylB* (31.3%), *cylA* (18.3%), and *efaAfm* (3.5%). The most prevalent virulence gene detected among *E. casseliflavus*, *E. faecium*, and *Enterococcus* spp. isolates was *gelE* with a frequency of 71.4%, 68.4%, and 84.4%, respectively. Only 9 *E. faecium* isolates (47.4%) tested positive for *efaAfm* while *E. gallinarum* had no virulence genes detected **(Table 5)**.

5. Clonal Relatedness

The evolutionary relationships of selected MDR *E. faecalis* isolates (n=99) and all *E. faecium* isolates (n=19) were determined using REP-PCR (**Figure 3** and **Figure 4,** respectively). The *E. faecalis* isolates

were chosen based on their antibiograms and source of isolation such that isolates with the same antibiogram from each sampling point were represented. *E. faecalis* displayed 35 REP-types (A-AI). There were 6 major REP-types which consisted of 47.5% (n=47) of *E. faecalis* isolates namely: type U (n=9), type AI (n=9), type T (n=8), type Z (n=7), type Q (n=7), and type AH (n=7). *E. faecalis* isolates from the "farm" were represented in all major REP-types. However, it must be noted that they were from different rounds of sampling on the farm (Round 1- Round 8). *E. faecalis* isolates from "transport" were also represented in major REP-type AI, while isolates from the "abattoir" and "retail" were quite diverse with representation in REP-types AH, T, Z, X and AD in the former and REP-types K, N, Q, T, U, V and Z in the latter. *E. faecium* displayed 7 REP-types (A-G). There were 2 major REP-types which consisted of 73.7% (n=14) of *E. faecium* isolates namely: type D (n=9) and type F (n=5). Of the 19 *E. faecium* isolates, 18 were from "farm," and 1 was from "abattoir." The clonal cluster D2 showed a similarity index of 100% and consisted of 5 isolates originating from faecal (Round 1) and wastewater (Round 2). There was less diversity in the source of *E. faecium* isolates. Isolates belonging to the same REP-types were isolated from the farm and its environments (feces and wastewater) but there was minimal evidence of transmission along the farm-to-form continuum.

Discussion

This study presented the results of the molecular characteristics of *Enterococcus* spp. isolated from pigs in food production along the farm-to-fork continuum over 4 months in KwaZulu Natal, South Africa. A total of 284 isolates were obtained and real time-PCR confirmed 79.2% of the isolates as *E. faecalis*, 6.7% as *E. faecium*, 2.5% as *E. casseliflavus*, 0.4% as *E. gallinarum,* and 11.2% as other *Enterococcus* spp. Antibiotic susceptibility testing revealed the highest resistance to sulfamethoxazole-trimethoprim (78.8%), tetracycline (76.9%), erythromycin (68.1%), and streptomycin (62.6 %) but no vancomycin, teicoplanin, tigecycline, or linezolid resistance. *E. faecium* displayed 44.4% resistance to quinupristin-dalfopristin. A total of 78% of *Enterococcal* isolates were MDR. Phenotypic resistance to tetracycline, aminoglycosides, and macrolides was corroborated by the presence of the *tetM*, *aph(3')-IIIa*, and *ermB* genes in 99.1%, 96.1%, and 88.3% of the isolates, respectively. The highest prevalence of virulence genes detected were: *gelE* (89.1%), *efaAfs* (78.5%), and *cpd* (77.1%). Clonality evaluated by REP-PCR revealed that *E. faecalis* isolates along the continuum are highly diverse, with major REP-types often consisting of isolates from the same sampling source but different sampling rounds (on the farm). In contrast, there was less diversity in the source of *E. faecium* isolates.

The incidence of enterococci with high resistance rates has been increasing recently(Hollenbeck *et al.,* 2012; Iweriebor *et al.,* 2015; Novais *et al.*, 2013; Molechan *et al.,* 2019). In the current study, the prevalence of *E. faecalis* (79.2%) dominated across all sampling points followed by undifferentiated *Enterococcus* species (11.2%) and *E. faecium* (6.7%). To a lesser extent, 2.5% and 0.4% of enterococcal isolates were identified as *E. casseliflavus* and *E. gallinarum,* respectively. Tan *et al.* (2018) conducted

a study in Malaysia which investigated the molecular epidemiology of 289 *Enterococcus* spp. recovered from pig (n=232), human (n=54) and environmental sources (n=3) from seven farms. Of this total, 73% and 25%, were identified as E. *faecalis* and E. *faecium*, respectively (Tan *et al*., 2018). Furthermore, a study done on two pig farms in Nigeria in 2016, revealed 268 enterococci isolates of which 79.5% were E. *Faecalis* and 20.5% were E. *Faecium* (Beshiru *et al.,* 2017). In these studies, *E. faecalis* was the predominant species which is consistent with the present study

In other works, *E. faecium* and was described as the more dominant species (Iweriebor *et al.,* 2015; de Jong *et al.,* 2019). The study done by de Jong *et al.* (2019) collected intestinal content from cattle, pigs, and chicken across Europe over 3 time periods. Out of 5334 *Enterococcus* strains recovered, 2435 E. *faecium* and 1389 E. *faecalis* were identified with 884 (36%) and 339 (24%) being isolated from pigs, respectively (de Jong *et al.,* 2019). Within South Africa, one study conducted on two pig farms reported that the most dominant enterococcal species (n=320) recovered from faecal samples from pigs was *E. faecium* (37.5%) followed by *E. hirae* (31.25%) (Iweriebor *et al.,* 2015). The reason for the relatively low prevalence of *E. faecium* (6.7%) in this study remains unclear. It may be possible to explain the differences in the composition of the enterococcal populations by the variation between geographical regions. However, it is difficult to draw firm conclusions that further highlights the significance of adding *Enterococcus spp.* in surveillance systems.

All enterococcal isolates (100%) were susceptible to ampicillin, imipenem, teicoplanin, vancomycin, tigecycline, and linezolid. According to the WHO CIA List, these antibiotics are considered critically important in human health (WHO, 2019). Susceptibility to several critically important antibiotics was also observed in other studies (de Jong *et al.,* 2018; Hasannejad *et al.,* 2015). The "highest priority" critically important antibiotic of significance in this study is vancomycin, a glycopeptide (WHO, 2019). The absence of resistance to "critically important" antibiotics for human medicines such as linezolid, imipenem, tigecycline, and vancomycin is encouraging. It should be noted that tetracycline and tylosin (one of four growth promoters banned in the EU) are still approved as antibiotics for growth promotion under the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947) in South Africa. The majority of antibiotics consumed in food animals in South Africa include tetracycline, sulfonamides/trimethoprim, macrolides, penicillins, and cephalosporins, all of which are of direct importance in human medicine (Eagar, Swan and Van Vuuren, 2012). Perhaps the high levels of resistance, displayed in enterococcal isolates in this study, to tetracycline (76.9%), sulfamethoxazole-trimethoprim (78.8%), erythromycin (68.1%) and streptomycin (62.6%) can be attributed to the extensive use of the above-mentioned antibiotics in food animals which exerts selection pressure for the development/escalation of resistance (Molechan *et al*., 2019).

The most frequently encountered tetracycline resistant determinant in enterococci is *tetM* which is consistent with the findings in this study as a majority of isolates that were positive for the *tetM* gene

(99.1%) showed phenotypic tetracycline resistance. Overall, 78 enterococcal isolates (34.7%) harboured both *tetM* (ribosomal protection) and *tetK* (efflux pump) resistance genes. The transferability of tetracycline resistance determinants has been regularly associated with conjugative transposons, mainly Tn916/Tn1545 carrying the *tetM* gene, usually in combination with *ermB*, although it has also been reportedly found on plasmids (Ayeni *et al.*, 2016; Torres *et al.,* 2018). In this study, 181 enterococcal isolates co-carried these genes. The most reported erythromycin-resistant determinant in enterococci is *ermB*. While a majority of enterococcal isolates harboured the *ermB* gene (88.3%), E. *faecalis* accounted for 67.8% of isolates. It is possible that other genes could be associated with erythromycin- resistant enterococcal isolates, such as *ermA*, *ermC*, *ermF,*or *ermT* or the macrolide efflux pump (*msrA*) (Van Hoek *et al.,* 2011).

Enterococci have intrinsic low-level resistance to aminoglycosides. A synergistic bactericidal combination of a cell wall- active agents like penicillin or a glycopeptide, with an aminoglycoside is commonly used to treat enterococcal infections. Enterococci that have acquired aminoglycoside resistance challenge this treatment option by eliminating this synergistic effect. High level aminoglycoside resistance was detected in enterococcal isolates (streptomycin 62.6% (n=204) and gentamicin 14% (n=39)). Two isolates from the high-level gentamicin resistance (HLGR) phenotype showed the presence of *aac(6')-e-aph(2")-Ia* gene. It is possible that other genes such as aph(2')-lc and aac(6')-li could be associated with HLGR (Van Hoek *et al.,* 2011). The aph(3')-IIIa gene was detected in 96.1% of isolates with a high-level streptomycin resistance phenotype, which shows a close association between phenotypic resistance and resistance determinants. Antibiotic resistance to quinupristin-dalfopristin was observed in 78.9% (n=15) of *E. faecium* isolates. This is of importance as streptogramins such as quinupristin-dalfopristin are used for the treatment of severe VRE. *faecium* infections associated with bacteremia (Isnard *et al.,* 2013).

It is important to identify the potential virulence factors of enterococcal strains as they may help to understand the complex pathogenic activity of these opportunistic bacteria (Chajęcka-Wierzchowska, Zadernowska and Łaniewska-Trokenheim, 2017). *E. faecalis (89.3%)* and *E. faecium (47.4%)* were positive for their corresponding cell wall adhesin genes, *efaAfs* and *efaAfm*. Gelatinase (*gelE*) and sex pheromone (*cpd*) genes were prevalent while cytolysin (*cylB* and c*ylA*) genes were detected to a lesser extent in *E. faecalis* and *E. faecium*. These genes confer pathogenicity by degrading host tissue and play a role in biofilm formation (*gelE*), promoting plasmid accumulation (*cpd*), haemolytic and bactericidal activity (*cylB* and *cylA*). This correlates with other studies that reported *gelE* gene as the most prevalent virulence determinant among *E. faecalis* isolates from pigs in China and Korea (Zou *et al.*, 2011; In Yeong *et al.,* 2011). Iweriebor *et al.* (2015) also reported the prevalence of *gelE* and *ace* genes from pigs in Eastern Cape, South Africa. It appears from this study that the incidence of virulence factors in

Enterococcus spp. is generally high, with majority of isolates carrying more than two virulence determinants (n=249; 87.7%).

REP-PCR was used to distinguish clonal relatedness among Enterococcal isolates. MDR *E. faecalis* isolates were diverse with 35 REP-types (A-AI), while *E. faecium* isolates showed less diversity with 7 REP-types (A-G). Concerning *E. faecalis*, it is interesting to note that REP-type T consisted of isolates from the farm that were ≥70% genetically related to those originating from the abattoir and retail meat. While this may suggest possible transmission of these isolates through the different stages of production, it must be noted that these isolates did not share the same resistance genes and virulence factors. REP-types Z, AH, AI comprised of faecal and wastewater isolates that were ≥70% genetically related, which may indicate possible enterococcal contamination of the associated environment. The outcome of "untreated wastewater and/or animal faeces on croplands could result in the dissemination of resistance and virulence determinants to home-grown soil bacteria via horizontal gene transfer, which could, in turn, disseminate resistance and virulence determinants back to animals or humans through crops" (Beshiru *et al.,* 2017). This reinforces the necessity for a multisectoral approach for AMR surveillance programs. Furthermore, REP-type AI consisted of isolates from the farm that were ≥70% genetically related to those originating from the transport site. These isolates were obtained from truck swabs after the pigs were loaded onto the truck. This may indicate possible transfer from the pigs to the truck however, these isolates showed no clonal relation to isolates from further along the continuum – abattoir and retail - which may highlight the importance of biosecurity measures as in-house decontamination protocols on the truck would be necessary. However, further studies that involve the use of more resolute typing methods such whole genome sequencing (WGS) will be needed to validate these claims. In comparison, *E. faecium* isolates showed a less diverse evolutionary relationship. However, of the 19 isolates analysed, it should be noted that 68.4% (n=13) were MDR with 57.9% (n=11) harboring at least two virulence genes and 84.2% (n=16) harboring at least one antibiotic resistance gene.

Conclusion

This is the first study in South Africa to investigate the molecular epidemiology of *Enterococcus* spp. isolated from pigs in food production along the farm-to-fork continuum in KwaZulu Natal. The results of this study highlight the prevalence of enterococcal species and MDR *Enterococcus* isolates which harbour resistance determinants that can serve as reservoirs for the possible transfer of these determinants from pigs to humans. The results highlight the importance of more robust guidelines for antibiotic use in intensive farming practices and the necessity of including *Enterococcus* spp. in food animal AMR monitoring systems.

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Table 2: Susceptibility profile of all *Enterococcus* spp. along the farm-to-fork continuum

	Susceptibility Profile																	
	E. faecalis (n=225)			*E. faecium (n=19)*			*E. casseliflavus (n=7)*			*E. gallinarum (n=1)*			Other *Enterococcus* spp. *(n=32)*			**Total % *(n=284)***		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin	225 (100%)	0	0	19 (100%)	0	0	7 (100%)	0	0	1 (100%)	0	0	32 (100%)	0	0	**100%**	0%	0%
Imipenem	0	225 (100%)	0	0	19 (100%)	0	0	7 (100%)	0	0	1 (100%)	0	0	32 (100%)	0	**0%**	100%	0%
Ciprofloxacin	204 (91%)	0	21 (9%)	16 (84%)	0	3 (16%)	7 (100%)	0	0	1 (100%)	0	0	30 (94%)	0	2 (6%)	**91%**	0%	9%
Gentamicin	191 (85%)	0	34 (15%)	19 100%)	0	0	6 (86%)	0	1 (14%)	1 (100%)	0	0	28 (88%)	0	4 (12%)	**86%**	0%	**14%**
Streptomycin	68 (30%)	0	157 (70%)	5 (26%)	0	14 (74%)	2 (29%)	0	5 (71%)	0	0	1 (100%)	5 (16%)	0	27 (84%)	**28%**	0%	**72%**
Teicoplanin	224 (99.6%)	1 (0.4%)	0	19 (100%)	0	0	7 (100%)	0	0	1 (100%)	0	0	32 (100%)	0	0	**100%**	0%	0%
Vancomycin	225 (100%)	0	0	19 (100%)	0	0	7 (100%)	0	0	1 (100%)	0	0	32 (100%)	0	0	**100%**	0%	0%
Quinupristin-Dalfopristin*	-	-	-	4 (21%)	0	15 (79%)	-	-	-	-	-	-	-	-	-	**21%**	0%	**79%**
Tigecycline	225 (100%)	0	0	19 (100%)	0	0	7 (100%)	0	0	1 (100%)	0	0	32 (100%)	0	0	**100%**	0%	0%
Linezolid	225 (100%)	0	0	19 (100%)	0	0	7 (100%)	0	0	1 (100%)	0	0	32 (100%)	0	0	**100%**	0%	0%
Nitrofurantoin	218 (97%)	0	7 (3%)	17 (89%)	0	2 (11%)	7 (100%)	0	0	1 (100%)	0	0	32 (100%)	0	0	**97%**	0%	**3%**
Sulfamethoxazole-trimethoprim	50 (22%)	0	175 (78%)	3 (16%)	0	16 (84%)	2 (29%)	0	5 (71%)	1 (100%)	0	0	13 (41%)	0	19 (59%)	**24%**	0%	**76%**
Erythromycin	8 (4%)	56 (25%)	161 (72%)	0	6 (32%)	13 (68%)	0	3 (43%)	4 (57%)	0	0	1 (100%)	1 (3%)	5 (16%)	26 (81%)	**3%**	**25%**	**72%**
Tetracycline	36 (16%)	10 (4%)	179 (80%)	7 (37%)	1 (5%)	11 (58%)	1 (14%)	0	6 (86%)	0	0	1 (100%)	1 (3%)	0	31 (97%)	**16%**	**4%**	**80%**
Chloramphenicol	126 (56%)	42 (19%)	57 (25%)	13 (68%)	4 (21%)	2 (11%)	4 (57%)	1 (14%)	2 (29%)	0	0	1 (100%)	17 (53%)	7 (22%)	8 (25%)	**56%**	**19%**	**25%**
Levofloxacin	215 (96%)	0	10 (4%)	19 (100%)	0	0	7 (100%)	0	0	1 (100%)	0	0	31 (97%)	0	1 (3%)	**96%**	0%	**4%**

*Q-D is reported for *E. faecium* isolates only.

S= Susceptible, standard dosing regimen; I= Susceptible, increased exposure; R= Resistant

Table 3: MDR profiles of *Enterococcus* isolates

Antibiogram	***E. faecalis* (n=225)**	***E. faecium* (n=19)**	***E. casseliflavus* (n=7)**	***E. gallinarum* (n=1)**	***Enterococcus* spp. (n=32)**
CIP-GEN-STR-SXT-ERY-TET	1	0	0	0	0
CIP-GEN-STR-SXT-ERY-TET-CHL	1	0	0	0	1
CIP-STR-ERY-TET	3	0	0	0	0
CIP-STR-ERY-TET-CHL	1	0	0	0	0
CIP-STR-NIT-ERY-TET	1	0	0	0	0
CIP-STR-NIT-SXT-ERY-TET	1	0	0	0	0
CIP-STR-Q-D-SXT-ERY-TET	0	1	0	0	0
CIP-STR-SXT-ERY-TET	2	0	0	0	0
CIP-STR-SXT-ERY-TET-CHL-LEV	2	0	0	0	0
CIP-STR-SXT-ERY-TET-LEV	2	0	0	0	0
CIP-SXT-TET	1	0	0	0	0
CIP-SXT-TET-CHL-LEV	1	0	0	0	0
CIP-SXT-TET-LEV	2	0	0	0	1
ERY-TET-CHL	2	0	0	0	0
GEN-STR-CHL	1	0	0	0	0
GEN-STR-ERY-TET	0	0	0	0	1
GEN-STR-NIT-SXT-ERY-TET	1	0	0	0	0
GEN-STR-SXT-CHL	1	0	0	0	0
GEN-STR-SXT-ERY-TET	27	0	0	0	2
GEN-STR-SXT-ERY-TET-CHL	2	0	1	0	0
NIT-ERY-SXT	1	0	0	0	0
NIT-SXT-ERY-TET	1	0	0	0	0
NIT-SXT-TET	1	0	0	0	0
Q-D-SXT-ERY-CHL	0	1	0	0	0
STR-ERY-CHL	1	0	0	0	0
STR-ERY-TET	16	0	1	0	7
STR-ERY-TET-CHL	7	0	0	1	2
STR-NIT-ERY-TET-CHL	1	0	0	0	0
STR-NIT-SXT-ERY-TET	2	0	0	0	0
STR-NIT-SXT-ERY-TET-CHL	1	0	0	0	0
STR-Q-D-ERY	0	1	0	0	0
STR-Q-D-ERY-TET	0	1	0	0	0
STR-Q-D-NIT-SXT-ERY-TET	0	1	0	0	0
STR-Q-D-NIT-SXT-ERY-TET-CHL	0	1	0	0	0
STR-Q-D-SXT-ERY-TET	0	6	0	0	0
STR-Q-D-SXT-TET	0	1	0	0	0
STR-SXT-CHL	1	0	0	0	0
STR-SXT-ERY	5	0	0	0	0
STR-SXT-ERY-TET	31	0	2	0	8
STR-SXT-ERY-TET-CHL	24	0	0	0	4
STR-SXT-ERY-TET-CHL-LEV	2	0	0	0	0
STR-SXT-TET	11	0	1	0	0
STR-SXT-TET-CHL	5	0	0	0	0
SXT-ERY-CHL	4	0	0	0	0
SXT-ERY-TET	8	0	0	0	0
SXT-ERY-TET-CHL	1	0	0	0	1
SXT-TET-CHL	1	0	0	0	0
Total (n=222; 78%)	176 (78%)	13 (68%)	5 (71%)	1 (100%)	27 (84%)

Table 4: Correlation of antibiotic resistance genes with phenotypic resistance in *Enterococcus spp*.

	Antibiotic resistance genes				
	ermB (n=205)	*aph(3')-IIIa* (n=204)	*tetK* (n=228)	*tetM* (n=228)	*aac(6')-Ie-aph(2")-Ia* (n=39)
E. faecalis	139 (67.8%)	155 (76.0%)	35 (15.4%)	177 (77.6%)	2 (5.1%)
E. faecium	13 (6.3%)	11 (5.4%)	1 (0.4%)	11 (4.8%)	0 (0.0%)
E. casseliflavus	2 (1.0%)	4 (2.0%)	0 (0.0%)	6 (2.6%)	0 (0.0%)
E. gallinarum	1 (0.5%)	0 (0.0%)	0 (0.0%)	1 (0.4%)	0 (0.0%)
Other Enterococcus spp.	26 (12.7%)	26 (12.7%)	3 (1.3%)	31 (13.6%)	0 (0.0%)
Total	**181 (88.3%)**	**196 (96.1%)**	**39 (17.1%)**	**226 (99.1%)**	**2 (5.1%)**

Table 5: Prevalence of virulence genes in all *Enterococcal spp.*

	Virulence genes					
	efaAfs	*gelE*	*cpd*	*cylB*	*cyl*A	*efaAfm*
E. faecalis (n=225)	201 (89.3%)	208 (92.4%)	186 (82.7%)	80 (35.6%)	49 (21.8%)	1 (0.4%)
E. faecium (n=19)	0 (0.0%)	13 (68.4%)	10 (52.6%)	1 (5.3%)	0 (0.0%)	9 (47.4%)
E. casseliflavus (n=7)	3 (42.9%)	5 (71.4%)	2 (28.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
E. gallinarum (n=1)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Other Enterococcus spp. (n=32)	19 (59.4%)	27 (84.4%)	21 (65.6%)	8 (25.0%)	3 (9.4%)	0 (0.0%)
Total (n=284)	***223 (78.5%)***	***253 (89.1%)***	***219 (77.1%)***	***89 (31.3%)***	***52 (18.3%)***	***10 (3.5%)***

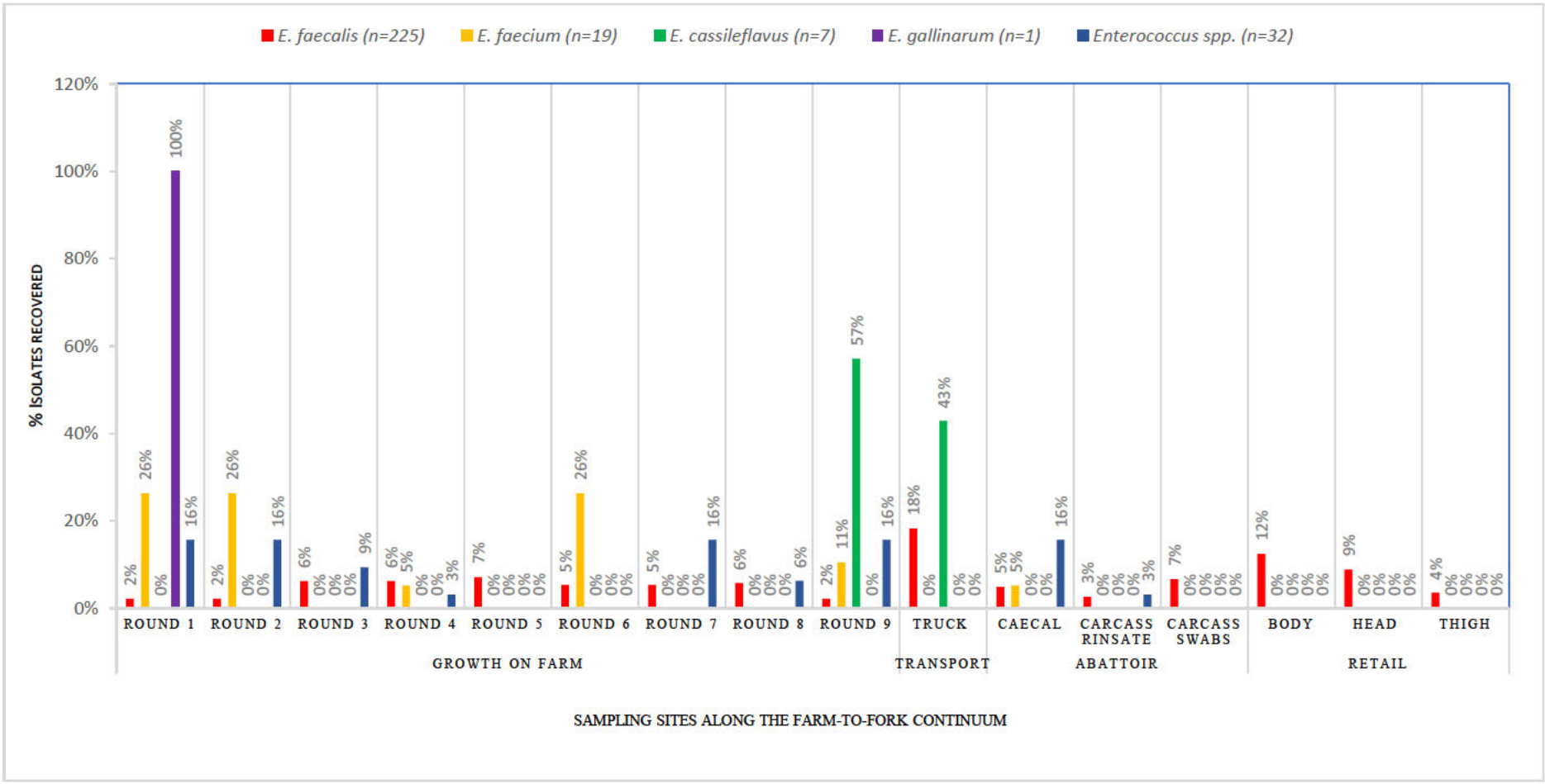

Figure 1: Distribution of *Enterococcus spp.* along the sampling points of the farm-to-fork continuum

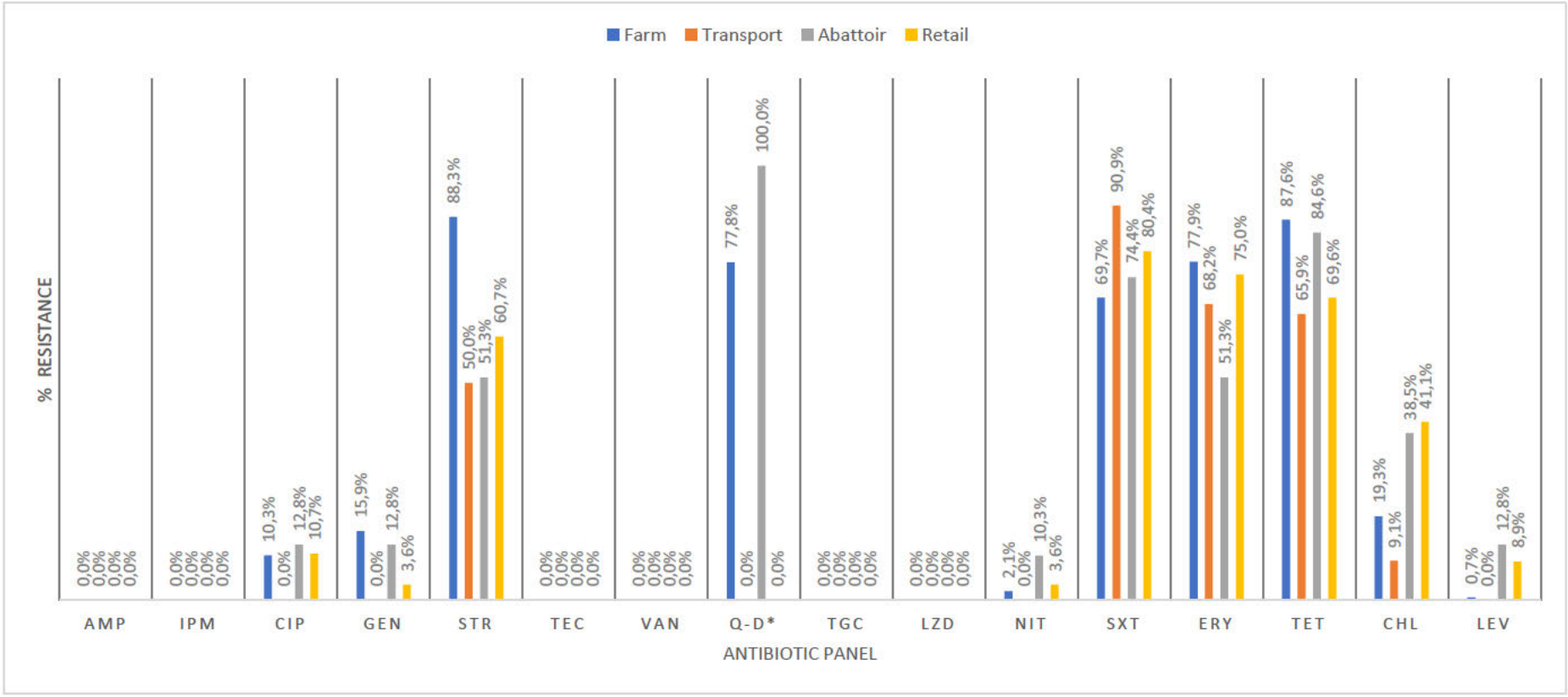

Figure 2: Antibiotic resistant profiles of *Enterococcus* spp. along the farm-to-fork continuum

*Q-D is reported for *E. faecium* isolates only.

Abbreviations: AMP:ampicillin; IPM: imipenem; CIP: ciprofloxacin; GEN: gentamicin; STR: streptomycin; TEC: teicoplanin; VAN: vancomycin; Q-D: quinupristin-dalfopristin; TGC: tigecycline; LZD: linezolid; NIT: nitrofurantoin; SXT: sulfamethoxazole-trimethoprim; ERY: erythromycin; TET: tetracycline; CHL: chloramphenicol; LEV: levofloxacin

E. faecalis

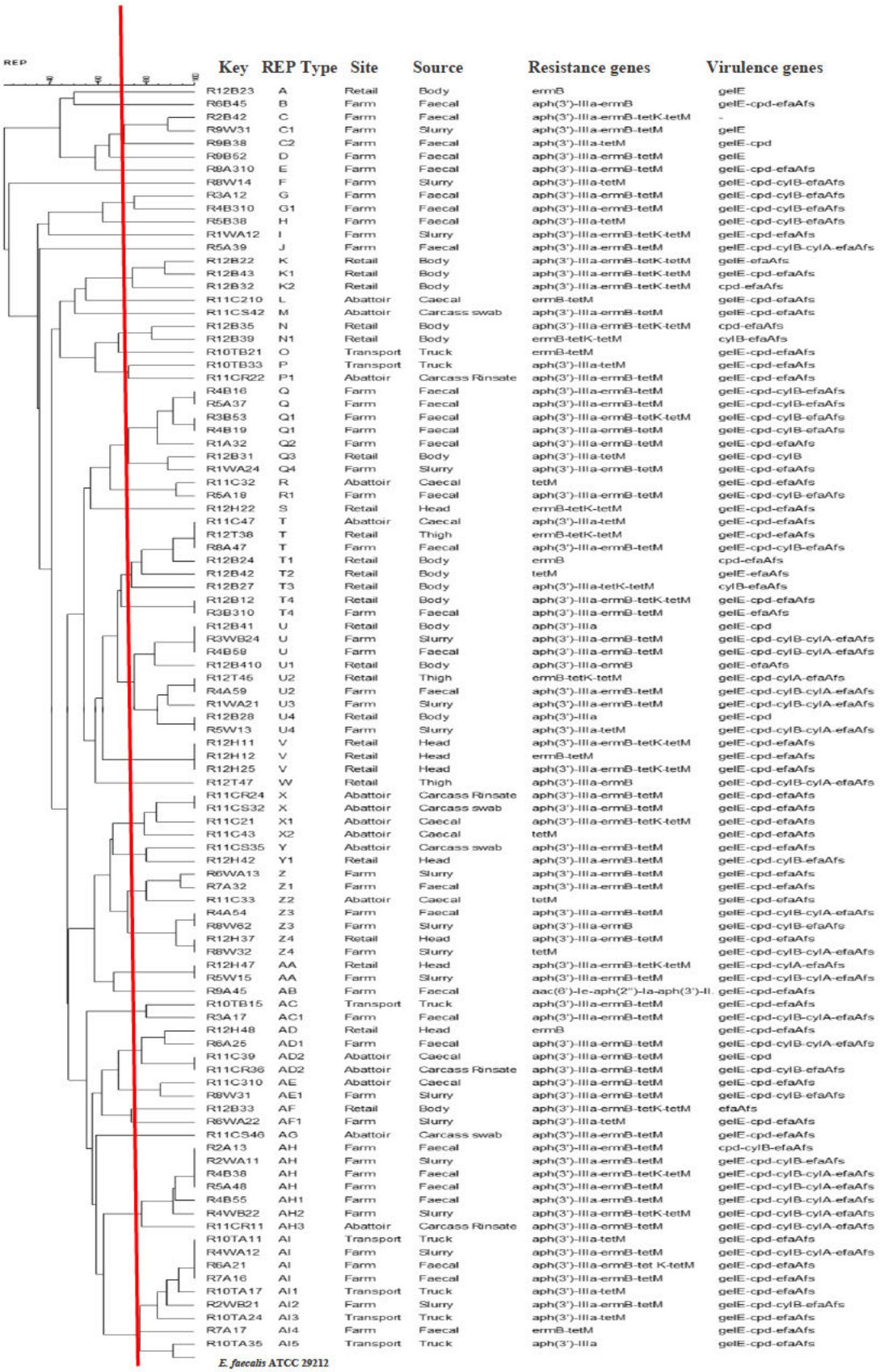

Figure 3: Dendrogram showing REP-types of *E. faecalis* isolates, based on ≥ 70% similarity index recovered along the farm-to-fork continuum. *E. faecalis* ATCC 29212 was used as the quality control strain.

E. faecium

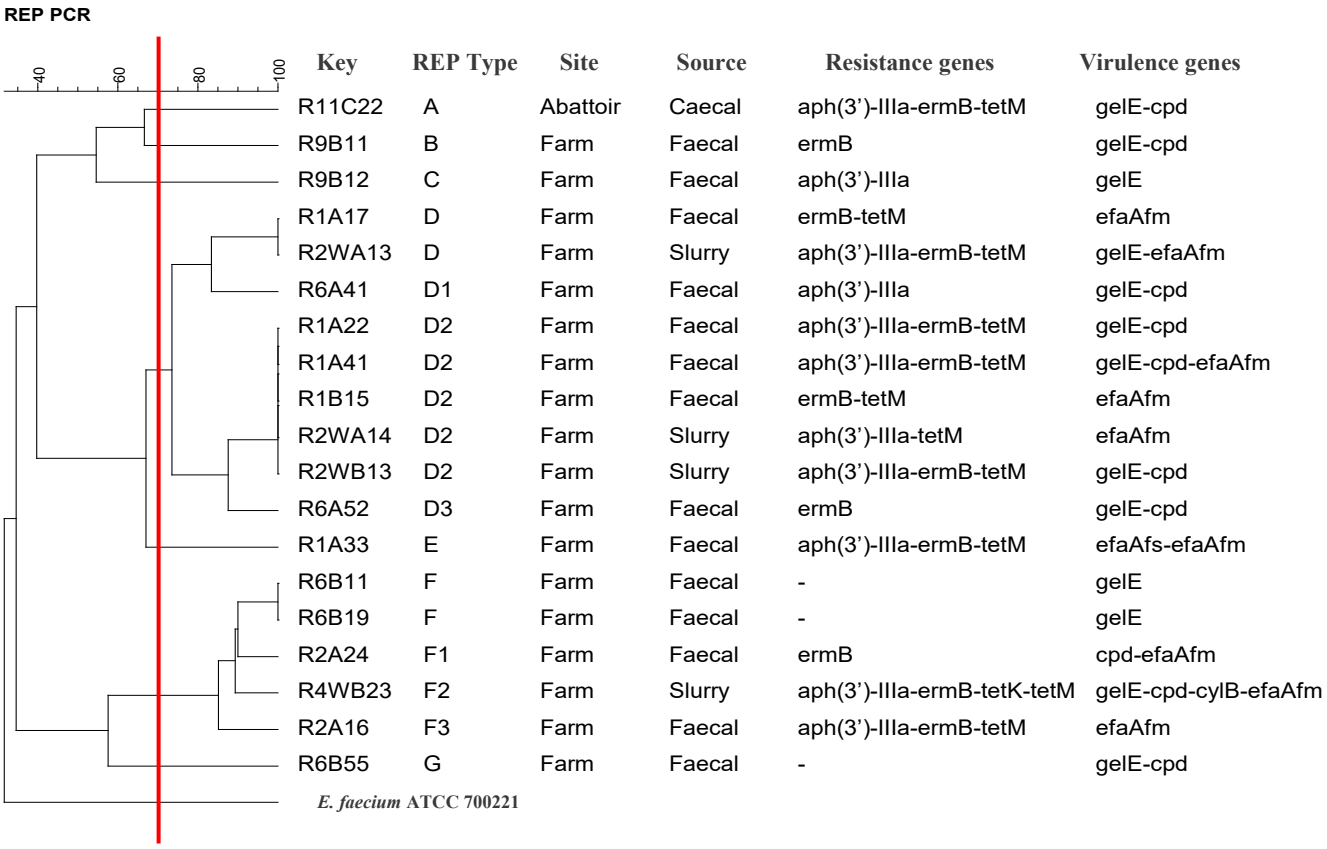

Figure 4: Dendrogram showing REP-types of *E. faecium* isolates, based on ≥70% similarity index recovered along the farm-to-fork continuum. *E. faecium* ATCC 700221 was used as the quality control strain.

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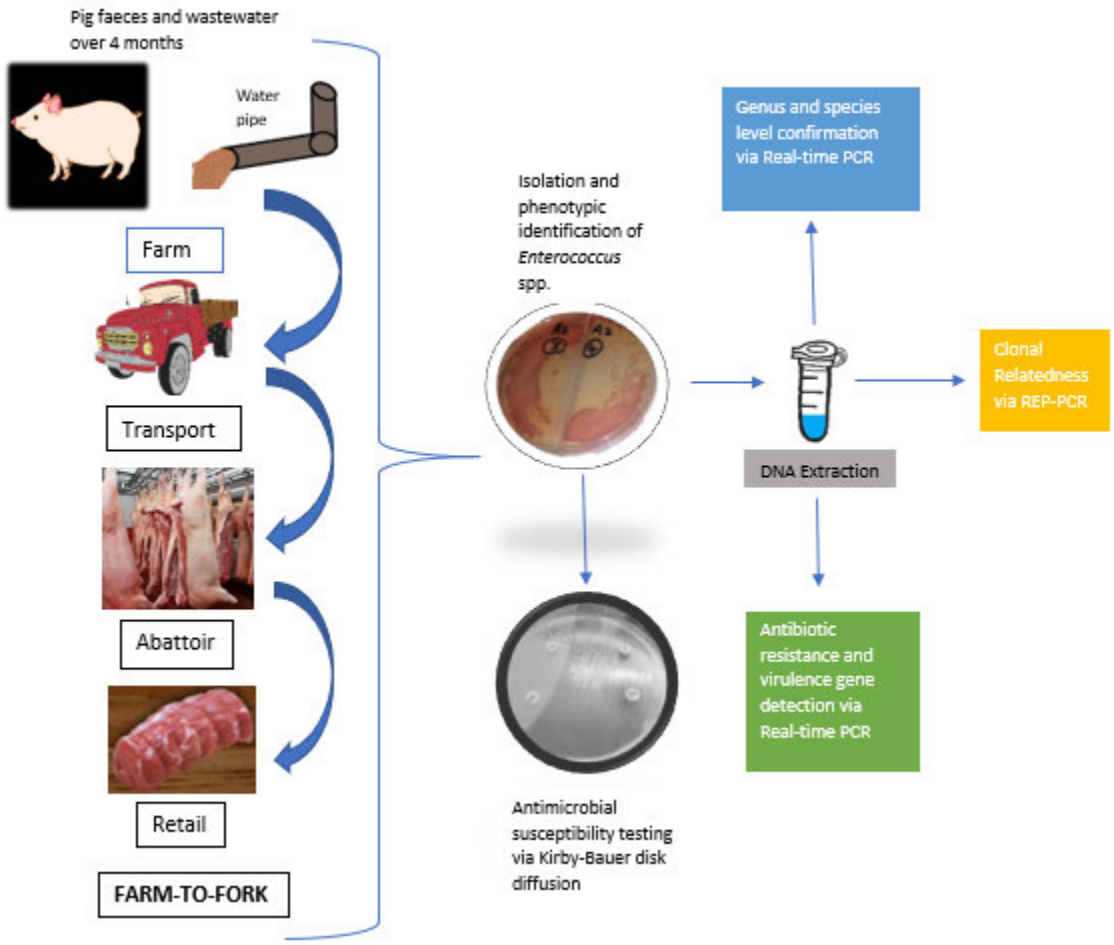
Graphical abstract



Supplementary material

Table S1: List of controls and primers used in molecular genus and species confirmation.

Control Strain	Primer	Primer sequence (5'-3')	Product size (bp)
E. faecalis ATCC 51299 (Genus)	ENT1 ENT2	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112
E. faecalis ATCC 51299	FA1 FA2	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360
E. faecium ATCC 35667	FM1 FM2	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	215
E. gallinarum Field strain (NHLS)*	GA1 GA2	TTACTTGCTGATTTTGATTCG TGAATTCTTCTTTGAAATCAG	173
E. casseliflavus ATCC 700327	CA1 CA2	TCCTGAATTAGGTGAAAAAAC GCTAGTTTACCGTCTTTAACG	288

Reference: (Molechan *et al*., 2019)

*Field strains were provided by the National Health Laboratory Services (NHLS), South Africa

Table S2: List of antibiotic resistance genes primers

Gene	Description	Positive control strains	Primer sequence (5'-3')
tetK	Tetracycline resistance	*S. aureus* clinical strain *	F- TTAGGTGAAGGGTTAGGTCC R-GCAAACTCATTCCAGAAGCA
tetM		*S. aureus* clinical strain *	F- GTTAAATAGTGTTCTTGGAG R- CTAAGATATGGCTCTAACAA
ermB	Erythromycin resistance	*E. faecalis* ATCC 51299	F-CTATCTGATTGTTGAAGAAGGATT R-GTTTACTCTTGGTTTAGGATGAAA
aac(6')-Ie-aph(2")-Ia	Gentamicin resistance	*E. faecalis* ATCC 51299	F- CAGGAATTTATCGAAAATGGTAGAAAAG R- CACAATCGACTAAAGAGTACCAATC
aph(3')-IIIa	Streptomycin resistance	*E. faecalis* ATCC 51299	F- GGCTAAAATGAGAATATCACCGG R- CTTTAAAAAATCATACAGCTCGCG

Reference: (Molechan *et al.,* 2019)

*Field strains were provided by the National Health Laboratory Services (NHLS), South Africa

Table S3: List of virulence genes primers

Gene	Description	Control strains	Primer sequence (5'-3')
gelE	Gelatinase production	*E. faecalis* ATCC 29212	F- ACCCCGTATCATTGGTTT R- ACGCATTGCTTTTCCATC
cpd	Sex pheromone	*E. faecalis* ATCC 51299	F- TGGTGGGTTATTTTTCAATTC R- TACGGCTCTGGCTTACTA
cylB	Cytolysin	*E. faecalis* ATCC 29212	F- ATTCCTACCTATGTTCTGTTA R- AATAAACTCTTCTTTTCCAAC
cylA		*E. faecalis* ATCC 29212	F-TGGATGATAGTGATAGGAAGT R- TCTACAGTAAATCTTTCGTCA
efaAfs	Cell wall adhesins	*E. faecalis* ATCC 29212	F- GACAGACCCTCACGAATA R- AGTTCATCATGCTGTAGTA
efaAfm		*E. faecium* ATCC 35667	F- AACAGATCCGCATGAATA R- CATTTCATCATCTGATAGTA

Reference: (Molechan *et al.,* 2019)

CHAPTER 3

3.1 Conclusions

The following conclusions were drawn from this study:

- A total of 284 isolates were obtained.
- Real-time PCR confirmed enterococcal isolates: 79.2% as *E. faecalis*, 6.7% as *E. faecium*, 2.5% as *E. casseliflavus*, 0.4% as *E. gallinarum* and 11.2% as other *Enterococcus* spp.
- Antibiotic susceptibility testing revealed resistance to sulfamethoxazole-trimethoprim (78.8%), tetracycline (76.9%), erythromycin (68.1%), streptomycin (62.6%), chloramphenicol (27.0%), ciprofloxacin (8.5%), gentamicin (8.1%), and levofloxacin (5,6%) but no vancomycin, teicoplanin, tigecycline or linezolid resistance. *E. faecium* displayed 44.4% resistance to quinupristin-dalfopristin.
- A total of 78% of *Enterococcal* isolates were MDR.
- Phenotypic resistance to tetracycline, aminoglycosides and macrolides was corroborated by the presence of the *tetM*, *aph(3')-IIIa*, and *ermB* genes in 99.1%, 96.1% and 88.3% of the isolates respectively.
- The highest prevalence of virulence genes detected were: *gelE* (89.1%), *efaAfs* (78.5%), and *cpd* (77.1%).
- Clonality evaluated by REP-PCR revealed that *E. faecalis* isolates along the continuum are highly diverse with major REP-types often consisting of isolates from the same sampling source but different sampling rounds (on the farm). In contrast, there was less diversity in the source of *E. faecium* isolates.

3.2 Limitations

- In this study, 11% of isolates were undifferentiated to the species level.
- The study sample was limited to a single farm and its associated abattoir in Kwa-Zulu Natal hence the results cannot be extrapolated to the whole of Kwa-Zulu Natal.

3.3 Recommendations

- Molecular confirmation of isolates to a species level should include E. *hirae* and E. *durans* as they have also been reported in pigs.

- To gain better understanding of the prevalence of virulence determinants present in isolates, additional virulence genes such as *asa, hyl* and *esp* should be studied.
- Whole-genome sequencing should be considered for a comprehensive representation of genomic profiles.
- More robust clonal typing methods should be used to elucidate the clonal complexes.
- To gain sufficient data to inform strategies for the containment of antibiotic resistance as well as gauge the burden of MDR enterococci on pig farms, further studies in the pig production system should be conducted, with a consideration of choosing farms from different geographical locations for a better representation of the South African situation.

APPENDICES

Appendix 1: Biomedical Research Ethics Committee (BREC) Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI
RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

17 May 2019

Prof SY Essack
Department of Pharmaceutical Sciences
School of Health Sciences
essacks@ukzn.ac.za

Dear Prof Essack

Title: One Health approach to the containment of antibiotic resistance.
Degree: Non-degree
BREC Ref No: BCA444/16

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 17 March 2019
Expiration of Ethical Approval: 16 March 2020

I wish to advise you that your application for Recertification received on 29 April 2019 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

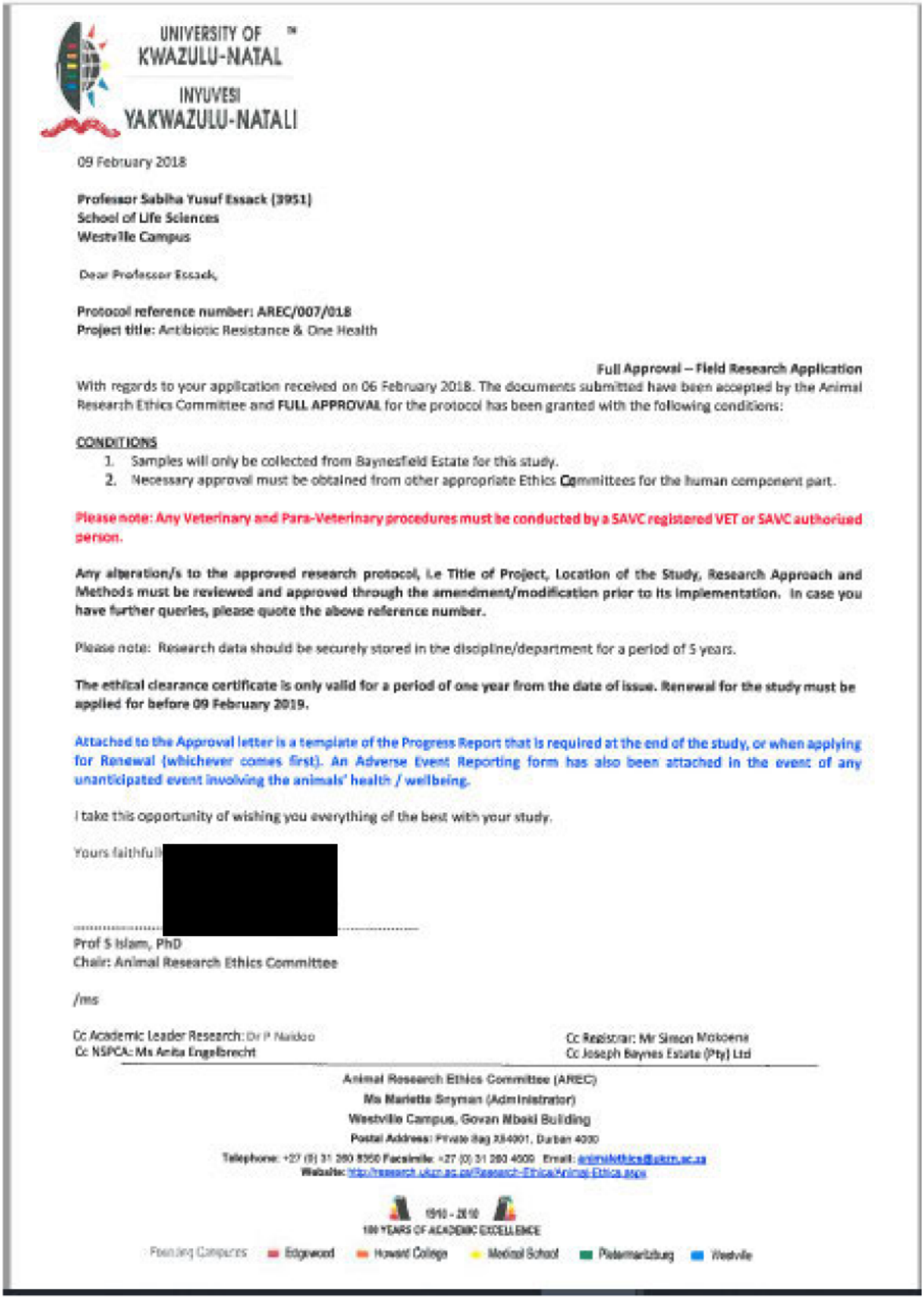
If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 11 June 2019.

Yo[redacted]

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

Appendix 2: Animal Research Ethics Committee (AREC) Approval



UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

09 February 2018

Professor Sabiha Yusuf Essack (3951)
School of Life Sciences
Westville Campus

Dear Professor Essack,

Protocol reference number: AREC/007/018
Project title: Antibiotic Resistance & One Health

Full Approval – Field Research Application

With regards to your application received on 06 February 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

CONDITIONS

1. Samples will only be collected from Baynesfield Estate for this study.
2. Necessary approval must be obtained from other appropriate Ethics Committees for the human component part.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorised person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 09 February 2019.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Academic Leader Research: Dr P Naidoo
Cc NSPCA: Ms Anita Engelbrecht
Cc Registrar: Mr Simon Mokoena
Cc Joseph Baynes Estate (Pty) Ltd

Animal Research Ethics Committee (AREC)
Ms Marietta Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 Facsimile: +27 (0) 31 260 4609 Email: animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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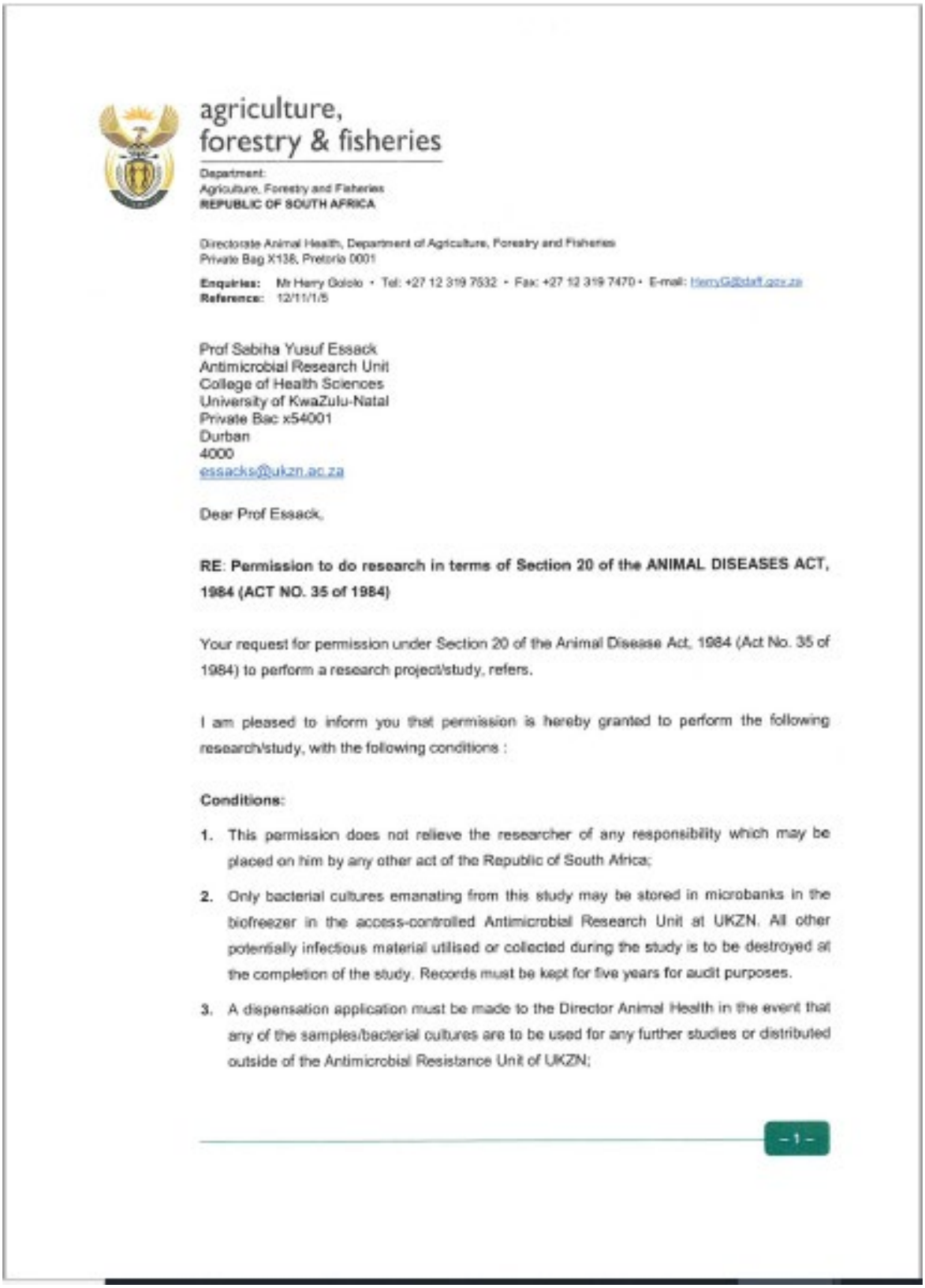
Appendix 3: Department of Agriculture, Forestry and Fisheries (DAFF) Section 20 Approval



agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Harry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HarryG@daff.gov.za
Reference: 12/11/1/5

Prof Sabiha Yusuf Essack
Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bac x54001
Durban
4000
essacks@ukzn.ac.za

Dear Prof Essack,

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your request for permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project/study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. Only bacterial cultures emanating from this study may be stored in microbanks in the biofreezer in the access-controlled Antimicrobial Research Unit at UKZN. All other potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes.
3. A dispensation application must be made to the Director Animal Health in the event that any of the samples/bacterial cultures are to be used for any further studies or distributed outside of the Antimicrobial Resistance Unit of UKZN;

– 1 –

4. The study is approved as per the application form dated 28 August 2018 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
5. Pig faecal and litter samples may only be collected from Baynesfield Farm for which a state veterinary letter has been provided;
6. Samples from pig holding pens may only be collected from Cato Ridge abattoir for which a state veterinary letter has been provided;
7. Caecal, carcass rinsates and carcass swabs may only be collected from Cato Ridge abattoir for which a state veterinary letter has been provided;
8. Removal of samples from the abattoir is subject to compliance with the provisions of the Meat Safety Act, 2000 (Act 40 of 2000), as well as written permission from the abattoir owner;
9. The study may only be performed in the Antimicrobial Resistance Unit laboratories at UKZN;
10. Should any aspect of the study change, please contact the Directorate Animal Health to enquire regarding the need for Section 20 permission;
11. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Antibiotic Resistance and One Health
Researcher (s): Prof Sabiha Yusuf Essack
Institution: Antimicrobial Research Unit, College of Health Sciences, University of KwaZulu-Natal
Your Ref./ Project Number: AREC/007/018, BCA444/16
Our ref Number: 12/11/1/5
Expiry date: 2019-04

Kind regards,

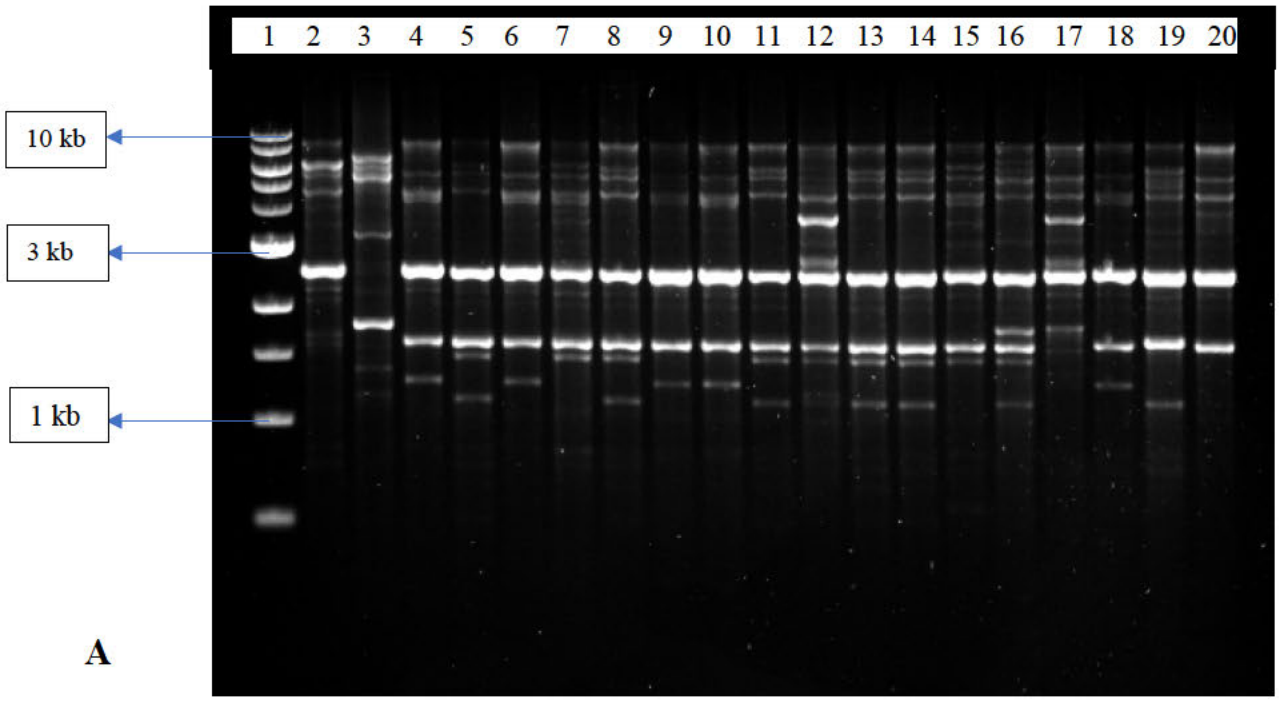
[redacted]

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -09- 17

CLASSIFICATION: CONFIDENTIAL

SUBJECT: SECTION 20 APPROVAL FOR: ANTIBIOTIC RESISTANCE AND ONE HEALTH - LIVR

Appendix 4: REP-PCR agarose gel electrophoresis image



A: Lane 1: 1 kb DNA molecular weight marker (NEB Quick-Load®, Massachusetts, USA), Lane 2-19: isolate ID's R12B33, R9B52, R2WA11, R2WB21, R2A13, R10TA24, R7A16, R4WB22, R4B55, R10TA11, R11C310, R6A21, R4WA12, R10TA17, R7A17, R11CS46, R4B38, R10TA35, Lane 20: E. *faecalis* ATCC 29212.