RÚZIVIA PIMENTEL OLIVEIRA

RESISTANCE TO CEPHALOSPORINS AND CARBAPENEMS IN GRAM-NEGATIVE BACTERIA ISOLATED FROM SWINE, SOIL, AND WASTEWATER OF A PIG PRODUCTION UNIT

Dissertation submitted to the Agricultural Microbiology Graduate Program in at the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Hilário Cuquetto Mantovani

Co-adviser: Denise Mara Soares Bazzolli

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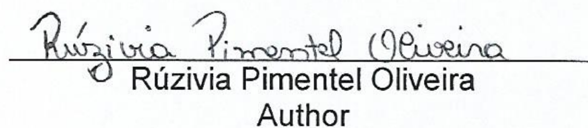

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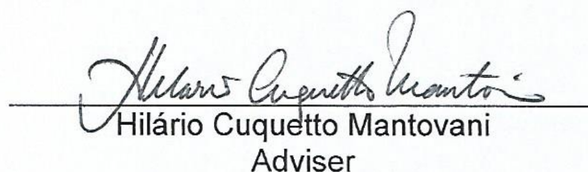

Hilário Cuquetto Mantovani
Adviser

To my parents, Raquel and Flávio for all their support and trust throughout their lives.

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“Conforme aprendemos, lemos, estudamos, convivemos com outras pessoas, vamos nos transformando. Não existe um eu permanente. A vida é movimento e o equilíbrio é fluído.”
(Monja Coen)

“A vida não é sobre metas, conquistas e linhas de chegada. É sobre quem você se torna durante a caminhada”
(Autor desconhecido)

ABSTRACT

OLIVEIRA, R. P., M.Sc., Universidade Federal de Viçosa, September, 2021. **Resistance to cephalosporins and carbapenems in Gram-negative bacteria isolated from swine, soil, and wastewater of a pig production unit.** Adviser: Hilário Cuquetto Mantovani. Co-adviser: Denise Mara Soares Bazzolli

Some antibiotics of veterinary importance belong to the same class and share the same mechanisms of action of antibiotics used for humans such as carbapenems, which can trigger the selection and spread of multidrug resistant bacteria from animals to the environment through waste and wastewater. The objective of this study was to evaluate the co-occurrence of genetic determinants conferring resistance to cephalosporins and carbapenems in Gram-negative bacteria isolated from finishing pigs, soil and wastewater from a swine production unit. For this, the different isolates obtained were characterized for the presence of genes encoding extended spectrum beta-lactamases (ESBL) and/or carbapenemases. After an initial screening in selective MacConkey agar supplemented with ceftiofur (8 mg/L), 77 isolates were obtained from the rectal swabs of finishing pigs, in addition to 58 isolates from soil and 52 isolates from wastewater, both collected from environments of the production unit. Susceptibility was evaluated against carbapenems (meropenem and imipenem) and cephalosporins of 1st (cefazolin), 3rd (cefotaxime and ceftriaxone) and 4th (cefepime) generations. The results indicated that 70 % of the isolates from all environments were resistant to imipenem and 20 % to meropenem. Regarding susceptibility to cephalosporins, more than 90 % of the swine and wastewater isolates and more than 81 % of the soil isolates were resistant to at least one of the cephalosporins tested. The highest frequencies of resistance to cephalosporins were observed in bacterial isolates from wastewater, with 95.5 % showing resistance to cefazolin, ceftriaxone and cefepime, and 90.9 % to cefotaxime. In addition to the resistance profile, genes associated with resistance to beta-lactams were identified, including bla_{CTX-M}, bla_{VIM} and bla_{IMP}. Approximately 85 % of the isolates from the three environments carried at least one resistance determinant. The bla_{VIM} gene was the most frequent among isolates from soil and wastewater, while bla_{CTX-M} was the most frequent gene in pig isolates, corroborating the hypothesis that genetic determinants of resistance to cephalosporins and carbapenems coexist in Gram-negative bacteria isolated from swine, soil and wastewater. Furthermore, the results suggest the existence of cross-

resistance between cephalosporins and carbapenems through resistance determinants between the three environments.

Keywords: Antibiotic resistance. Food-producing animals. ESBL. Swine. Cross-resistance.

RESUMO

OLIVEIRA, R. P., M.Sc., Universidade Federal de Viçosa, setembro de 2021. **Resistance to cephalosporins and carbapenems in Gram-negative bacteria isolated from swine, soil, and wastewater of a pig production unit**. Orientador: Hilário Cuquetto Mantovani. Coorientadora: Denise Mara Soares Bazzolli

Alguns antibióticos de importância veterinária pertencem à mesma classe e compartilham os mesmos mecanismos de ação dos antibióticos de uso humano, como os carbapenêmicos, podendo desencadear a seleção e disseminação de bactérias multirresistentes dos animais para o meio ambiente por meio de dejetos e águas residuais. O objetivo deste estudo foi avaliar a co-ocorrência de determinantes genéticos para resistência à cefalosporinas e carbapenêmicos em bactérias Gram-negativas isoladas de suínos em fase de terminação, solo e água residual da unidade de produção. Para isso, os isolados bacterianos obtidos foram caracterizados em relação à presença de genes que codificam beta-lactamases de espectro estendido (ESBL) e/ou beta-lactamases carbapenemase. Após uma triagem inicial em meio seletivo MacConkey suplementado com ceftiofur (8 mg/L) foram obtidos 77 isolados a partir de swab retal de 92 suínos em fase de terminação, além de 58 isolados de solo e 52 isolados de água residual, provenientes de amostras coletadas de ambientes da unidade de produção. A susceptibilidade aos carbapenêmicos (meropenem e imipenem) e a outras cefalosporinas de 1° (cefazolina), 3° (cefotaxima e ceftriaxona) e 4° (cefepime) geração também foi avaliada. Os resultados indicaram que 70 % dos isolados de todos ambientes foram resistentes ao imipenem e 20 % ao meropenem. Com relação à susceptibilidades às cefalosporinas, mais de 90 % dos isolados de suínos e de água residual e mais de 81 % dos isolados de solo foram resistentes a pelo menos uma das cefalosporinas testadas. As maiores frequências de resistência às cefalosporinas foram observadas nos isolados bacterianos de água residual, com 95.5 % apresentando resistência à cefazolina, ceftriaxona e cefepime, e 90.9 % à cefotaxima. Além do perfil de resistência, foram identificados genes associados à resistência aos beta-lactâmicos (*bla*$_{CTX-M}$, *bla*$_{VIM}$ e *bla*$_{IMP}$), sendo que 85 % dos isolados dos três ambientes carreavam pelo menos um determinante de resistência. O gene *bla*$_{VIM}$ foi o mais frequente entre os isolados do solo e da água residual, enquanto *bla*$_{CTX-M}$ foi o gene mais frequente nos isolados de suínos, corroborando

com a hipótese de que determinantes genéticos de resistência às cefalosporinas e aos carbapenêmicos coexistem em bactérias Gram-negativas recuperadas de suínos, solo e água residual. Além disso, os resultados sugerem a existência de resistência cruzada entre cefalosporinas e carbapenêmicos por meio de determinantes de resistência entre os três ambientes.

Palavras-chave: Resistência antimicrobiana. Animais de produção. ESBL. Suínos. Resistência cruzada.

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LIST OF ACRONYMS AND ABBREVIATIONS

ABPA – Associação Brasileira de Proteína Animal

ABCS – Associação Brasileira de Criadores de Suínos

ARG – Antibiotic Resistance Genes

BrCAST – Brazillian Committee on Antimicrobial Susceptibility Testing

CEAUP/UFV - Comissão de Ética no Uso de Animais de Produção/Universidade Federal de Viçosa

CFZ – Cefazolin

CFU - Colony Forming Units

CIM – Carbapenem inactivation method

CLSI – Clinical and Laboratory Standards Institute

CLSI VET – Clinical and Laboratory Standards Institute Veterinary Antimicrobial Testing

CMY – Cefamicinases

CON - Control

CRO – Ceftriaxone

CTX – Cefotaxime

CTX-M – Cefotaximase

EFSA – European Food Safety Authority

ESVAC – European Medicines Agency

EUCAST - European Committee on Antimicrobial Susceptibility Testing

ESBL – Extended spectrum beta-lactamase

FEP – Cefepime

GDP - Gross domestic product

HGT – Horizontal Gene Transfer

IBGE – Instituto Brasileiro de Geografia e Estatística

IMP - Imipenem

iTOL – Interactive Tree of Live

KPC – Klebsiella pneumoniae carbapenemase

LB - Luria Bertani

MALDI-TOF - Matrix Assisted Laser Desorption Ionization - Time Of Flight

MBL – Metallo-β-lactamase

mCIM – Modified carbapenem inactivation method

MIC - Minimal Inhibitory Concentration

NDM – New Delhi-metallo- β-lactamase

PBP – Protein binding penicillin

OD600nm - Optical Density at 600nm

OIE – World Organization for Animal Health

OXA - Oxacilinases

RDP - Ribosomal Database Project website

TSB – Tryptic Soy Broth

VIM – Verona integron-encoded metallo- β-lactamase

WHO – World Health Organization

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1. GENERAL INTRODUCTION

Pork consumption has increased worldwide and in 2020, the consumption of pork products reached 16 kg/inhabitant in Brazil, with pork production reaching 4,436 million tons, the highest in the last 10 years (ABPA, 2021). The growing demand for pork products is inducing changes in management practices and promoting the intensification of the pig production systems, aiming to improve feed efficiency and to increase meat production (AARESTRUP, 2015).

Feed additives have been used in swine production systems to improve the productivity, health, and welfare of pigs (RUTZ and LIMA, 1994). The use of antibiotics in feeds has been associated with reduced mortality, disease prevention and growth promotion in livestock (JACELA *et al.* 2010). In addition to growth promotion, antibiotics are used for therapeutic purposes, as well as prophylaxis and metaphylaxis. However, in recent years there has been a growing concern about the overuse and misuse of these antibiotics in farm animals, associated with the emergence of antibiotic-resistant bacteria (ESVAC, 2011; AARESTRUP, 2015; MANYI-LOH *et al.* 2018).

The concern associated with the use of antibiotics in livestock is because some antibiotics of veterinary importance belong to the same class of medically relevant antibiotics, such as the beta-lactams (SAIFUL *et al.* 2016). Beta-lactams are widely used worldwide and have activity against both Gram-positive and Gram-negative bacteria. These molecules act inhibiting bacterial cell wall synthesis by binding to penicillin-binding proteins (PBP), which are enzymes responsible for catalysing the final steps of the cell wall biosynthesis (GEORGOPAPADAKOU and LIU, 1980). The class of beta-lactams includes penicillins, cephalosporins, carbapenems and monobactams. The classification scheme is based on the chemical nature of the compound that binds to the beta-lactam ring (CHAIN, 1979; SELWYN, 1979). In veterinary medicine, beta-lactams such as cephalosporins, are widely used in swine to control respiratory and gastrointestinal infections and to treat septicemias (OIE, 2018).

The mechanisms of resistance to the beta-lactams involve inhibition of the interaction between PBP and the antibiotic, the presence of efflux pumps that extrude the drug, or hydrolysis of the antibiotic molecule through enzymes called beta-lactamases. Beta-lactamases are the main cause of resistance against beta-lactams among Gram-negative bacteria. These enzymes hydrolyze the beta-lactam ring causing its opening and its inactivation (REYGAERT, 2018). These enzymes are

classified into four classes. Enzymes of classes A (CTX-M, KPC, GES), C (CMY, AmpC) and D (OXA-type) have a serine residue in their active sites while enzymes belonging to the class B (VIM, NDM, IMP) are dependent on zinc (BUSH and BRADFORD, 2016; CODJOE and DONKOR, 2018). The genes encoding these enzymes are normally located in mobile genetic elements and can be transferred to opportunistic or commensal bacteria present in animals and humans. However, genes encoding some of the enzymes conferring resistance to beta-lactams (e.g. cephalosporinase AmpC) can also be found in the bacterial chromosome (JACOBY, 2009). The enzymes SHV, TEM, CTX-M and CMY-2 confer resistance primarily to 2nd, 3rd and 4th generation cephalosporins (RUPPÉ *et al.* 2015).

The production of beta-lactamases by bacteria can confer resistance to all beta-lactam antibiotics and the presence of such antibiotics in the environment could be a driving force for the co-selection of antibiotic resistance (TAL-JASPER *et al.* 2016). Mechanisms associated with co-selection of antibiotic resistance include both co-resistance and cross-resistance. Co-resistance typically occurs when different resistance determinants are located in the same mobile genetic element (e.g. transposons, plasmids) or when the bacteria accumulate mutations in different genetic loci. In contrast, cross-resistance occurs when the resistance mechanism (acquired by horizontal transfer or mutations) protects the bacteria against different inhibitors often belonging to the same antibiotic class. Additionally, overexpression of efflux pumps can offer protection against antibiotics from different classes (CANTÓN and RUIZ-GARBAJOSA, 2011). Thus, the use of an antibiotic of a class can trigger the co-selection of resistance to inhibitors from the same class or to antibiotics with different mechanisms of action (PAL *et al.* 2017).

Considering the gaps in the literature regarding the co-selection of resistance to beta-lactams in pigs, soil and wastewater, this study aimed to evaluate the co-occurrence of cephalosporins and carbapenems resistance in Gram-negative bacteria isolated from pigs in the finishing phase, in addition to soil and wastewater from the swine production unit.

2. CHAPTER 1

2.1. Literature review

2.1.1. Economic and social impacts of pig production

Pork was the second most important source of animal protein in the world in 2018, with an average of 120 million tons of meat consumed and just behind chicken meat (123 million tons) (ABCS, 2020). According to IBGE, in 2019 the number of pig breeders in Brazil was 40,556,892 heads. Brazil ranked fourth in pork production worldwide in 2020, with an average of 4 million tons of meat produced, behind China, the European Union and the United States (ABCS, 2020). Pork meat production in Brazil is predominantly destined for the domestic market. In 2020, 77 % of the pork meat produced in the country was consumed nationally and only 23 % was exported (ABPA, 2021). Pork consumption in 2020 was 16 kg/hab (ABPA, 2020), and the swine production chain contributed with US$ 22 million to the Brazilian GDP (ABCS, 2020). Brazilian pig production is concentrated in the Southern region of the country, especially in the states of Santa Catarina, with an average production of more than 1 million tons of carcasses in 2019, followed by Rio Grande do Sul and Paraná. The Southeast region of the country is the second largest producing region, and the state of Minas Gerais occupies the fourth position in the national rank with an average of 497 thousand tons of carcasses (IBGE, 2019).

Pig production systems vary according to the geographical region into intensive or extensive systems (ABCS, 2014). In the extensive systems the animals are raised outdoors (free-range pig farming), the technological level is low as well as the productivity. In the 1990s, it was estimated that extensive systems accounted for 32% of the pig farms in Brazil (SOBESTINSKY *et al.* 1998; ABCS, 2014). Intensive systems focus on improving productivity through the control of genetic, nutritional, and health conditions (NICOLAIEWSKY *et al.* 1998), to promote faster animal weight gain. In intensive systems, the animals are raised in small spaces, and sanitary management and genetic background is improved to optimize production (SOBESTINSKY *et al.* 1998). However, intensive systems also have disadvantages, such as higher installation costs and environmental impact, and reduced well-being of the animals (CARVALHO and VIANA, 2011).

To improve the quality of life of pigs, animal performance, as well as the quality of the meat produced, additives have been added to the diet of livestock in Brazil since 1976 in accordance with decree 76.986, which regulated law 6198 of 1974. Feed additives can include amino acids, vitamins, prebiotics, probiotics and antibiotics (LORENZONI, 2010). Additives such as antibiotics are added to animal diets to

promote growth and improve reproductive performance, and to reduce morbidity and mortality rates, without being used as a nutrient by the animal (JACELA *et al.* 2010). In addition of acting as growth promoters that are continuously included in the feed at subtherapeutic doses to improve animal performance, antibiotics are largely applied for therapeutic and prophylactic purposes, especially when animals are under stress, a condition where even the healthy individuals can develop diseases. The metaphylactic use of antibiotics in livestock is also a common practice among pig farmers. In this latter case, if one or a few animals show clinical signs of disease, the entire herd will be subjected to the same drug treatment (ESVAC, 2011; AARESTRUP, 2015). However, in recent years, the use of antibiotics for growth promotion purposes has been a matter of much debate worldwide (MANYI-LOH *et al.* 2018, OLIVEIRA *et al.* 2020).

2.1.2. The pig production cycle and the usage of antibiotics

In pig production, the growth stages are classified according to the age or the productive phase of the animal. Sow gestation lasts an average of 115 days, and the lactation phase starts after giving birth to the piglets. The period between birth and weaning lasts from 21 to 28 days, where the piglet feeds colostrum and milk. The nursery phase occurs after weaning with an average duration of 30 days. This phase is considered an adaptation period for the piglets both in terms of housing and feeding. In the growth or fattening phase, the objective is to gain weight maintaining a balanced diet until the pigs reach an average body weight of 55 kg (FERREIRA, 2017). Finally, in the finishing phase, the animals reach an average of 110 kg after approximately 140 days and are considered ready for slaughter or reproduction (HOAR and ANGELOS, 2014).

Normally, the weaning of piglets is a stressful process because these animals must adapt quickly to drastic social and physical changes. The emergence of gastrointestinal disorders such as diarrhea is one of the consequences of such stressful conditions (LI *et al.* 2017). Therefore, the addition of antibiotics to the feeds of young pigs during the weaning period is a common practice to reduce mortality, and to prevent and treat infectious diseases. Lekagul *et al.* (2019) reported that gastrointestinal infections were reported more frequently in piglets and nursery pigs, with *Escherichia coli* and *Salmonella* spp. as the main pathogens. The authors reported

that amoxicillin was the most used oral and injectable antibiotic among the farms surveyed, being used both for prophylaxis and treatment of infections. Li *et al.* (2017) also reported that weaned piglets fed zinc bacitracin and colistin for 28 days increased daily weight gain and showed minor gastrointestinal disturbances compared to the group that did not receive antibiotics.

In contrast, the use of antibiotics during the growth and fattening phases has been associated with improved growth and feed efficiency of pigs, in addition to promoting growth (LEKAGUL *et al.* 2019). Other benefits associated with the use of antibiotics during these phases include the improvement of the appetite and overall animal appearance, in addition to faster growth depending on the nutritional strategy, living conditions, and the type of antibiotic used (LI *et al.* 2017). Lekagul *et al.* (2019) reported that farmers consider the use of antibiotics during this phase, such as lincomycin, tiamulin and tylosin, as crucial for the prevention of common livestock diseases.

The diseases affecting the respiratory and gastrointestinal tract of pigs are the most common ones in pig production systems. *Streptococcus suis*, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae* are the main pathogens associated with respiratory diseases in pigs (ROBBINS *et al.* 2014). Such infections can occur at any stage of the animal life, but are often recurrent in the weaning and fattening phases (BUROW *et al.* 2019). Piglets are particularly susceptible to gastrointestinal (GI) disorders, although the animals in other phases can also be afflicted. The most common GI disorders are swine dysentery (*Brachyspira* spp.), gastroenteritis (*E. coli*), and epidemic diarrhea (coronavirus) (ROBBINS *et al.* 2014). Antibiotics are usually added to the swine feed or water to treat such infections (RAJIĆ *et al.* 2006; MERLE *et al.* 2013). The aminoglycosides, beta-lactams, polypeptides, macrolides, and tetracyclines are some of the antibiotic classes most used for the treatment of the GI diseases (OIE, 2018). Burow *et al.* (2019) demonstrated that the beta-lactams, macrolides, tetracyclines, and trimethoprim were the classes/antibiotics most frequently used to control respiratory diseases in pig farms. Regarding gastrointestinal disorders, such as diarrhea, the most common classes/antibiotics used are the macrolides, colistin, tiamulin, and beta-lactams at the farm level (BUROW *et al.* 2019). According to Burow and co-authors (2019), beta-lactams are also commonly used to control streptococcal infections and arthritis.

2.1.3. Dynamics of antimicrobial resistance in pig production systems

Antibiotics are compounds that kill bacteria or inhibit their growth. These molecules are classified as bactericidal, when they cause the death of the bacteria, or bacteriostatic when they inhibit bacterial growth. Antibiotics can be categorized according to their mechanism of action, which may be through inhibition of peptidoglycan synthesis, inhibition of DNA gyrase activity or protein synthesis, among other mechanisms (GOLDSTEIN, 2011; REYGAERT, 2018).

The mechanisms of antibiotic resistance in Gram-negative bacteria involve four main strategies: limitation of antibiotic uptake; modification of antibiotic target; antibiotic inactivation and expression of active efflux pumps. These mechanisms may occur not only due to intrinsic resistance caused by chromosomal mutations (REYGAERT, 2018) but also due to the acquisition of resistance genes through horizontal gene transfer mediated by mobile genetic elements, such as conjugative plasmids (RUPPÉ, *et al.* 2015; MUNITA and ARIAS, 2016). Among the resistance mechanisms in *Enterobacteriaceae*, as shown in Figure 1, the inactivation of antibiotics through enzymes has been one of the best strategies used by bacteria and one of the best-known mechanisms of acquired resistance (MUNITA and ARIAS, 2016). The enzymes responsible for this strategy are called β-lactamases, acting in the hydrolysis of the β-lactam rings, making the antibiotic ineffective. In some Gram-negative bacteria, beta-lactamases act in conjunction with other resistance mechanisms, such as the loss of a porin or the overexpression of efflux pumps (GUERRA *et al.* 2014).

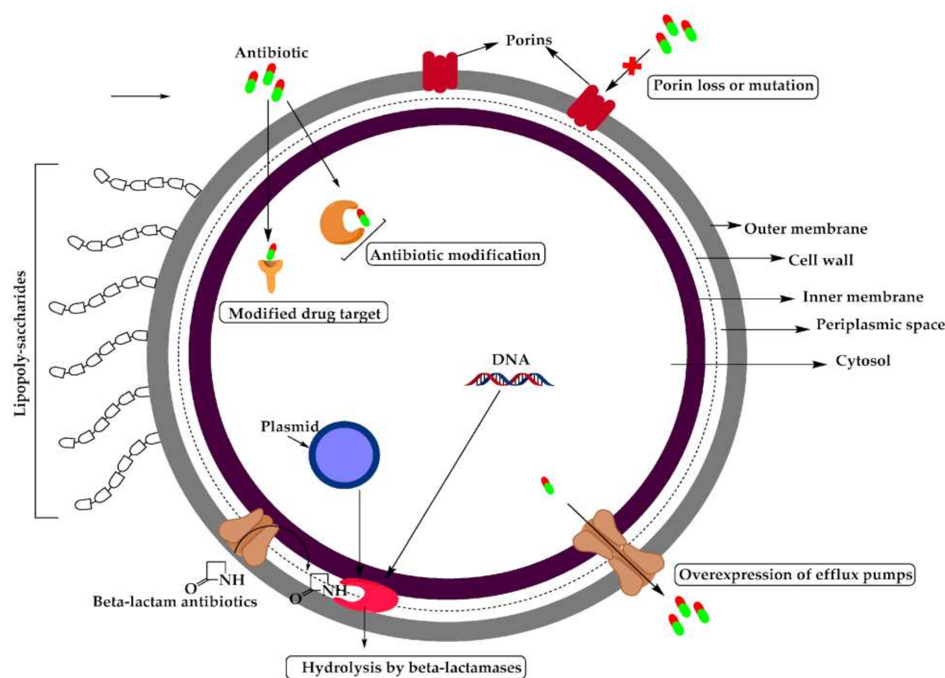

Figure 1. Schematic representing the mechanisms of antibiotic resistance in Gram-negative bacteria (Adapted from BREIJYEH *et al*. 2020).

The misuse and overuse of antibiotics in agriculture, food production, veterinary and clinical practice sectors have been considered the main factor in the emergence and spread of antibiotic resistance (OIE 2016; MCEWEN and COLLIGNON, 2018). However, in the veterinary and agriculture sectors, antibiotic use in food animals has been associated with increased antibiotic resistance in human pathogens, as well as the direct transfer of antibiotic-resistant bacteria from animals to humans (ZHU *et. al.* 2013). In 2018, the OIE (World Organization for Animal Health) reported that the beta-lactams, including cephalosporins and penicillins, are the antibiotics most intensively used in veterinary medicine and in food-producing animals. Ceftiofur, cefalexin, ceftriaxone and cefquinome are antibiotics considered as critically important for veterinary use and represent the most used cephalosporins in swine production. Ceftiofur is a 3rd generation cephalosporin used to treat respiratory diseases and diarrhea in pigs, and for the prophylaxis against other bacterial diseases in newly weaned piglets (CALLENS *et al*. 2012).

Normally, cephalosporin-resistance is linked to genes encoding extended spectrum beta-lactamases (ESBL), such as $\textit{bla}_{\text{CTX-M}}$ (PITOUT and LAUPLAND, 2008) and $\textit{bla}_{\text{CMY-2}}$ (Class C) (JACOBY, 2009). Aaresptrup *et al.* (2006) reported that two *E. coli* isolates recovered from infections in pigs harbored the gene $\textit{bla}_{\text{CTX-M}}$ and showed

resistance to cephalosporins and other antibiotics such as ampicillin and tetracycline. The authors reported that these isolates were recovered from farms with a history of usage of both cefquinome and ceftiofur. Hammerum *et al.* (2014) demonstrated in their study a higher prevalence of the bla_{CTX-M} gene in *E. coli* recovered from pigs from farms with a history of use of 3rd and 4th generation cephalosporins, and a low frequency of ESBL-producing *E. coli* in farms with no usage history. Being able to associate the use of cephalosporins with the emergence of this beta-lactamase. However, Abraham *et al.* (2018) reported that significant reduction or elimination of cephalosporin-resistant *Escherichia coli* in pig farms may take a long time to occur (at least 4 years), even after the withdrawal of the selective pressure imposed using antibiotics. Furthermore, Abraham *et al.* (2018) demonstrated that the persistence of the cephalosporin resistance in the pig population was attributed to a highly transmissible plasmid found in *Escherichia coli* strains.

Reports in the last years have indicated a significant rate of antibiotic-resistant bacteria in pig farms (LIU *et al.* 2016; FISCHER *et al.* 2017; MOLLENKOPF *et al.* 2018; ABRAHAM *et al.* 2018). Even so, the mechanisms involved in the selection of resistance genes in farm animals are still unclear. A previous report by Liu *et al.* (2018) revealed the coexistence of carbapenemases genes (bla_{NDM-1}, bla_{KPC-2}, bla_{OXA-48}) and beta-lactamases genes (bla_{SHV}, $bla_{CTX-type}$, $bla_{TEM-type}$) in *E. coli* isolates recovered from healthy and diarrheal pigs. These bacteria showed different antibiotic resistance profiles between beta-lactams and other classes of antibiotics tested (fluoroquinolones, aminoglycosides), demonstrating a possible co-selection of resistance. A recent study by Chaudhry *et al.* (2020) demonstrated the existence of carbapenem-resistant *K. pneumoniae* in veterinary and wastewater environments, revealing a high prevalence of resistance in wastewater samples. Antibiotic resistance genes (ARGs) were also detected in isolates from wastewater and fecal samples (bla_{CTX-M}, bla_{SHV}, bla_{TEM}, bla_{OXA-48}, bla_{NDM-1}) that were also resistant to all cephalosporins tested (cefepime, ceftriaxone, cefuroxime, cefoxime) and to meropenem. These results suggested a possible horizontal gene transfer between niches in the pig production units.

The spread of resistance genes and multi-resistant pathogens may occur through direct contact between animals and farm facilities or workers, or indirectly

through the products of animal origin (milk, meat) and through contaminated soils and water bodies with waste from these animals (SCHMITHAUSEN *et al.* 2018). Therefore, residual contamination has been considered one of the main sources of antibiotic release into the environment (SCHWARTZ *et al.* 2003; BAQUERO *et al.* 2008; LUPO *et al.* 2012). These antibiotic residues can be deposited in the soil and may represent a high risk to human health (OLIVEIRA *et al.* 2020). Resistant-bacteria and resistance genes can also spread through other pathways, such as air, manure, plants and soils irrigated with treated wastewater (SCHMITHAUSEN *et al.* 2015; VON SALVIATI *et al.* 2015). The spread of this resistance to water bodies can be worse if this contaminated water is ingested or used to irrigate crops. Furthermore, in the wastewater environment the non-pathogenic bacteria that can acquire resistance genes and become reservoirs (BAQUERO *et al*. 2008; COLEMAN *et al*. 2012). Antibiotics that are administered and not fully metabolized by the animal are released into the environment, especially into the water or soil. Thus, the amount and frequency of antibiotic released into the environment depends on the specific antibiotic used, the dosage administered, and the species and age of the animals (ZHAO *et al.* 2010; MANYI-LOH *et al.* 2018).

In a recent study, Gao *et al.* (2020) showed that untreated swine wastewater could change the composition of the bacterial community in soils. The authors also detected the presence of resistance genes (sulfonamides, tetracyclines, and chloramphenicol) in the soils treated with swine wastewater. Lu *et al.* (2017) characterized the presence of resistance genes in feces of piglets fed with and without additives and antibiotic contents in manure through HPLC analysis. They observed that the antibiotic content in manure from pigs fed with additives was higher than in animals fed with a grain-based diet without additives. Likewise, the authors reported that bacteria found in swine manure are resistant to sulfonamide, β-lactam, fluoroquinolone, and tetracycline antibiotics, among others and contained most of the β-lactam resistance genes (bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX}). Another study by Zhu *et al.* (2019) revealed that an isolate classified as *Enterobacter cloacae* recovered from the rectal swab of pigs had high MIC values for a variety of antibiotics, including carbapenems, monobactams, penicillins, and fluoroquinolones. In addition, the bacterial isolate carried the bla_{IMP-4} gene located on a conjugative plasmid.

2.1.4. Resistance to cephalosporins and carbapenems in Gram-negative bacteria of animal origin

Beta-lactams are bactericidal agents that block the synthesis of bacterial cell wall by binding to the penicillin binding proteins (PBPs). The PBPs are transpeptidase enzymes required for the terminal stage of the bacterial cell wall synthesis. On average, most bacterial species have at least four PBP's (BUSH and BRADFORD, 2016).

Beta-lactams share a specific core structure consisting of a four-sided β-lactam ring (Figure 2) (BREIJYEH *et al.* 2016; REYGAERT, 2018). The β-lactam group includes penicillin, cephalosporin, monobactam and carbapenem. This classification depends on the chemical nature of the ring attached to the region of the molecule where the receptor binds (pharmacophore). In cephalosporins, the β-lactam ring is attached to a 6-membered sulfur-containing ring, while in penicillin it is attached to a 5-membered sulfur-containing ring. Carbapenems are attached to a cyclopentene ring and monobactams have no ring attached to their β-lactam ring (CHAIN, 1979; SELWYN, 1979).

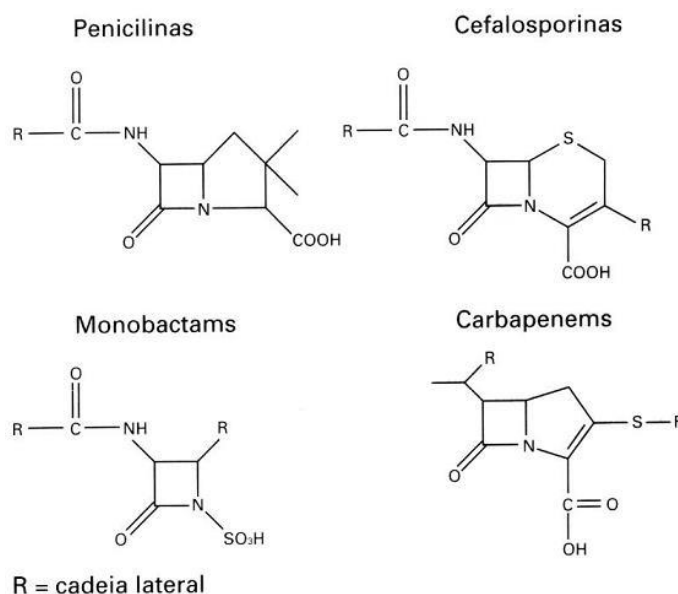

Figure 2. Molecular structure of β-lactam antibiotics. The classification of the β-lactams depends on the chemical structure of the molecule that binds to the β-lactam ring.

The β-lactamases enzymes are grouped into four classes according to the Ambler classification (AMBLER, 1980): β-lactamases class A, β-lactamases class B, β-lactamases class C, and β-lactamases class D. Class A β-lactamases include the Extended Spectrum Beta-lactamase (ESBL), which confer resistance to extended-

spectrum cephalosporins but are sensitive to the action of beta-lactamases inhibitors, such as, clavulanic acid and tazobactam (WELDHAGEN *et al.* 2003). The enzymes in classes A, C and D share similar structures and the presence of a serine residue in their active catalytic site (MUNITA and ARIAS, 2016). Enzymes of the TEM and SHV-types, which hydrolyze penicillin belong to this group. The TEM-type and SHV-type enzymes are widely distributed among members of the *Enterobacteriaceae* family and the genes encoding them have been found in plasmids since the 1970s (MATTHEW, 1979).

Currently, the CTX-M-type is the ESBL most found worldwide (BUSH and BRADFORD, 2019). The enzyme confers resistance to penicillin and cephalosporin and the gene encoding it is associated with insertion sequences or transposons located in plasmids. The enzyme CTX-M-type has been reported in *Escherichia coli*, *Klebsiella pneumoniae,* and other Gram-negative bacteria (DOHMEN *et al.* 2015; ZHAO *et al.* 2020; GOUDARZI and SHAKIB, 2020). The $bla_{\text{CTX-M}}$ gene encodes an enzyme that is highly effective in hydrolyzing cefotaxime, ceftriaxone and aztreonam (POIREL *et al.* 2002; BUSH and BRADFORD, 2019). The $bla_{\text{CTX-M}}$ gene has been widely reported in livestock and in the environment (DAHMS *et al.* 2015; SHIN *et al.* 2017; FURLAN, 2017; FACCONE *et al.* 2019; DEVI *et al.* 2020; SADEK *et al.* 2021).

Other clinically relevant enzymes of class A (GES, PER and VEB), are mostly encoded by mobile genetic elements found among members of the *Enterobacteriaceae* family and other Gram-negative bacteria such as *Acinetobacter* spp. (POTRON *et al.* 2015). KPC-like enzymes are serine carbapenemases, frequently detected in *K. pneumoniae* but also found in species of Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter* spp. and *Salmonella* spp. (KAZMIERCZAK *et al.* 2016). The KPC-type is commonly found in plasmids and has a broad-spectrum activity against all β-lactams but is inhibited by clavulanic acid. KPC enzymes were first reported in 1996 when they were recovered from a clinical sample in the USA from human. However, a growing number of studies report that KPC enzymes are widely spread worldwide being mainly associated with infection outbreaks in hospitals (LAU *et al.* 2014; KOPOTSA *et al.* 2019).

Class B β-lactamases, also called metallo-β-lactamases (MBL), have activity against carbapenems and other β-lactams except for monobactams, and are not inhibited by conventional β-lactamases inhibitors, but by metal ion chelators (WALSH

et al. 2005; NORDMANN *et al.* 2012). Their activity is dependent on the interaction with the zinc ion in their active site rather than a serine residue as in classes A, C and D. Normally, the class B is widely reported in clinical strains of *Enterobacteriaceae* family and *Acinetobacter* spp. (MUNITA and ARIAS, 2016) and are commonly found in plasmids that harbor genes to other antibiotics. Among the acquired MBLs (encoded by genes located on plasmids), IMP, VIM and NDM enzymes show widespread distribution and are detected in several Gram-negative pathogens (KAZMIERCZAK *et al.* 2016). The IMP-type (imipenem-resistance), which was first reported in the 1990s in Asia, is widely reported in lactose and non-lactose fermenting Gram-negative bacteria and is normally present in large plasmids (NORDMANN, 2011; REYGAERT, 2018). VIM-type (Verona integron-encoded metallo-β-lactamase) commonly occurs in *Pseudomonas* spp. and was first discovered in Verona (Italy) in the 1990s. NDM-type (New Delhi-metallo-β-lactamase) was first reported in *K. pneumoniae* and *E. coli* in clinical settings in 2009 (WALSH *et al.* 2011). The NDM enzyme also seems to have worldwide distribution in mobile elements, such as plasmids (CODJOE and DONKOR, 2017). Metallo-β-lactamases enzymes have been reported in the environment and livestock and became a concern due to their potential dissemination to other environments such as hospital settings (ZHU *et al.,* 2019; TEIXEIRA *et al.* 2020; DIACONU *et al.* 2020; CHAUDHRY *et al.* 2020; PAULY *et al.* 2021).

Class C beta-lactamases include AmpC-type enzymes that have cephalosporinase activity (POTRON *et al.* 2015). Many species of Gram-negative bacteria expressed chromosomal β-lactamases and are normally found in clinical isolates, such as *Enterobacter* spp., *Serratia marcescens*, *P. aeruginosa*, *Acinetobacter baumannii* have the gene that encodes this AmpC enzyme, however, is expressed at a low level but can be induced with exposure to beta-lactams, being active on pecinilins and cephalosporins (JACOBY, 2009; BUSH and BRADFORD, 2019). CMY-type are expressed from genes located in plasmids and confer resistance to cephalosporins. This CMY-type enzyme are known as cephamycinases due to their activity hydrolyzing cephalosporins (PHILIPPON *et al.* 2002; JACOBY, 2009).

Finally, class D β-lactamases, known as oxacillinases (OXA), hydrolyze penicillin and some extended-spectrum cephalosporin and have intrinsic activity against carbapenems (POIREL *et al.* 2007; POIREL *et al.* 2010). The OXA-48 enzyme is broadly spread and was recovered in 2001 from a *K. pneumonia* isolate in Turkey.

Since then, many other types of OXA showing a variety of hydrolytic profiles have been characterized, and the genes are mainly encoded in plasmids (MUNITA and ARIAS, 2016).

Regarding the carbapenems, the main mechanism of bacterial resistance is the production of carbapenemase (FISCHER *et al.* 2017). Most of the carbapenem-resistance genes are located in mobile genetic elements that can facilitate the horizontal transfer of genes among potential pathogens and commensal bacteria, representing a potential threat to public health (NORDMANN *et al.* 2012). However, the production of carbapenemase is not the only mechanism of resistance to carbapenems. Alterations in the permeability of the cell membrane or outer membrane that limit the diffusion of antibiotics to the cytoplasm can also trigger resistance to carbapenems or decrease the susceptibility of target bacteria (EFSA, 2013; GUERRA *et al*. 2014). Resistance to carbapenems can trigger resistance to other beta-lactam antibiotics, making them ineffective. As such, co-selection can arise from the accumulation of combined resistances to several classes of antibiotics or different molecules of the same class (TAL-JASPER *et al.* 2016; OLIVEIRA *et al.* 2020).

Different mechanisms explaining the co-selection phenotype have been described in the scientific literature, including co-resistance and cross-resistance. Co-resistance occurs when there different resistance determinants are encoded in the same mobile genetic element (e.g., transposon, plasmid), or when mutations conferring resistance to antibiotics are acquired in different genetic loci, thus providing advantages to the bacteria under selection pressure. Likewise, cross-resistance occurs when a resistance mechanism mediated by the acquisition of resistance genes or mutations confers resistance to different molecules belonging to the same class of antibiotics. However, the resistance may involve different classes of antimicrobial agents, as a result of mutations that render an efflux pump with a broad range of activity (CANTÓN and RUIZ-GARBAJOSA, 2011).

Thus, the use of an antibiotic of a class can trigger the co-selection of resistance affecting molecules of the same class or molecules with distinct mechanisms of action (PAL *et al.* 2017). Pauly *et al*. (2021) characterized a carbapenem-resistant *Escherichia coli* harboring an IncHI2 plasmid harboring the bla_{VIM-1}, bla_{SHV-12} and bla_{ACC-1} genes. This was the first report of such plasmid in bacterial isolates of animal origin, indicating that further studies are needed to improve the understanding of

transmission routes, gene dissemination and factors contributing to the persistence of these plasmids in livestock. Studies by Singh *et al.* (2020), in turn, reported isolates of *E. coli*, *Klebsiella* spp, *Stenotrophomonas maltophilia* and *Ochrobactrum anthropi* recovered from retail goat meat, carrying genes encoding ESBL and carbapenemases and showing high MIC values to meropenem, imipenem, cefotaxime, and ceftazidime. The *S. maltophilia* and *O. anthropi* isolates co-harbored the bla_{TEM}, bla_{SHV} and bla_{NDM} genes and showed resistance to four antibiotics tested in their study.

Co-selection of antibiotic resistance in bacteria isolated from commercial chicken has also been reported. Xiang *et al.* 2018, reported a strain of *K. pneumoniae* isolated from a commercial chicken farm in China that co-harbored *mcr-3* and bla_{NDM-5} in plasmids of different sizes. In addition, the strain carried 33 resistance genes to different classes of antibiotics (aminoglycosides, beta-lactams, quinolones, and phenicols). In a study carried out in Tunisia, Dhaouadi *et al.* (2020) reported *E. coli* isolates recovered from broiler chickens that were resistant to four antibiotic classes or more and co-harbored the bla_{CTX-M}, bla_{TEM}, bla_{SHV} genes. The *mcr-1*, *tetA*, *floR* and *sul1* genes were also found in some isolates that harbored the genes mentioned above, suggesting the co-selection of resistance.

The increasing detection of these resistance genes in isolates of clinical and veterinary origin is a matter of concern for scientists seeking to develop faster and more effective methods to monitor the resistance phenotypes. Typically, phenotypic tests are used for rapid detection of bacterial antibiotic resistance in hospital environments and routine laboratories. Some standardized tests to detect beta-lactamases are recommended by BrCAST, CLSI, and EUCAST. These tests include the combined disc test, in which cefotaxime, ceftazidime, and cefepime discs are used alone and combined with clavulanic acid. In the double disc synergism test, the cephalosporin discs (cefotaxime, ceftazidime, and cefepime) are applied to the plate next to the clavulanic acid disc (BrCAST, 2018).

Carbapenemase-producing strains can also be detected with commercial colorimetric tests (e.g., CARBA NP), which are based on a color change reaction according to the pH of the solution. The carbapenem inactivation test (CIM) and its modifications (mCIM), are based on a disk assay that allows the detection of meropenem degradation by the tested strain. The meropenem disc is incubated in water (CIM) or TSB medium (mCIM) for 2-4 h with the bacterial inoculum and placed

on a plate containing an indicator microorganism susceptible to meropenem (*E. coli* ATCC 25922). The result is evaluated according to the diameter of the inhibition halo. Detection of carbapenemase can also be performed using mass spectrophotometry (MALDI-TOF), where the result is based on the reduction or disappearance of certain carbapenem peaks in isolates previously incubated with carbapenems. However, despite having good specificity and sensitivity, this methodology requires expensive pieces of equipment and has not yet been tested and confirmed on a large scale (LASSERRE *et al.,* 2015; BRCAST, 2018).

Given the public health concern and current reports of resistance in animals and livestock, it is important to investigate the mechanism of resistance between these environments and detect related genetic determinants to improve our understanding of the routes that mediate the spreading of antibiotic-resistance genes.

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1. CHAPTER 2

1.1. Introduction

The use and misuse of antibiotics in animal production systems as feed additives or therapeutic agents are considered a matter of public health concern worldwide, due to the increasing evidence of direct or indirect transmission of multidrug-resistant bacteria from livestock animals to humans (MANYI-LOH *et al.* 2018; SCHMITHAUSEN *et al.* 2018). Both commensal and pathogenic bacteria carrying resistance determinants can contaminate products of animal origin consumed by humans or spread into the environment through animal waste and wastewater from livestock production units (SCHMITHAUSEN *et al.* 2015; OLIVEIRA *et al.* 2020).

Some antibiotics of veterinary importance (e.g., ceftiofur) belong to the same class and share similar mechanisms of action of antimicrobials used for clinical practice, such as the carbapenems (SAIFUL *et al.* 2016; MANYI-LOH *et al.* 2018). Co-selection of antibiotic resistance genes in microbial communities can lead to phenotypes of co- and cross-resistance among bacterial species (PERICHON, 2015; OLIVEIRA *et al.* 2020). Isolates showing co-resistance phenotypes often contain genes located in the same mobile genetic element (e.g., plasmids) or in different loci in the chromosome that confer resistance to more than one class of antibiotics. Cross-resistance occurs when a resistance mechanism protects the bacteria from different antimicrobial agents or classes of molecules that share similarities in their structures or mechanism of action (CANTÓN, 2011).

Previous studies hypothesized that the use of cephalosporins in livestock represents a selective pressure for the emergence and persistence of resistance to

carbapenems and other classes of antibiotics (JØRGENSEN *et al.* 2007; AGERSØ *et al.* 2012; AGERSØ, 2013; MOLLENKOPF *et al.* 2017; ABRAHAM *et al.* 2018). Carbapenems and cephalosporins are β-lactam antibiotics that can bind to penicillin-binding proteins and inhibit bacterial cell wall synthesis (WAXMAN *et al.* 1980). The World Health Organisation (WHO) considers these classes of antibiotics as critically important for human medicine (WHO, 2019). Therefore, prudent use of cephalosporins is licensed in livestock animals, whereas carbapenems are not licensed for use in veterinary medicine (WHO, 2018). Furthermore, genes that confer resistance to cephalosporins seem to spread by horizontal transfer among enterobacteria isolated from pigs, but some reports also indicate the presence of these genes in identical bacterial clones isolated on the same farm in different years, suggesting the persistence of cephalosporin-resistance in the production unit over the years. (JAHANBAKHSH *et al.* 2016). Nonetheless, little is known about the prevalence of cephalosporins and carbapenem resistance in pig production systems without the routine use of cephalosporins.

The bacterial resistance to β-lactams (cephalosporin, carbapenem, monobactam, and penicillin) is often mediated by the expression of β-lactamases enzymes encoded by genes of the *bla* family. These enzymes are divided into classes A, B, C, or D according to their molecular structure and/or functional properties (PARTRIDGE, 2015; REYGAERT, 2018). Class A includes enzymes of the groups TEM-type and SHV-type that confer resistance to penicillin, CTX-M-type that confers resistance to extended-spectrum β-lactams (e.g. cephalosporins), and KPC-type that confers resistance to carbapenems. The Class B enzymes depend on zinc ions for their activity and are classified as metallo-β-lactamases while other classes of enzymes contain a serine residue in their active site (CORNAGLIA, 2011). The enzymes encoded by the genes bla_{IMP}, bla_{VIM}, and bla_{NDM} are considered the most clinically important from this group given their worldwide distribution (MUNITA and ARIAS, 2016). The Class C enzymes are found in many species of *Enterobacteriaceae* conferring resistance to β-lactamase inhibitors such as clavulanic acid, as well as cephalosporins. The most clinically relevant enzyme, AmpC, is chromosomally encoded (JACOBY, 2009; PARTRIDGE, 2015). Finally, class D enzymes (OXA), known as oxacillinase, differ from class A due to their ability to hydrolyze oxacillin. These enzymes can degrade cephalosporins and carbapenems (EVANS, 2014), and

the resistance genes encoding them are usually located in mobile transposable elements, such as plasmids and transposons.

Plasmids play a major role in horizontal gene transfer (HGT) and the spread of resistance genes among bacterial species and between the components of the one health triad (human, animal, and the environment). Elnekave *et al.* (2019) reported the presence of plasmid-borne ESBL genes ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}27}$, and $bla_{CMY\text{-}2}$) in bacteria isolated from pig samples that showed high MIC values to ceftiofur. Moreover, Zelendova *et al.* (2019) demonstrated that multidrug-resistant *E. coli* isolates obtained from pig samples in the Czech Republic carried the $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}15}$ genes. More recently, Irrgang *et al.* (2020) reported that an *E. coli* strain recovered from swine feces showed a phenotype of resistance to beta-lactams, including the carbapenems, and harbored a plasmid carrying the $bla_{OXA\text{-}48}$ gene.

Therefore, investigating the prevalence of extended-spectrum cephalosporin-resistant bacteria in livestock is relevant to understanding the pathways driving the selection of ESBL as well as developing strategies to maintain the therapeutic efficacy of antibiotics against multidrug-resistant bacteria. Here we hypothesize that genetic determinants conferring resistance to cephalosporins and carbapenems are co-selected in *Enterobacteriaceae* and other Gram-negative bacteria from food-producing animals. In this study, we evaluate the co-selection of resistance to ceftiofur, meropenem, and imipenem in bacteria isolated from swine, soil, and wastewater in a pig production system without a history of usage of these antibiotics for therapeutic purposes.

2. Material and Methods

2.1. Sample collection

Animal samples

Animal samples were obtained directly from the rectum of ninety-two commercial hybrid castrated male pigs (DB Genética Suína; Patos de Minas, Brazil) using sterile cotton swabs (Olen/Kasvi, São José dos Pinhais, Brazil). The average age and weight of the animals were 120 days and 77 ± 8 kg at the time of sample collection and the average feed consumption was 2.9 ± 0.3 kg of feed/day. All pigs

were allocated in pens housing four animals per pen and were fed with a diet without antibiotics (CON group, 46 animals) or added with 55 ppm of Zinc Bacitracin and 5 ppm of Enramycin (ATB group, 46 animals). Finishing pigs were kept on these diets for 26 days up to one week before sample collection. The rectal swabs were immediately stored in Stuart medium as recommended by the manufacturer until the samples were taken to the laboratory for bacterial isolation (described below). All the animal handling procedures were carried according to guidelines approved by the Ethics Committee on the Use of Production Animals of the Universidade Federal de Viçosa (CEUAP/UFV, Protocol n° 027/2021).

Soil and wastewater samples

Soil samples (10 g) were collected at depths of 5–10 cm and 10 distinct locations adjacent to the pig pens using sterile conical plastic tubes and tools (hand shovel, mattock, spatula, etc.) cleaned with ethanol 70% for each sampling site. The wastewater from the pig unit was collected in quadruplicate (10 mL) into sterile tubes at sampling points located before the anaerobic digestion. All samples were stored under cooling conditions and transported to the laboratory in less than 2 h for further analysis.

2.2. Screening of ceftiofur-resistant isolates

The presence of Gram-negative bacteria showing resistance to cephalosporins in the swine, soil, and wastewater samples was screened in MacConkey agar selective medium (Kasvi, Conda Laboratories, Spain) containing 8 mg/L ceftiofur (Ceftiomax, Biogénesis Bagó, Buenos Aires, Argentina). As there is not a single ceftiofur concentration that can be used for all Gram-negative bacteria, this study followed the ceftiofur breakpoint value established for *Enterobacteriales* by the Clinical and Laboratory Standards Institute Veterinary Antimicrobial Testing (CLSI VET01, 2020).

Swabs of the swine samples were streaked directly onto the surface of the MacConkey Agar. Soil samples (1 g) were weighted and serially diluted up to 1000-fold into 10 mL of 0.85 % saline solution and spread plated (100 microliters) on MacConkey agar. Similarly, the wastewater samples were plated (100 microliters) without dilution or diluted 10 and 100-fold in 0.85 % saline solution. The plates were incubated under aerobic conditions at 37 °C for 24 h and isolated colonies showing differences in phenotypic traits such as colony size and morphology, and pattern of

lactose and non-fermentation were selected and sub-cultured in the same media to obtain pure cultures. The ceftiofur-resistant isolates were then transferred to Luria Bertani (LB) broth (Kasvi, São José dos Pinhais, Brazil) added with 40% glycerol and stored at -20 °C for further characterization.

2.3. Selection and characterization of carbapenem-resistant isolates

To evaluate if the ceftiofur-resistant isolates also exhibited resistance to carbapenems, the susceptibility of the isolates to meropenem and imipenem (European Pharmacopoeia, Strasbourg, France) was determined. The cultures were initially grown in fresh media for 24 h and then transferred (15 microliters) to a 96-well microplate containing 200 µL of Mueller-Hinton broth (Kasvi, São José dos Pinhais, Brazil). Plates were incubated at 37 °C for 24 h and activated again in the same media. The isolates were then transferred with a colony replicator to a plate containing Mueller-Hinton agar (Kasvi, São José dos Pinhais, Brazil) supplemented with meropenem (8 mg/L) or imipenem (4 mg/L). The method followed the standard breakpoints concentrations established by the Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST, 2021), as carbapenems are not licensed for use in swine, BrCAST was used with the breakpoints for these antibiotics. *Escherichia coli* 25922 was used as negative quality control, following the BrCAST (2021) recommendations.

The carbapenem-resistant isolates were evaluated for the production of carbapenemases both genotypically and phenotypically. The phenotypic characterization of the carbapenem-resistant isolates was performed using the modified carbapenem inactivation method as described by Pierce *et al.* (2017). Each isolate was grown for 18 h and 1 µL of the culture was added into a microtube containing 2 ml of tryptic soy broth (TSB) (Kasvi, São José dos Pinhais, Brazil) and mixed for 15 s. Next, a disk of meropenem (10 µg) (Laborclin, Pinhais, Brazil) was added into the microtube containing the bacterial suspension, and the tube was incubated for 4 h±15 min at 35 °C. To evaluate the inactivation of the antibiotic, a fresh suspension of the susceptible indicator microorganism, *E. coli* ATCC 25922, was prepared in saline solution to a density equivalent to 0.5 of the MacFarland scale and streak plated with a sterile swab (Labor Import, Itajaí, Brazil) in a Mueller-Hinton agar (MHA) plate. The meropenem disk was removed from the microtube with the bacterial

suspension and placed on the surface of the inoculated MHA. The plates were then incubated at 35 °C for 18 to 24 h. The interpretation of the results followed recommendations from Pierce *et al.* (2017) and was based on the diameter of the zone of inhibition around the meropenem disk. Isolates in which the diameter of the zone of inhibition ranged from 6 to 10 mm were classified as carbapenemase producers. Zones of inhibition varying from 11 to 19 mm were considered indeterminate, requiring further tests to confirm the production of carbapenemases, and zones of inhibition ≥ 20 mm were considered as negative (absence of carbapenemase).

For the genotypic characterization, the genes encoding carbapenemases were amplified using one loci or multiple loci as targets by multiplex PCR assay or single PCR. Three separate PCR assays were designed to detect a family of class A carbapenemases (KPC), three families of class B carbapenemases (IMP, VIM, and NDM), and a family of class D carbapenemases (OXA-48). Genes encoding CTX-M-type β-lactamases were also evaluated to assess the association between cephalosporin and carbapenem resistance. The PCR assays were performed with pairs of specific primers targeting fragments of different sizes (Table 1). Approximately 50 ng of DNA was used in the amplification reactions. The amplification reactions for OXA-48 and NDM enzymes were performed separately using the following reagents (final concentration): 2.5 µL of Super Taq Reaction Buffer (1 x), 1 µL dNTP's (100 µM), 1 µL of each primer (0.5 µM), 0.3 µL of Super Taq Pol (1.5 U/µL) (Cellco Biotec, Brazil), 1.25 µL of $MgCl_2$ (2.5 mM) and 0.62 µL of DMSO (5 %).

The conditions for the PCR assays were established according to each primer set used in the assays. The *bla*$_{OXA\text{-}48}$ gene was amplified with an initiation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 10 min. For the *bla*$_{NDM}$ gene, the conditions were: initiation step of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension of 72 °C for 10 min. A multiplex PCR assay was performed for the detection of *bla*$_{KPC}$, *bla*$_{VIM}$, and *bla*$_{IMP}$ genes. The amplification reaction contained (final concentration): 2.5 µL of Taq Reaction Buffer complete (1 x), 1.5 µL of dNTP (100 µM), 1 µL of IMP and KPC primers (10 µM), 0.75 µL of VIM primer (10 µM) and 0.3 µL of Taq Pol (1.5 U/µL) (Cellco Biotec, São Carlos, Brazil). The PCR assay conditions were as follows: initiation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min, with a final

extension of 72 °C for 8 min. The amplification products were analyzed by electrophoresis in 1.5 % agarose gel.

Table 1. Sequence of primers used for detecting resistance genes encoding carbapenemases and ESBL in Gram-negative bacteria.

Targeted gene	Primer name	Sequence (5' to 3')	Length (pb)	Reference
*bla*VIM	*bla*VIM-F	GTTTGGTCGCATATCGCAAC	389	Doyle *et al*. 2012
	*bla*VIM-R	AATGCGCAGCACCAGGATAG		
*bla*IMP	*bla*IMP-F	GAAGGCGTTTATGTTCATAC	587	Doyle *et al*. 2012
	*bla*IMP-R	GTACGTTTCAAGAGTGATGC		
*bla*NDM-1	*bla*NDM-1-F	TGCATTGATGCTGAGCGGGTG	621	Hou *et al*. 2015
	*bla*NDM-1-R	ATCACGATCATGCTGGCCTTG		
*bla*KPC	*bla*KPC-F	TGTCACTGTATCGCCGTC	900	Doyle *et al*. 2012
	*bla*KPC-R	CTCAGTGCTCTACAGAAAACC		
*bla*OXA-48	*bla*OXA48-F	GCGTGGTTAAGGATGAACAC	438	Nordmann *et al.* 2011
	*bla*OXA48-R	CATCAAGTTCAACCCAACCG		
*bla*CTX-M	*bla*CTX-M-F	CGATGTGCAGTACCAGTAA	585	Batchelor *et al.* 2019
	*bla*CTX-M-R	AGTGACCAGAATCAGCGG		

2.4. Minimum Inhibitory Concentration of resistant isolates

To determine the minimum inhibitory concentration (MIC) of the carbapenem-resistant isolates, the broth microdilution method was performed as recommended by BrCAST (2021). Serial dilutions were performed in 96-well microplates containing 150 µL of Muller Hinton broth and the antibiotic concentrations ranged from 0.125 mg/L to 256 mg/L. The fresh culture used as inoculum was adjusted to a cell density equivalent to 0.5 of the MacFarland scale in saline solution, followed by two serial dilutions (1:10 increments) to obtain a final population of approximately 10^{-6} CFU mL^{-1}. Fifty microliters of the adjusted inoculum were added to each well. *Escherichia coli* 25922 (MIC range 0.008 - 0.06 mg/L) was used as the negative quality control for meropenem as established by BrCAST (2021). *Staphylococcus aureus* 29213 (MIC range 0.015 - 0.06 mg/L) was the quality control used for imipenem, following recommendations from the CLSI VET (2020), since BrCAST has no defined imipenem MIC value for this strain. Interpretive criteria for meropenem (< 2 mg/L, susceptible; between 4 - 8 mg/L, intermediate; > 8 mg/L, resistant) and imipenem (< 2 mg/L, susceptible; 4 mg/L, intermediate; > 4 mg/L, resistant) were based on breakpoints established by the Brazillian Committee on Antimicrobial Susceptibility Testing (BrCAST) (2021).

The microplates were incubated at 37 °C for 24 h and growth was determined by monitoring the optical density (DO_{600nm}). Cell viability was verified at the end of the incubation with the addition of 30 microliters of 0.1% resazurin to each well (BAKER *et al.* 1994).

2.5. Cephalosporins susceptibility testing

To evaluate the antimicrobial susceptibility of the bacterial isolates to other cephalosporins, the standard disk diffusion method was used according to the Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST, 2021). The following antibiotics were tested (µg/disc): cefazolin (30), cefepime (30), cefotaxime (5), ceftriaxone (30). *E. coli* ATCC 25922 was used as quality control. The tests were performed in triplicate.

2.6. DNA extraction and 16S rRNA sequencing

The resistant isolates (n= 131) were subjected to taxonomic identification by sequencing the 16S rRNA gene, using the total DNA extraction method with phenol-chloroform with modifications (OLIVEIRA *et al.* 2002). The isolates were grown at 37 °C for 24 h in LB broth. The cultures were centrifuged at 10,600 x g for 5 min and the pellet was resuspended in 500 µL TE buffer (10 mM Tris-HCl; pH 8.0, 1 mM EDTA) and centrifuged again. The supernatant was discarded and the pellet was resuspended in 200 µL TE buffer and vortexed. Approximately 5 µL of lysozyme (50 mg/mL) was added and the tubes were incubated at room temperature for 30 min. Then, 100 µL of 10 % SDS and 5 µL of proteinase K (20 mg/mL) were added and the samples were incubated at 55 °C for 30 min. Next, 500 µL of sodium acetate (5 M) and 700 µL of isopropanol were added and centrifuged for 5 min at 10,600 x g. The supernatant was discarded, and the pellet was washed with 70% ethanol. Tubes containing the DNA pellets were allowed to air dry and then the DNA was resuspended in 600 µL of Milli-Q water. Next, 600 µL of phenol-chloroform (1:1), pH 8.0, was added and the samples were incubated on ice for 5 min, followed by centrifugation for 10 min. The aqueous phase (upper phase) was transferred to a new microtube, and the process was repeated. The DNA was precipitated with 200 µL of sodium acetate (5 M) and 600 µL of isopropanol and homogenized. Finally, the samples were incubated at - 20 °C for 30 min and centrifuged for 10 min. The supernatant was discarded and the pellet was washed with 1 ml of cold 70 % ethanol. The DNA pellet was resuspended in 200 µL TE buffer and stored at -20 °C until use.

The amplification reaction was carried out with approximately 50 ng of total DNA and the reaction mixture contained (final concentration): 5 µL of Super *Taq* Reaction Buffer (1 x), 1 µL dNTP's (100 µM), 1.25 µL of each primer (0.5 µM), 0.3 µL of Super Taq Pol (1.5 U/µL) (Cellco Biotec, São Carlos, Brazil), 2.5 µL of $MgCl_2$ (2.5 mM). The following primer pairs targeting the 16S rRNA used were: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (LANE, 1991), with an amplicon size of approximately 1,500 bp. The conditions of the amplification reaction were 95 °C for 3 minutes, followed by 30 cycles of 95 °C for 1 minute, 56 °C for 30 seconds, and 72 °C for 2 minutes, with a final extension step of 72 °C for 5 minutes. The PCR products were purified using the enzyme ExoSap-IT (Cellco Biotec, São Carlos, Brazil) as instructed by the manufacturer. The PCR

products were subjected to Sanger sequencing at the Instituto René Rachou - Fiocruz Minas (Belo Horizonte, Brazil).

2.7. Phylogenetic analysis

The quality of the forward and reverse sequences obtained from each isolate was performed using the SnapGene Viewer 5.2.4 software. The forward and reverse sequences obtained from each isolate were aligned using BLASTn (https://blast.ncbi.nlm.nih.gov/) to generate a consensus sequence. The 16S rRNA consensus sequence from each isolate was analyzed using the Basic Local Alignment Search Tool (BLAST). Sequences were compared with 16S ribosomal RNA sequences of bacteria and archaea deposited in the GenBank sequence database (https://www.ncbi.nlm.nih.gov/) and the Ribosomal Database Project (https://rdp.cme.msu.edu/). Multiple 16S rRNA sequence alignments from isolates that obtained ≥ 97% identity for the reconstruction of the phylogenetic tree using ClustalW (Thompson *et al.* 1994) were performed using the software MEGA 7 (Kumar *et al.* 2016). The maximum likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa *et al.* 1985) was used for the inference of the phylogenetic tree and bootstrapping of 1,000 replicates was applied for determining the reliability of branches/trees. The Interactive Tree of Life (iTol) interface v6 (https://itol.embl.de/) (Letunic and Bork, 2021) was used to generate the output file (.tree).

2.8. Statistical analysis

The experiments were carried out with at least two biological and two technical replicates. Significant differences between the resistance profile to carbapenems across sites, and the number of isolates resistant to carbapenems between sampling sites, were evaluated by the non-parametric Kruskal-Wallis test. To assess whether there was a significant difference between the number of isolates resistant to carbapenems between sites and the number of resistant isolates of cephalosporins in each site, the two-way ANOVA test and the Tukey test for multiple comparisons were applied. Both tests were performed using the GraphPad Prism version 8.4.3 program (GraphPad Software, San Diego, CA). The level of statistical significance of the tests was $P < 0.05$.

3. Results

3.1. Frequencies of bacterial resistance to cephalosporins and carbapenems

The screening of Gram-negative bacteria in selective MacConkey agar medium with established ceftiofur breakpoint resulted in 187 ceftiofur-resistant bacterial isolates. From these, 77 were isolated from swine samples (40 from the ATB group and 37 from the CON group), 58 were from soil and 52 from wastewater. When these ceftiofur-resistant isolates were tested for susceptibility to carbapenems, 131 isolates (70 %) showed resistance to imipenem, and 20 isolates (15 %) were resistant concomitantly to meropenem and imipenem. Twenty-nine isolates (37 %) were obtained from swine samples (12 from the ATB group and 17 from the CON group), 44 isolates from wastewater (84 %) and all the soil isolates were resistant to imipenem. Out of the 20 isolates that showed resistance to meropenem, 17 were soil isolates and 3 were wastewater isolates. All bacterial isolates obtained from swine were susceptible to meropenem.

Analysis of the MIC values of bacterial isolates revealed that 12 soil isolates had high MIC values for imipenem (ranging from 128 - 256 mg/L). However, the majority (77.5 %) of the soil isolates had MIC values that were below the resistance threshold for imipenem (between 0.25 - 4 mg/L). The dispersion of MIC values to imipenem among isolates from swine samples was lower compared to the bacteria from other sites (Figure 1). Overall, the frequency of isolates resistant to imipenem was highest in swine (37.7 %) followed by soil (22.4 %) and wastewater (5.8 %) (Table 2). Twenty-nine isolates from swine were resistant to imipenem (MIC > 4 mg/L) (Figure 2A). In contrast, assays evaluating the susceptibility to meropenem revealed only 17 soil isolates (29.6 %) that were resistant to this antibiotic. All other isolates were classified either as susceptible (MIC < 2 mg/L) or intermediate resistant (MIC between 4-8 mg/L) to meropenem (Figure 2B).

Table 2. Number and frequency of resistant bacterial isolates from different sites showing resistance to carbapenems.

Antibiotic	Sampling sites			P-value (P <0.05)
	Swine	Soil	Wastewater	

Meropenem	0 (0 %)	17 (29.3 %)	0 (0 %)	0.0161
Imipenem	29 (37.7 %)	13 (22.4 %)	3 (5.8 %)	0.7841

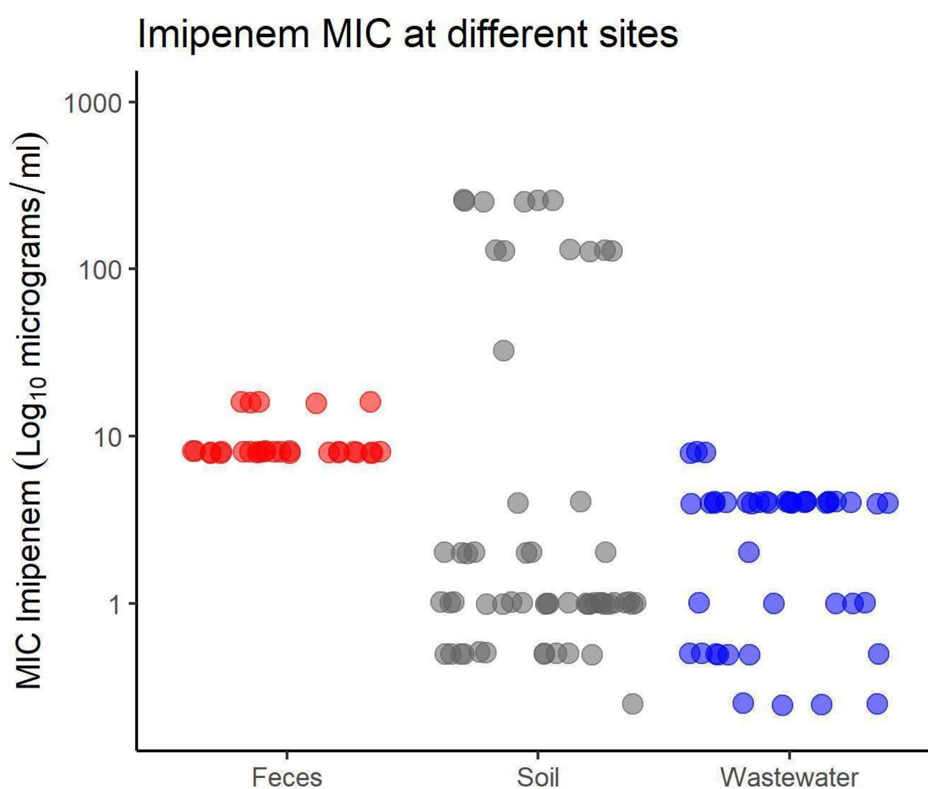

Figure 1. Minimum inhibitory concentration (MIC) to imipenem in bacterial isolates from different sites. MIC was evaluated by the broth microdilution method in Muller-Hinton broth following standard procedures (BrCAST, 2021). The colored dots represent the distribution of indivivual bacterial isolates at each site according to their MIC values, where red dots are from swine, gray dots are from soil and blue dots from wastewater.

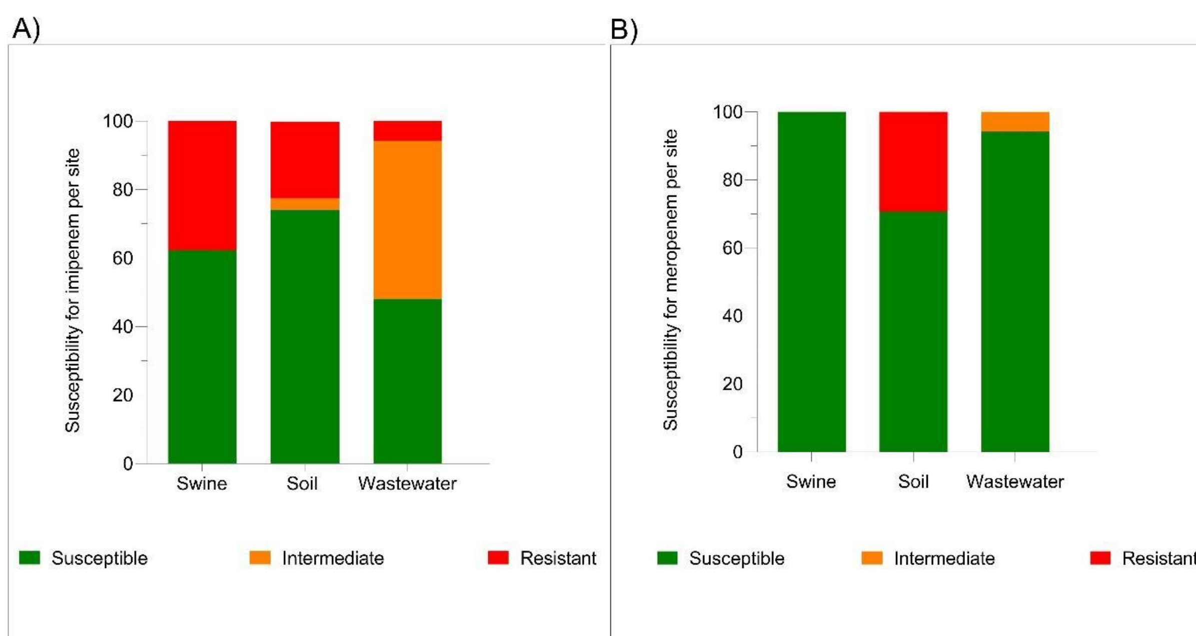

Figure 2. Frequency of resistance to carbapenems (A) imipenem, (B) meropenem among bacterial isolates obtained from different isolation sites, feces, soil and wastewater. Bacterial isolates were classified based on the interpretive criteria established by BrCAST (2021). The results obtained show that meropenem was more effective than imipenem against isolates.

The susceptibility profiles among the soil isolates indicated that five isolates were resistant to all antibiotics tested (Figure 3). Among these, three isolates belonged to the genus *Klebsiella*, one was classified as *Pseudomonas* and the other as *Stenotrophomonas,* identified through RDP and BLASTn. These isolates showed high MIC values for carbapenems (ranging from 32 to 256 mg/L). One isolate classified as *Pseudomonas putida* (S8.5) harbored the *bla*$_{VIM}$ gene and was susceptible only to meropenem. Thirteen isolates from soil belonging to the genera *Klebsiella* spp., *Pseudomonas* spp. and *Enterobacter* spp. were susceptible to carbapenems, but showed resistance to the four cephalosporins tested.

The susceptibility profile of the swine isolates to carbapenems indicated that all isolates (n = 29) were resistant to imipenem, but not to meropenem. Among the resistant isolates, 22 were resistant to all tested cephalosporins. One isolate classified as *Shigella flexneri* (F2.234) was resistant only to imipenem and ceftriaxone. Four isolates classified as *Shigella flexneri* were susceptible only to meropenem and cefotaxime (Figure 4). Three isolates from wastewater were resistant to imipenem and all the tested cephalosporins. These isolates were classified as members of the genera

Enterobacter (n = 1) and *Escherichia / Shigella* (n = 2). Furthermore, it was observed that 37 isolates from wastewater that were susceptible to imipenem showed resistance to all cephalosporins tested in the present study (Figure 5). One isolate from wastewater classified as *Arcobacter cloacae* (W3.20) showing resistance to cefepime was susceptible to all the other antibiotics tested.

On average, more than 90 % of the carbapenem-resistant isolates from pig feces and wastewater and > 81 % of the isolates from soil were also resistant to at least one of the cephalosporins evaluated, including cefazolin (CFZ, first-generation), cefotaxime (CTX, third generation), ceftriaxone (CRO, third generation), and cefepime (FEP, fourth generation) (Table 3). In general, there was a significant difference (P < 0.05) for the frequency of resistant isolates between sites, but not between the cephalosporins tested (P > 0.05). Multiple comparisons (Tukey's test) showed that there was a significant difference among the frequencies of isolates from feces and soil for all cephalosporins tested (P < 0.05). However, there was no difference between the frequencies of resistant isolates obtained from other sites.

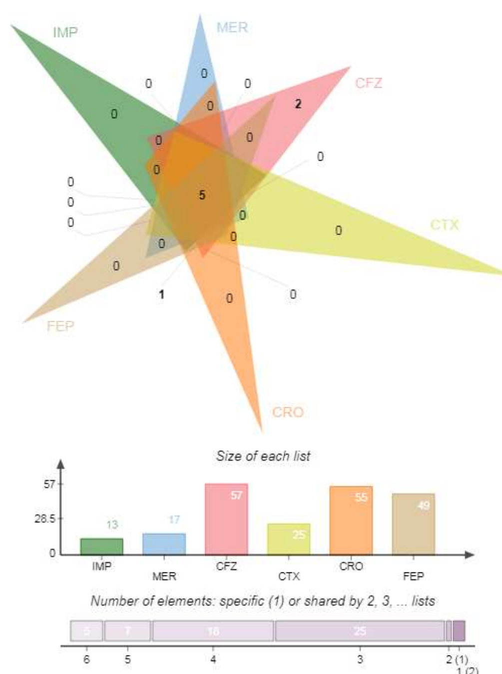

Figure 3. Venn diagram showing the number of isolates with multiple resistances to carbapenem antibiotics and cephalosporins in soil isolates. IMP: Imipenem; MER: meropenem; CFZ: cefazolin; CTX: cefotaxime; CRO: ceftriaxone and FEP: cefepime. In this Venn diagram, only isolates that were resistant to all six antibiotics are shown in the central part of the figure.

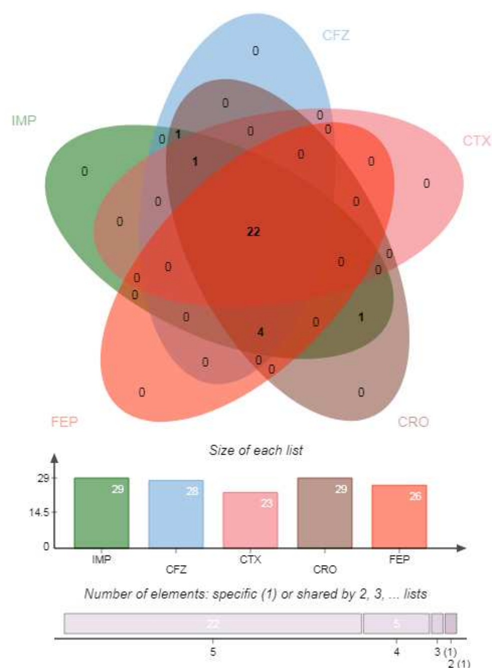

Figure 4. Venn diagram showing the number of isolates with multiple resistances to carbapenem antibiotics and cephalosporins in swine isolates. IMP: Imipenem; MER: meropenem; CFZ: cefazolin; CTX: cefotaxime; CRO: ceftriaxone and FEP: cefepime.

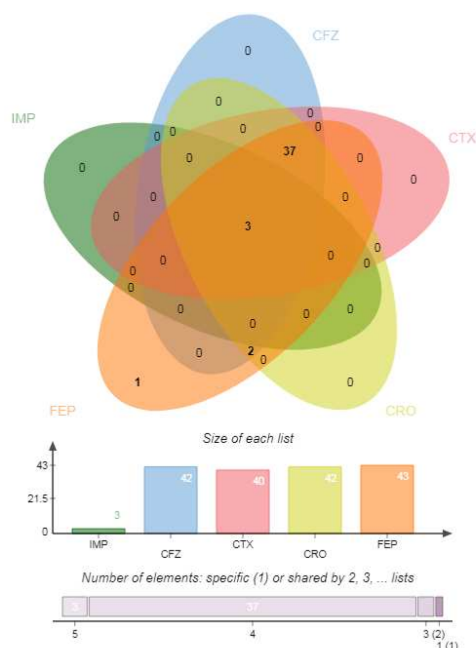

Figure 5. Venn diagram showing the number of isolates with multiple resistances to carbapenem antibiotics and cephalosporins in swine isolates. IMP: Imipenem; MER: meropenem; CFZ: cefazolin; CTX: cefotaxime; CRO: ceftriaxone and FEP: cefepime.

Table 3. Number and frequency (%) of carbapenem-resistant bacterial isolates from different sites also showing resistance to different cephalosporins.

Antibiotic	Cephalosporin classification	Zone diameter for resistance (mm)	Sampling sites			P-value (P <0.05)
			Feces	Soil	Wastewater	
Cefazolin	First generation	< 20	28 (96.6 %)	58 (100 %)	42 (95.5 %)	0.1913
Cefotaxime	Third generation	< 17	23 (79.3 %)	26 (44.8 %)	40 (90.9 %)	0.1773
Ceftriaxone	Third generation	< 22	29 (100 %)	55 (94.8 %)	42 (95.5 %)	0.1856
Cefepime	Fourth generation	< 24	26 (89.7 %)	49 (84.5 %)	42 (95.5 %)	0.0863
Average frequency of resistant isolates			91.4 %	81.0 %	94.4 %	

3.2. Identification and characterization of resistant isolates

Phylogenetic analyses of 16S rRNA sequences revealed that all fecal isolates belonged to the genus *Escherichia/Shigella* (Figure 6). Among these, *Shigella flexneri* (69 %, n = 20) was the most abundant species, followed by *Escherichia albertii* (n = 3), *Shigella sonnei* (n = 3), *Atlantibacter hermannii* (n = 2) and *Escherichia marmotae* (n = 1). Analysis of the bacterial diversity across sampling sites indicated that fecal samples were less diverse (Shannon index = 1.026) compared to soil (Shannon index = 2.097) and wastewater (Shannon index = 2.311) ($P < 0.05$).

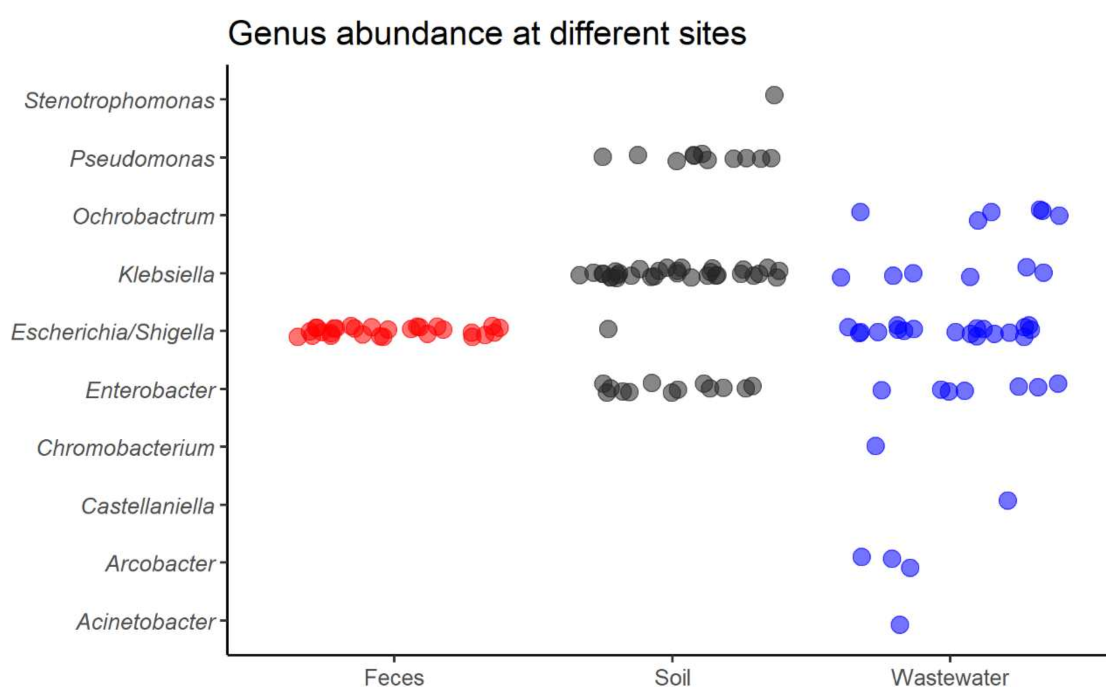

Figure 6. Abundance at genus level of the fecal, soil, and wastewater carbapenem-resistant bacterial isolates. *Escherichia* / *Shigella* was the most abundant genus in fecal and wastewater isolates, while *Klebsiella* was the most abundant genus in soil isolates.

Among the soil isolates *Klebsiella* (55 %, n = 32) was the most frequent genus, followed by *Enterobacter* (n = 13), *Pseudomonas* (n = 11), *Escherichia/Shigella* (n = 1) and *Stenotrophomonas* (n = 1). For the genera *Klebsiella, Klebsiella quasipneumoniae* subsp*. similipneumoniae* (n = 18) and *Klebsiella pneumoniae* (n = 14) were the most abundant species, followed by *P. putida* (n = 7) and *Enterobacter bugandensis* (n = 6). Other species were found in lower frequency including

Pseudomonas fulva (n = 3), *Enterobacter cancerogenus* (n = 3), *Enterobacter ludwigii* (n = 2), *Enterobacter cloacae* (n = 2), *Pseudomonas monteilii*, *S. flexneri* and *S. maltophilia* (one isolate of each) (Figure 7).

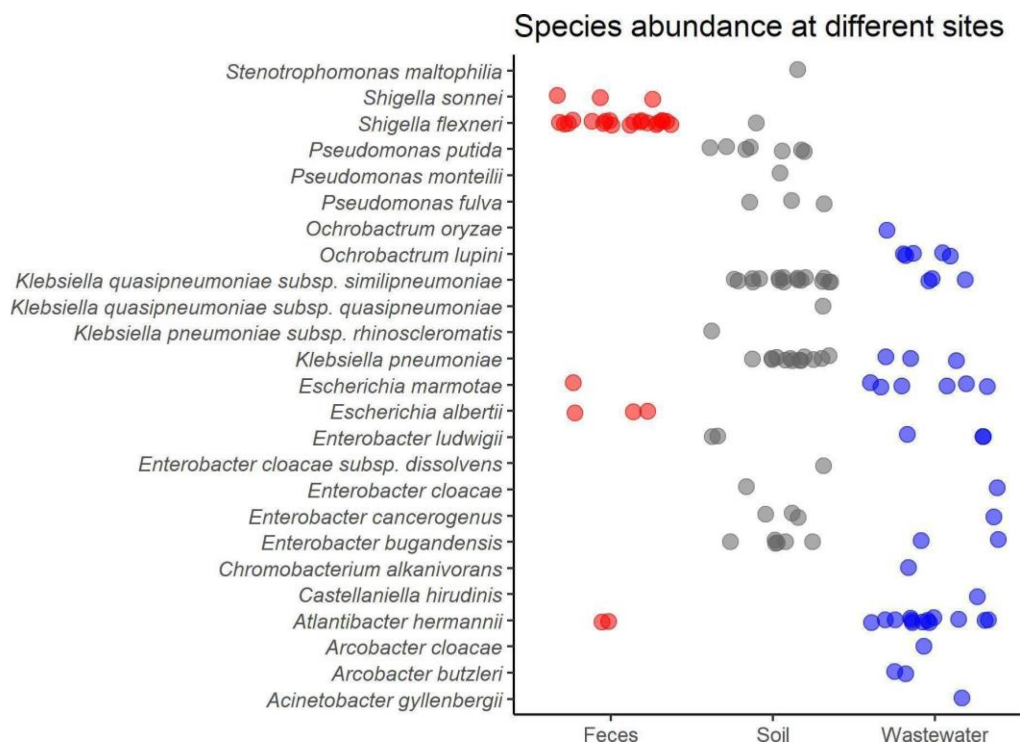

Figure 7. Abundance at the species level in the fecal, soil, and wastewater carbapenem-resistant bacterial isolates. *Shigella flexneri* was the most prevalent species in fecal isolates, while *K. quasipneumoniae* subsp. *similipneumoniae* was the most prevalent in soil isolates and *A. hermannii* species in wastewater isolates.

In wastewater, *Escherichia / Shigella* (43 %, n = 19) was the most abundant genus, followed by *Enterobacter* (n = 7), *Klebsiella* (n = 6), *Ochrobactrum* (n = 6), and *Arcobacter* (n = 3) in addition to *Acinetobacter, Castellaniella* and *Chromobacterium* with one isolate from each genera (Figure 4). *A. hermannii* (n = 13) was the most frequent bacterial species in wastewater samples. However, other species were also isolated from wastewater, including *E. marmotae* (n = 6), *Ochrobactrum lupini* (n = 5), *K. quasipneumoniae* subsp. *similipneumoniae* (n = 3), *K. pneumoniae* (n = 3), *E. ludwigii* (n = 3), *E. bugadensis* (n = 2), *Arcobacter butzleri* (n = 2) and *E. cancerogenus, E. cloacae, Chromobacterium alkanivorans, Ochrobactrum oryzae, Castellaniella hirudinis, Arcobacter cloacae* and *Acinetobacter gyllenbergii* with one isolate each (Figure 5).

PCR-screening of the bla_{IMP} and bla_{VIM} (class B metallo-beta-lactamase), and the bla_{CTX-M} (extended-spectrum beta-lactamase) genes demonstrated a high frequency of *Escherichia/Shigella* isolates from fecal (82 %, n = 24) and wastewater (43 %, n = 19) samples harboring resistance determinants. The bla_{CTX-M} gene was the gene found with higher frequency among the swine isolates (76 %, n = 22), with a higher prevalence in *Shigella*. Although the bla_{VIM} gene was found less frequently (34 %, n = 10) (Table 4), at least eight fecal isolates resistant to imipenem co-harbored both resistance genes, and six of them were also resistant to all the tested cephalosporins. Nonetheless, when these isolates were subjected to a phenotypic test to evaluate the inactivation of carbapenems, all the results turned indeterminate.

Table 4. Number, frequency and co-occurrence of resistance genes found in pig, soil and wastewater isolates.

Resistance gene	Swine	Soil	Wastewater
bla_{VIM}	10 (34 %)	38 (65 %)	31 (70 %)
bla_{CTX-M}	22 (76 %)	34 (59 %)	18 (41 %)
bla_{IMP}	0 (0%)	3 (5 %)	0 (0 %)
bla_{VIM} + bla_{CTX-M}	8 (28 %)	21 (36 %)	14 (32 %)
bla_{VIM} + bla_{IMP}	0 (0 %)	3 (5 %)	0 (0 %)
bla_{CTX-M} + bla_{IMP}	0 (0%)	2 (3 %)	0 (0 %)

Among the soil isolates, the resistance genes were mostly found in isolates of the genus *Klebsiella* (n = 29), with a prevalence of the bla_{VIM} gene (34 %, n = 20 isolates) followed by bla_{CTX-M} (32 %, n = 19 isolates). Two *Klebsiella* isolates (S10.3 and S10.4) carrying the bla_{CTX-M} gene were resistant to all six antibiotics tested, showing high MIC values for imipenem (128 mg/L) and meropenem (64 mg/L). Eighteen *Klebsiella* isolates harboring the bla_{CTX-M} gene were resistant to at least three of the tested cephalosporins, and six of these were also resistant to meropenem. Six *Enterobacter* isolates carried bla_{VIM} and bla_{CTX-M}. Despite being susceptible to carbapenems, these six *Enterobacter* isolates were resistant to at least three of the cephalosporins tested. Two of these isolates classified as *E. bugadensis* and *E.*

cancerogenus, also co-harbored the bla_{IMP} gene. One *Enterobacter* isolate (S2.2) co-harbored the bla_{VIM} and bla_{IMP} genes. One isolate classified as *P. putida* (S5.6) was resistant only to cefazolin and harbored the bla_{VIM} gene. Overall, the bla_{VIM} gene was found in 38 soil isolates, being the most frequent resistance gene found among isolates from this site (Figure 8).

According to the phenotypic test (mCIM), twenty-one soil isolates were considered non-carbapenemase producers, while all the other isolates showed indeterminate resistance, requiring further tests for confirmation. Twenty-two isolates from soil harbored at least two genes and despite being susceptible to carbapenems, 20 isolates were resistant to three or more cephalosporins tested.

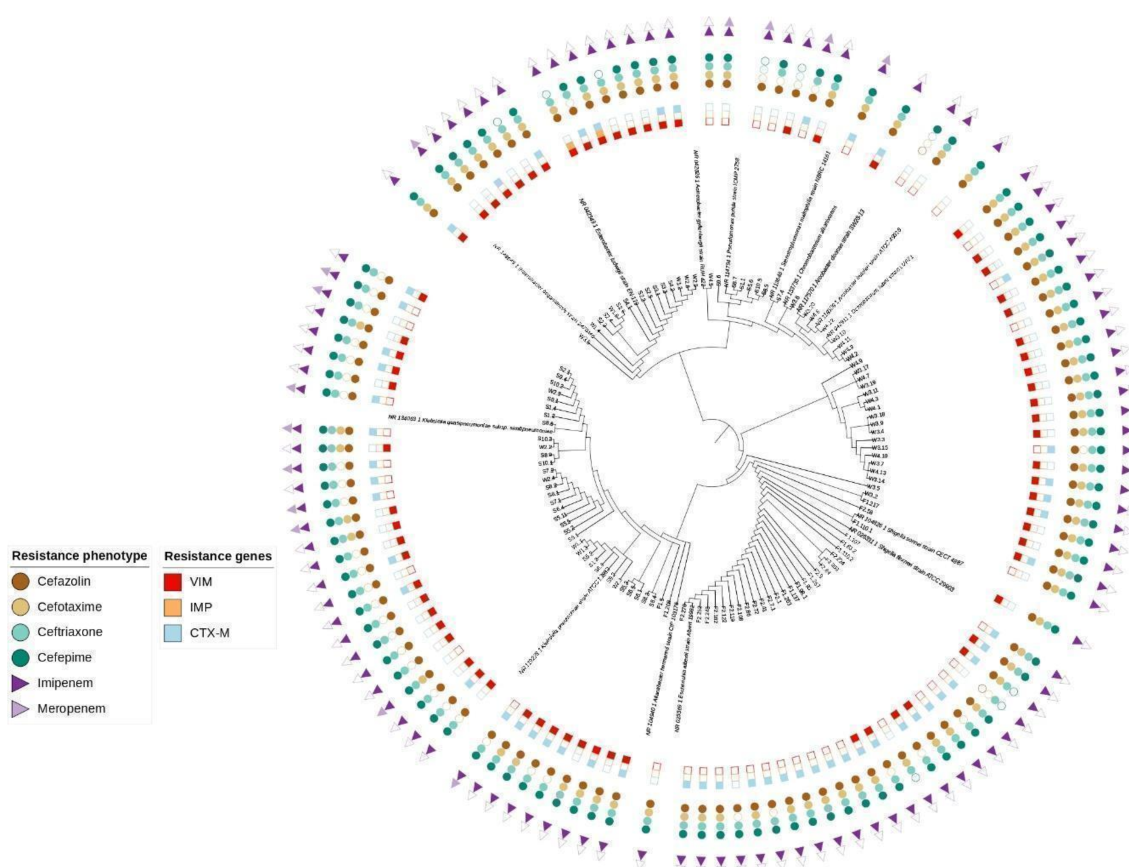

Figure 8. Phylogenetic tree of Gram-negative isolates from swine, soil, and wastewater samples that obtained ≥ 97% identity, and their respective phenotypes and resistance genotypes. The phylogenetic tree was generated with the maximum likelihood method based on the Hasegawa-Kishino-Yano model, using MEGA 7 (1000 replicates), and visualized and annotated using iTOL.

Thirty-five wastewater isolates harbored at least one resistance gene. The resistance genes were detected in nineteen *Escherichia / Shigella* isolates. Eight

Escherichia / Shigella isolates also carried the *bla*$_{VIM}$ and *bla*$_{CTX-M}$ genes and were resistant to all cephalosporins tested, with two also showing resistance to imipenem. Two *Escherichia / Shigella* isolates (W3.12 and W3.18) harboring *bla*$_{VIM}$ showed a positive result to carbapenemase production. However, these isolates were susceptible to carbapenems, although they showed resistance to all cephalosporins tested. Two isolates from the genus *Klebsiella* and three from the genus *Enterobacter* that co-harbored the *bla*$_{VIM}$ and *bla*$_{CTX-M}$ genes were also resistant to all cephalosporins. One *Enterobacter* isolate (W2.6) was also resistant to imipenem. Another *Enterobacter* isolate (W3.6) from wastewater that co-harbored the *bla*$_{CTX-M}$ and *bla*$_{VIM}$ genes showed a positive result for carbapenemase production, even though it was not resistant to carbapenems.

Overall, the *bla*$_{VIM}$ gene was found in 31 isolates from wastewater, and the *bla*$_{CTX-M}$ was found in 18 isolates. Contingency table analysis indicated that the probability of the bacterial isolates to carry the *bla*$_{CTX-M}$ gene was 54 %, while the probability of an isolate to carry *bla*$_{CTX-M}$ and *bla*$_{VIM}$ simultaneously was 95 % (Table 5). In addition, our data suggest a low probability (4 %) of the *bla*$_{IMP}$ gene appearing simultaneously with the *bla*$_{CTX-M}$ gene. The *bla*$_{CTX-M}$ gene (cefotaximase) was found in high frequency across isolation sites, showing highest abundance among the bacterial isolates from the soil, which may explain the high levels of resistance to cephalosporins in this study.

Table 5. Contingency and probability table showing the number and frequency of isolates presenting the *bla*$_{VIM}$ and *bla*$_{IMP}$ genes simultaneously with the *bla*$_{CTX-M}$ gene.

ESBL Gene	*bla*IMP	*bla*VIM	Total
Non-*bla*CTX-M	1	36	37
*bla*CTX-M	2 (4 %)	43 (95 %)	45 (45 %)
Total	3	79	82

Contingency table analysis (Table 6) also indicated that the probability of isolates carrying the *bla*$_{CTX-M}$ and resistance to imipenem or meropenem was 43 % and

16 %, respectively. Nonetheless, other carbapenemases genes from class A (*bla*$_{KPC}$), B (*bla*$_{NDM}$) and D (*bla*$_{OXA-48}$) were not found among the bacterial isolates evaluated.

Table 6. Contingency and probability table demonstrating the number and frequency of isolates resistant to carbapenem (imipenem and meropenem) and the presence or absence of the *bla*$_{CTX-M}$ gene.

Carbapenem antibiotic	***bla*CTX-M**			**Non - *bla*CTX-M**		
	Resistance	Susceptible	Total	Resistance	Susceptible	Total
Imipenem	32 (43 %)	42	74	13	44	57
Meropenem	12 (16 %)	62	74	5	52	57

The phenotypic test (modified carbapenem inactivation method) showed that three wastewater isolates carrying the *bla*$_{VIM}$ gene could also produce carbapenemase, even though they showed intermediate resistance to imipenem and were susceptible to meropenem. Ten soil isolates showed negative results to carbapenemase production but carried carbapenem resistance genes. Analysis of the zone of inhibition for the other isolates from all isolation sites showed indeterminate results, requiring further tests to confirm if these microorganisms are potential carbapenemase producers.

4. Discussion

In recent years, there have been increasing reports of bacterial resistance to β-lactams, becoming a major public health concern because this class contains antibiotics considered the last source for the treatment of multidrug-resistant infections, such as carbapenems (HAWKEY and LIVERMORE, 2012; EL-GAMAL *et al.* 2017). Although β-lactam-resistant bacteria are frequently found among clinical isolates, increased detection of β-lactam resistance genes has also been observed in bacteria isolated from farm animals and the environments near the production unit (CHIKA *et*

al. 2017; MOBASSERI *et al.* 2019; TEIXEIRA *et al.* 2020; PAULY *et al.* 2021). However, little is known about the ability of some resistance genes to spread and the possible co-selection of resistance between different groups of antibiotics.

In this study, we investigated the occurrence of resistance to carbapenems (meropenem and imipenem) and 1st (cefazolin), 3rd (ceftiofur, ceftriaxone, cefotaxime), and 4th (cefepime) generation cephalosporins among Gram-negative bacteria isolated from swine in the finishing phase, as well as from soil and wastewater from the production unit. Our results showed that although the animals were not treated with these antibiotics, the majority (85 %) of bacterial isolates obtained from the three environments carried at least one resistance determinant and 74 % of isolates (n = 98) were resistant to four or more antibiotics tested. In addition, 3.8 % (n = 5) of the isolates obtained from soil were resistant to all 6 antibiotics tested.

Although the sampled animals were fed a diet that contained enramycin (5 ppm) and zinc bacitracin (55 ppm), there is no evidence that these antibiotics contribute to the selection of cephalosporins and carbapenems tested in the present work. Enramycin and zinc bacitracin belong to the polypeptide class and inhibit the synthesis of the bacterial cell wall showing activity primarily against Gram-positive bacteria (SMITH and WEINBERG, 1962; CALVO and MARTÍNEZ, 2009; BELOTE *et al.* 2018). Moreover, enramycin is not licensed for therapeutic use in humans or veterinary medicine. However, according to Technical Note No. 9/2018, enramycin can be used as a growth promoter and improvement in feed efficiency in monogastric animals, such as pigs and poultry. To date, there are no reports in the literature of resistant bacteria to this antibiotic or of cross-resistance with other antibiotics, despite enramycin being commonly used in all growth stages of broilers and swine (OIE, 2018).

Bacitracin is licensed for human and veterinary use and is indicated in the topical treatment of skin infections caused by *Staphylococcus aureus*, and bacterial skin and mucosal infections in humans (NGUYEN *et al.* 2020). In animals, it is used for the treatment of necrotic enteritis in poultry, as well as treatment of septicemia and urinary tract infections in swine, poultry, cattle, and sheep (OIE, 2018). Acquired bacitracin resistance has been associated with overproduction of the BceAB transporter, acting to remove bacitracin by a mechanism that is still unclear (OHKI *et al.* 2003). It has also been reported that the presence of a two-component system called BceSR, whose genes are located upstream of the BceAB genes, assists by controlling the expression of the transporter gene, detecting when bacitracin is present in the extracellular

medium (RADECK *et al.* 2016). Bacitracin has also been associated with the selection of cross-resistance to colistin via the *mcr-1* gene (XU *et al.* 2018). However, there are still no reports in the literature of bacitracin cross-resistance with β-lactams.

The resistance to carbapenems and cephalosporins observed in isolates from pigs, soil and wastewater raise concerns about the origin of resistance genes in these environments since the animals evaluated in this study had no history of exposure to cephalosporins, and carbapenems are not licensed for use in animal production. One hypothesis would be the selective pressure caused by other antibiotics of the same or other classes used in animals, which could promote horizontal gene transfer and the spread of resistance determinants to soil and wastewater (VISWANATHAN, 2014).

In addition, workers who directly handle these animals may also represent a potential source of resistance genes (LANDERS *et al.* 2012). It is noteworthy that soil is an environment with high microbial diversity and many microorganisms are antibiotic producers that show intrinsic resistance to various antimicrobials. For example, *Stenotrophomonas maltophilia* has intrinsic resistance to aminoglycosides, beta-lactams, and quinolones due to the expression of drug efflux system proteins (BROOKE, 2012). The genus *Acinetobacter* spp. has intrinsic resistance to ampicillin and glycopeptides (REYGAERT, 2018), as well as to beta-lactams due to chromosomal mutations (RUPPÉ *et al.* 2015). It is also recognized that *Pseudomonas aeruginosa* has intrinsic resistance to 1st and 2nd generation cephalosporins and other beta-lactams (FAJARDO, *et al.* 2008; REYGAERT, 2018), in addition to fluoroquinolones (RUPPÉ *et al.* 2015). Metagenomic analyses performed by Forsberg and co-authors (2014) demonstrated that agricultural and grassland soils are an important reservoir of resistance genes. These authors demonstrated that different soil types harbor distinct resistomes in addition to an abundance of resistance genes. In this study, we observed an abundance of Gram-negative bacteria from soil showing intrinsic resistance to beta-lactams, which sparks the interest to investigate in detail the mechanisms of intrinsic resistance observed in different bacterial species isolated from the swine production unit.

The results of the present study indicate a lower frequency of bacterial resistance to meropenem when compared to imipenem with significant differences between the environments evaluated (P=0.0053). However, for the imipenem, no differences were observed between the frequency of resistant isolates obtained from the three environments sampled (P=0.1737). These differences in carbapenem

resistance may be explained by differences in carbapenem binding affinity to penicillin-binding proteins (PBPs) among the bacterial isolates. Meropenem has a higher binding affinity for some types of PBPs present in the cell wall of bacteria. For example, in *E. coli* and *Pseudomonas aeruginosa* meropenem has strong binding to PBP types 2, 3, and 4 (MERREM, 2007; BALDWIN *et al.* 2008). Imipenem in turn has an affinity for *Pseudomonas aeruginosa* PBPs 4 and 5 and inhibits *E. coli* PBPs 1, 2, 4, and 5. In addition, alterations in porins of the outer membrane may also be associated with decreased susceptibility to carbapenems (EL-AMIN *et al.* 2005; OCAMPO-SOSA *et al.* 2012). These changes occur due to chromosomal mutations that result in decreased porin expression or changes in porin function or type (MUNITA and ARIAS, 2016). OprD, for example, present in *P. aeruginosa* is associated with acquired resistance to imipenem and also with increased MIC values for carbapenems (EL-AMIN *et al.* 2005; GHAI and GHAI, 2018). These results suggest that alterations in the porins of the isolates tested in this work could account for the higher resistance to imipenem compared to meropenem. These alterations were not tested in this study but should be considered in future studies through conventional PCR and sequencing to evaluate the conservation of these sequences or by real-time PCR to evaluate the level of porin expression in the isolates under test.

In this study, a soil isolate classified as *Stenotrophomonas maltophilia*, showed high MIC values for carbapenems (64 - 256 mg/L) and was resistant to all cephalosporins tested, however, only the $bla_{\text{CTX-M}}$ gene was identified in this isolate. Previous studies have shown that this bacterial species has intrinsic resistance to beta-lactams due to low outer membrane permeability (AKOVA *et al.* 1991), in addition to the synthesis of efflux pumps (AL-HAMAD *et al.* 2009) and the production of β-lactamases L1 and L2 encoded from the chromosome (MERCURI *et al.* 2002; GIL-GIL, *et al.* 2020). *S. maltophilia* has been associated with hospital infections in recent years and is considered an emerging pathogen (AL-HAMAD *et al.* 2009; BROOKE, 2012; ADEGOKE *et al.* 2017; GRÖSCHEL *et al.* 2020). *S. maltophilia* has been isolated from patients with chronic obstructive pulmonary diseases (NSEIR *et al.* 2006), patients with bacteremia (LAI *et al.* 2006; ARAOKA *et al.* 2010), cystic fibrosis (ALCARAZ *et al.* 2021), and is also found in the soil, plants (BERG *et al.* 2005; BERG, 2009), urban wastewater (IVANOV *et al.* 2005) and other environments. However, there are still few reports of the isolation of this bacterium from pigs and poultry (HAN *et al.* 2020). In addition to intrinsic resistance to β-lactams, the presence of *S.*

maltophilia in the soil also raises the question about the possible acquisition of resistance genes/mechanisms not evaluated in the present study. It is possible to infer that this bacterial species is a reservoir of resistance genes acquired from the soil microbiota, which could be disseminated to commensal microorganisms and animal pathogens. *S. maltophilia* has its pathogenicity mainly related to the production of pili / flagella that contribute to adhesion and aggregation, membrane lipopolysaccharide (LPS), which promotes biofilm formation, and other virulence factors related to quorum sensing that contribute to antibiotic resistance (KALIDASAN et al. 2018). Therefore, the recent emergence of this bacteria outside the hospital environment requires special attention.

In some *enterobacterial* species, such as *Shigella flexneri* and *Enterobacter cloacae*, resistance to carbapenems has been associated with the production of AmpC-type enzymes (SCHUMACHER *et al.* 1992; GALLENI *et al.* 1988) while extended-spectrum β-lactamases (ESBL) have been identified, for example, in CTX-M-producing *E. coli* (SHAFIQ *et al.* 2019). It is also known that in *Pseudomonas aeruginosa, E. coli* (REYGAERT, 2018), and *E. cloacae* (THIOLAS *et al.* 2004) the overexpression of efflux pumps and porin alterations are associated with carbapenem resistance (WALSH, 2000; WILKE *et al.* 2005; CODJOE and DONKOR, 2018). Ma and co-authors (2020) reported clinical isolates of *K. aerogenes* that were obtained from urine, sputum, and bile from patients at a tertiary care hospital in China showing MIC values for imipenem and meropenem ranging from 16 to 32 mg/L, in addition to resistance to ceftazidime, cefepime, and ceftriaxone. Although *K. aerogenes* isolates do not possess genes for carbapenemases, genes encoding ESBL, AmpC, and efflux pumps have been identified in these isolates. In the current work, bacteria of the genus *Klebsiella* spp. obtained from soil presented MIC values for imipenem and meropenem ranging from 32 to 256 mg/L. Seven isolates carried the gene $bla_{CTX\text{-}M}$, which encodes for an extended-spectrum beta-lactamase. Therefore, other determinants of resistance that were not evaluated in this study may also be involved in the resistance to carbapenems in *Klebsiella* spp. obtained from soil samples of the swine production unit.

We also observed a high frequency of resistance to 1st, 3rd, and 4th generation cephalosporins in bacteria from all sampling sites, a phenotype that has been frequently reported in *Enterobacteriaceae* isolated from healthy and diseased pigs (JAHANBAKHSH *et al.* 2016; ABRAHAM *et al.* 2018; BACCI *et al.* 2020). More than

90% of the isolates obtained from pigs and wastewater were resistant to cephalosporins and around 81% of the soil isolates were resistant to at least one of the cephalosporins tested, with significant differences for the frequencies of resistant isolates between environments ($P < 0.05$). The highest frequencies of resistance to cephalosporins were observed in isolates from wastewater samples, where 95.5% of the isolates were resistant to cefazolin, ceftriaxone, and cefepime, and 90.9% were resistant to cefotaxime. These results are consistent with the results found by Savin *et al.* (2020), who reported that isolates from swine slaughterhouse wastewater in Germany showed a high frequency of resistance to all cephalosporins tested (ceftazidime, cefotaxime). The higher frequency of wastewater isolates classified as *Escherichia/Shigella* and *Klebsiella* spp. in this study agrees with the frequencies reported by Savin *et al.* (2020). The gene $bla_{CTX\text{-}M}$ was detected in 78% of the isolates classified as *Escherichia/Shigella* and *Klebsiella* spp. Regarding the $bla_{CTX\text{-}M}$ gene, 58% of *Escherichia/Shigella* isolates (n = 11) from wastewater carried the resistance determinant, and 72% (n = 8) of these isolates had both bla_{VIM} and $bla_{CTX\text{-}M}$ genes simultaneously. The $bla_{CTX\text{-}M}$ gene was identified at a lower frequency (4.5 %) in the genus *Klebsiella* spp. in agreement with the results obtained by Savin *et al.* (2020), where only the variant $bla_{CTX\text{-}M\text{-}15}$ was reported among *Klebsiella* spp. isolates. These results indicate that the $bla_{CTX\text{-}M}$ gene has a wide geographic distribution in wastewater (KORZENIEWSKA, 2013; LIEN *et al.* 2017; ARISTIZÁBAL-HOYOS *et al.* 2019). However, the origin of the $bla_{CTX\text{-}M}$ gene in wastewater remains uncertain, and further studies are needed to explore the phylogenetic relationships and pathways of the spread of this gene in the environment and swine production systems.

The bla_{VIM} gene is less frequently investigated than $bla_{CTX\text{-}M}$ in bacteria isolated from swine wastewater, being primarily reported in clinical isolates (KHAN *et al.* 2018) and surface water (KIEFFER *et al.* 2016; ZARFEL *et al.* 2017). However, our results showed that 70 % (n = 31) of the wastewater isolates had bla_{VIM}, and of these, 32 % also carried the $bla_{CTX\text{-}M}$ gene, potentially suggesting that this aquatic environment may be a favorable reservoir for the spread of these resistance determinants. The resistance mechanism associated with $bla_{CTX\text{-}M}$ and bla_{VIM} is the inactivation of the antibiotic, and our results elucidated high frequencies of resistance to carbapenems and cephalosporins, suggesting that these genes are being expressed and exerting their hydrolytic activities. Although they were not present in all isolates, when present, these results may be contributing to our hypothesis that genetic determinants of

resistance related to cephalosporins and carbapenems contribute to co-selection of resistance. Other genes not yet tested may also be associated with this co-selection.

These results highlight the high incidence of resistance genes in the wastewater before entering the swine production unit effluent treatment system. This fact reinforces the need for proper treatment of these effluents to reduce the frequency and potential spread of antibiotic resistance genes in the environment. Wastewater from livestock production is typically rich in nutrients and organic matter. These characteristics, coupled with the high diversity of microorganisms in these effluents, may provide an ideal environment for horizontal gene transfer (SUMMERS, 2006; BENGTSSON-PALME *et al.* 2018).

All the soil isolates (100 %) were resistant to cefazolin, while 94.8 % were resistant to ceftriaxone, 84.5 % to cefepime, and 44.8 % to cefotaxime. In addition, 12 soil isolates showed high MIC values for the carbapenems (range 128 - 256 mg/L). These results agree with the results reported by Furlan and Stehling (2017), where *Ochrobactrum* and *Achromobacter* isolates recovered from pasture soils, coffee, sugarcane, avocado, and corn crops from all regions of Brazil showed high levels of resistance to meropenem and imipenem, as well as resistance to the cephalosporins tested (ceftazidime, ceftriaxone, cefotaxime, and cefepime). In addition, the bacterial isolates from the cited study carried genes encoding beta-lactamases, such as *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{KPC}$. In our study, the *bla*$_{VIM}$ gene was the most frequent (65 %, n = 38) in the soil isolates, followed by *bla*$_{CTX-M}$ (58 %, n = 34), and three isolates also carried the *bla*$_{IMP}$ gene. Soil is an ecosystem with high species diversity and consequently serves as a reservoir for antibiotic-resistant bacteria and resistance genes that can be potentially spread to other ecosystems such as water, air, animals, and humans (MONIER *et al.* 2011; NESME and SIMONET, 2015). However, Allen *et al.* (2010) suggest that genetic determinants conferring antibiotic resistance are naturally distributed among bacteria in the environment due to the presence of compounds that impose selective pressure. Thus, the high frequency of resistance genes observed in the soil isolates from this study agree with the idea that genetic determinants exist spontaneously in nature (Allen *et al.* 2010). However, it is difficult to affirm that the resistance genes reported in this environment resulted spontaneously or were acquired by the microorganisms by horizontal gene transfer. Martinez (2012) emphasizes that the maintenance of resistance genes generates an adaptive cost for the microorganism, and it is unlikely that these genes will be maintained without

selective pressure (e.g. antibiotics). Therefore, further studies are needed to investigate the origin of these genes and their maintenance and dissemination strategies in this environment.

In isolates recovered from swine, bla_{CTX-M} was the gene most frequently found in the bacterial isolates. The cultures also showed 100 % resistance to ceftriaxone, 96.6 % to cefazolin, 89.7 % to cefepime, and 79.3 % to cefotaxime. Among the cephalosporins tested, cefotaxime showed the lowest frequencies of resistance among the isolates from the three environments, even though we observed a high frequency of the bla_{CTX-M} gene. Recently, Hayer and co-authors (2020) reported that 18 % of *E. coli* isolates recovered from diseased pigs were resistant to ceftiofur and carried the bla_{CTX-M} gene on plasmids. Our data showed that 76 % of the isolates from swine carried the bla_{CTX-M} gene and were resistant to at least three of the cephalosporins tested (cefazolin, cefotaxime, ceftriaxone, and cefepime). A high frequency of the bla_{CTX-M} gene in pigs was also reported by Dahms and co-authors (2015) in a study where they evaluated the occurrence of ESBL-producing *Escherichia* spp. recovered from fecal samples, cloacal swabs, and inguinal swabs from cattle, pigs, poultry, and the workers of the respective farms. The results showed that the highest frequency of bla_{CTX-M} was of swine origin and was characterized by conventional PCR and by phenotypic testing using cefotaxime and ceftazidime discs as established by EUCAST, as well as E-test using a beta-lactamase inhibitor (clavulanic acid). However, in this study, the susceptibility profile to cephalosporins was not evaluated, and the susceptibility of these isolates to β-lactams should be assessed in future studies. The high frequency of the bla_{CTX-M} gene and cephalosporin resistance reported in the swine feces isolates highlight the importance of monitoring these resistance determinants in production animals, particularly in swine production.

The high frequency of resistance to cephalosporins and carbapenems reported in the three environments evaluated may be related to the phenomenon called cross-resistance. Cross-resistance occurs when there is the acquisition of resistance genes or mutations that confer resistance to antibiotics of the same class (CANTÓN, 2011). Studies have reported that swine bacterial isolates can show multiple phenotypes of resistance to antibiotics belonging or not to the same class (JAHANBAKHSH *et al.* 2016; SILVA *et al.* 2016). However, cross-resistance also occurs when a mechanism confers resistance to several compounds (e.g. efflux pumps) (CHAPMAN, 2003), including antibiotic resistance genes and heavy metals. This mechanism may occur

naturally in some bacteria or there may be overexpression of genes that result in cross-resistance (DEPARDIEU *et al.* 2007). In this study, the concern about cross-resistance mechanisms between cephalosporins and carbapenems observed in swine, soil, and wastewater is due to the fact that the beta-lactam class is widely used in both veterinary medicine and clinical practice. This resistance to beta-lactams can hinder the treatment of common infections in both animal husbandry and clinical practice and consequently contribute to the spread of resistance genes and resistant bacteria.

In this study, the phenotypic test for the detection of carbapenemase-producing bacteria was performed. The test chosen was the modified carbapenemase inactivation test (mCIM). Our results indicated three wastewater isolates (W3.6, W3.12, W3.18) showing positive results for carbapenemase production. Despite carrying the *bla*$_{VIM}$ gene, the phenotypic profiles of these isolates indicated intermediate resistance to imipenem, susceptibility to meropenem, and resistance to the four cephalosporins tested (cefotaxime, cefazolin, ceftriaxone, and cefepime). One explanation for such results would be the low hydrolytic activity of the carbapenemases encoded by these genes. On the other hand, these isolates may also carry other mechanisms of resistance to the carbapenems (e.g., changes in porins and decreased outer membrane permeability). Another explanation would be a low level of expression of the genes encoding the metallo-β-lactamases found in the isolates. The expression of these genes is often regulated because resistance is most advantageous for the microorganism during exposure to the antimicrobial agent. Therefore, controlling the expression of these genes contributes to reducing energy expenditure and enables rapid adaptation of the bacterium (DEPARDIEU *et al*. 2007).

In conclusion, in this study, a high frequency of resistance to cephalosporins and carbapenems was observed in Gram-negative isolates recovered from swine, soil, and farm wastewater. High frequencies of the *bla*$_{VIM}$ and *bla*$_{CTX-M}$ genes were also observed in all three environments, with a lower frequency of the *bla*$_{IMP}$ gene, suggesting a possible spread of genes between these environments through horizontal gene transfer. Possible co-selection of resistance was represented by cross-resistance between cephalosporins and carbapenems mediated by beta-lactam resistance genes. However, further studies are needed for a better understanding of the origin of these resistance genes and the mechanisms promoting their emergence in livestock production systems.

5. Conclusions

This study reported a connection between genetic determinants of resistance and resistance phenotypes associated with cephalosporins and carbapenems in Gram-negative bacteria obtained from swine, soil and wastewater from a pig production unit. Furthermore, genes that confer resistance to cephalosporins and carbapenems were found widely distributed among these environments even without a history of antibiotic use in the pig production unit. Our findings support the hypothesis that there is a possible co-selection of resistance between cephalosporins and carbapenems through cross-resistance.

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8. Supplemental material

Table S1. Sequence similarity searching results for comparison of the 16S ribosomal RNA sequences from Gram-negative bacteria isolated from swine, soil and wastewater with the 16S rRNA sequences of bacteria in the GenBank sequence database. F = Swine ; S = Soil; W = Wastewater.

ID	Genus	Species	Site	Length (bp)	Query Cover (%)	E-value (21,826*)	% ID
F1.5	*Escherichia/Shigella*	*Atlantibacter hermannii*	Feces	788	100	0.0	99.37
F1.63.2	*Escherichia/Shigella*	*Shigella sonnei*	Feces	871	100	2e-158	99.35
F1.85	*Escherichia/Shigella*	*Shigella sonnei*	Feces	895	100	0.0	99.89
F1.98.1	*Escherichia/Shigella*	*Escherichia albertii*	Feces	788	100	0.0	99.75
F1.107	*Escherichia/Shigella*	*Shigella sonnei*	Feces	892	100	0.0	99.44
F1.110.1	*Escherichia/Shigella*	*Escherichia albertii*	Feces	751	100	0.0	98.54

F1.110.2	*Escherichia/Shigella*	*Shigella flexneri*	Feces	814	100	0.0	99.39
F1.137	*Escherichia/Shigella*	*Shigella flexneri*	Feces	822	100	0.0	99.76
F1.208	*Escherichia/Shigella*	*Atlantibacter hermannii*	Feces	749	100	0.0	99.07
F1.217	*Escherichia/Shigella*	*Escherichia marmotae*	Feces	866	100	0.0	99.19
F1.263	*Escherichia/Shigella*	*Shigella flexneri*	Feces	835	100	0.0	99.76
F1.267	*Escherichia/Shigella*	*Escherichia albertii*	Feces	733	100	0.0	99.18
F2.1	*Escherichia/Shigella*	*Shigella flexneri*	Feces	848	100	0.0	99.76
F2.7.1	*Escherichia/Shigella*	*Shigella flexneri*	Feces	882	100	0.0	99.44
F2.9	*Escherichia/Shigella*	*Shigella flexneri*	Feces	823	100	0.0	99.39
F2.41	*Escherichia/Shigella*	*Shigella flexneri*	Feces	834	100	0.0	99.76
F2.58	*Escherichia/Shigella*	*Shigella flexneri*	Feces	900	100	0.0	99.33
F2.72	*Escherichia/Shigella*	*Shigella flexneri*	Feces	875	100	0.0	99.77
F2.84	*Escherichia/Shigella*	*Shigella flexneri*	Feces	843	100	0.0	99.76
F2.86	*Escherichia/Shigella*	*Shigella flexneri*	Feces	845	100	0.0	99.73
F2.108	*Escherichia/Shigella*	*Shigella flexneri*	Feces	858	100	0.0	99.42
F2.119	*Escherichia/Shigella*	*Shigella flexneri*	Feces	834	100	0.0	99.64

F2.122	*Escherichia/Shigella*	*Shigella flexneri*	Feces	830	100	0.0	99.64
F2.182	*Escherichia/Shigella*	*Shigella flexneri*	Feces	878	100	0.0	99.66
F2.202	*Escherichia/Shigella*	*Shigella flexneri*	Feces	850	100	0.0	99.65
F2.234	*Escherichia/Shigella*	*Shigella flexneri*	Feces	876	100	0.0	99.20
F2.243	*Escherichia/Shigella*	*Shigella flexneri*	Feces	826	100	0.0	99.52
F2.252	*Escherichia/Shigella*	*Shigella flexneri*	Feces	846	100	0.0	99.76
F2.270	*Escherichia/Shigella*	*Shigella flexneri*	Feces	837	100	0.0	99.41
S1.1	*Pseudomonas*	*Pseudomonas putida*	Soil	854	100	0.0	98.95
S1.2	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	840	100	0.0	99.52
S1.3	*Klebsiella*	*Klebsiella pneumoniae*	Soil	854	100	0.0	99.77
S1.4	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	839	100	0.0	99.41
S2.1	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	845	100	0.0	99.17

S2.2	*Enterobacter*	*Enterobacter ludwigii*	Soil	924	100	0.0	99.46
S2.3	*Enterobacter*	*Enterobacter bugandensis*	Soil	843	100	0.0	99.17
S2.4	*Enterobacter*	*Enterobacter bugandensis*	Soil	820	100	0.0	99.39
S2.5	*Enterobacter*	*Enterobacter ludwigii*	Soil	843	100	0.0	98.70
S2.6	*Enterobacter*	*Enterobacter bugandensis*	Soil	850	100	0.0	99.18
S2.7	*Enterobacter*	*Enterobacter bugandensis*	Soil	811	100	0.0	95.57
S3.1	*Enterobacter*	*Enterobacter cancerogenus*	Soil	841	100	0.0	99.52
S3.2	*Enterobacter*	*Enterobacter bugandensis*	Soil	749	99	0.0	95.85
S3.3	*Enterobacter*	*Enterobacter cancerogenus*	Soil	815	100	0.0	99.63
S4.1	*Enterobacter*	*Enterobacter bugandensis*	Soil	780	99	0.0	99.10
S4.2	*Enterobacter*	*Enterobacter cancerogenus*	Soil	835	100	0.0	99.05
S5.1	*Klebsiella*	*Klebsiella pneumoniae*	Soil	837	100	0.0	99.40
S5.2	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	880	100	0.0	99.43
S5.3	*Klebsiella*	*Klebsiella pneumoniae*	Soil	822	100	0.0	98.79
S5.4	*Escherichia/Shigella*	*Shigella flexneri*	Soil	841	100	0.0	94.11

S5.5	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	835	100	0.0	99.28
S5.6	*Pseudomonas*	*Pseudomonas putida*	Soil	872	100	0.0	99.66
S5.7	*Enterobacter*	*Enterobacter cloacae*	Soil	858	98	0.0	84.44
S5.8	*Enterobacter*	*Enterobacter cloacae* subsp. *dissolvens*	Soil	742	99	0.0	92.58
S5.9	*Pseudomonas*	*Pseudomonas fulva*	Soil	851	99	2e-87	70.62
S5.10	*Pseudomonas*	*Pseudomonas monteilii*	Soil	799	97	0.0	90.96
S5.11	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	866	100	0.0	99.31
S6.1	*Klebsiella*	*Klebsiella pneumoniae*	Soil	839	100	2e-150	99.02
S6.2	*Klebsiella*	*Klebsiella pneumoniae*	Soil	854	100	0.0	99.65
S6.3	*Klebsiella*	*Klebsiella pneumoniae*	Soil	888	100	8e-70	99.33
S6.4	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	883	100	0.0	99.43

S6.5	*Klebsiella*	*Klebsiella pneumoniae*	Soil	833	100	9e-130	98.53
S6.6	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	825	99	0.0	87.88
S7.1	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	800	100	0.0	99.25
S7.2	*Klebsiella*	*Klebsiella pneumoniae*	Soil	879	100	0.0	99.09
S7.3	*Klebsiella*	*Klebsiella pneumoniae*	Soil	709	98	0.0	85.57
S7.4	*Stenotrophomonas*	*Stenotrophomonas maltophilia*	Soil	832	99	0.0	97.58
S8.1	*Klebsiella*	*Klebsiella quasipneumoniae subsp. similipneumoniae*	Soil	791	100	0.0	99.62
S8.2	*Klebsiella*	*Klebsiella quasipneumoniae subsp. similipneumoniae*	Soil	870	100	0.0	98.63
S8.3	*Klebsiella*	*Klebsiella pneumoniae*	Soil	833	100	4e-148	99.01
S8.4	*Klebsiella*	*Klebsiella pneumoniae*	Soil	819	100	7e-111	99.56

S8.5	*Pseudomonas*	*Pseudomonas putida*	Soil	748	99	0.0	98.52
S8.6	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *quasipneumoniae*	Soil	771	100	0.0	99.22
S8.7	*Pseudomonas*	*Pseudomonas putida*	Soil	869	100	0.0	99.31
S8.8	*Pseudomonas*	*Pseudomonas fulva*	Soil	853	98	0.0	83.49
S8.9	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	835	100	0.0	99.05
S9.1	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	762	100	0.0	99.61
S9.2	*Klebsiella*	*Klebsiella pneumoniae* subsp. *rhinoscleromatis*	Soil	647	100	0.0	98.46
S9.3	*Pseudomonas*	*Pseudomonas putida*	Soil	831	99	0.0	97.35
S9.4	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	722	100	0.0	99.17

S9.5	*Klebsiella*	*Klebsiella pneumoniae*	Soil	850	94	0.0	81.50
S9.6	*Pseudomonas*	*Pseudomonas putida*	Soil	866	100	0.0	80.96
S10.1	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	833	100	0.0	98.92
S10.2	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *Similipneumoniae*	Soil	817	99	0.0	99.15
S10.3	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Soil	819	100	0.0	99.39
S10.4	*Klebsiella*	*Klebsiella pneumoniae*	Soil	630	93	5e-119	77.72
S10.5	*Pseudomonas*	*Pseudomonas putida*	Soil	816	100	0.0	98.41
S10.6	*Pseudomonas*	*Pseudomonas fulva*	Soil	845	91	0.0	81.53
W1.1	*Klebsiella*	*Klebsiella pneumoniae*	Waste water	734	100	0.0	99.59
W1.2	*Enterobacter*	*Enterobacter ludwigii*	Waste water	821	100	0.0	99.03
W1.3	*Klebsiella*	*Klebsiella pneumoniae*	Waste water	800	100	0.0	99.63
W1.4	*Enterobacter*	*Enterobacter bugandensis*	Waste water	792	100	0.0	99.62

W1.6	*Enterobacter*	*Enterobacter cancerogenus*	Waste water	809	100	0.0	99.38
W1.7	*Enterobacter*	*Enterobacter cloacae*	Waste water	748	97	0.0	85.66
W2.1	*Klebsiella*	*Klebsiella pneumoniae*	Waste water	874	100	0.0	98.52
W2.2	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Waste water	871	100	0.0	98.52
W2.3	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Waste water	755	100	0.0	99.08
W2.4	*Klebsiella*	*Klebsiella quasipneumoniae* subsp. *similipneumoniae*	Waste water	880	100	0.0	99.21
W2.5	*Enterobacter*	*Enterobacter ludwigii*	Waste water	869	100	0.0	99.08
W2.6	*Enterobacter*	*Enterobacter ludwigii*	Waste water	828	100	0.0	99.28
W2.8	*Ochrobactrum*	*Ochrobactrum lupini*	Waste water	775	28	2e-18	70.54
W3.2	*Escherichia/ Shigella*	*Escherichia marmotae*	Waste water	793	100	0.0	99.50
W3.3	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	774	100	0.0	99.36
W3.4	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	694	100	0.0	98.57

W3.5	*Escherichia/ Shigella*	*Escherichia marmotae*	Waste water	811	100	0.0	98.89
W3.6	*Enterobacter*	*Enterobacter bugandensis*	Waste water	747	100	0.0	98.13
W3.7	*Escherichia/Shigella*	*Escherichia marmotae*	Waste water	794	100	0.0	97.11
W3.8	*Chromobacterium*	*Chromobacterium alkanivorans*	Waste water	819	100	0.0	99.76
W3.9	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	759	100	0.0	98.02
W3.10	*Ochrobactrum*	*Ochrobactrum oryzae*	Waste water	759	100	0.0	98.95
W3.11	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	757	100	0.0	98.95
W3.12	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	789	99	0.0	96.82
W3.14	*Escherichia/ Shigella*	*Escherichia marmotae*	Waste water	875	100	0.0	99.09
W3.15	*Escherichia/ Shigella*	*Escherichia marmotae*	Waste water	828	100	0.0	99.04
W3.16	*Castellaniella*	*Castellaniella hirudinis*	Waste water	759	73	1e-133	79.82
W3.17	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	821	100	0.0	98.30
W3.18	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	826	100	0.0	98.43
W3.19	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	736	100	0.0	98.92
W3.20	*Arcobacter*	*Arcobacter cloacae*	Waste water	733	100	0.0	99.05
W4.1	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	757	100	0.0	99.34

W4.2	*Ochrobactrum*	*Ochrobactrum lupini*	Waste water	720	100	0.0	99.86
W4.3	*Escherichia/ Shigella*	*Escherichia marmotae*	Waste water	830	100	0.0	99.04
W4.5	*Acinetobacter*	*Acinetobacter gyllenbergii*	Waste water	768	100	0.0	99.61
W4.6	*Arcobacter*	*Arcobacter butzleri*	Waste water	757	100	0.0	98.41
W4.7	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	780	100	0.0	98.98
W4.8	*Ochrobactrum*	*Ochrobactrum lupini*	Waste water	762	100	0.0	99.61
W4.9	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	769	100	0.0	98.96
W4.10	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	787	100	0.0	99.37
W4.11	*Ochrobactrum*	*Ochrobactrum lupini*	Waste water	810	100	0.0	99.88
W4.12	*Arcobacter*	*Arcobacter butzleri*	Waste water	752	100	0.0	98.40
W4.13	*Escherichia/ Shigella*	*Atlantibacter hermannii*	Waste water	771	100	0.0	99.61
W4.14	*Ochrobactrum*	*Ochrobactrum lupini*	Waste water	763	100	0.0	96.99