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Phenotypic and genetic characterization of *Escherichia coli* isolated from clinically healthy swine and the role of plasmids and extracellular vesicles in the dissemination and reservoir of antimicrobial resistance genes

Rúzivia Pimentel Oliveira
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Thesis submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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“All our dreams can come true if we have the courage to pursue them.”
(Peter Pan)

ABSTRACT

OLIVEIRA, Rúzivia Pimentel, D.Sc., Universidade Federal de Viçosa, September, 2025. **Phenotypic and genetic characterization of *Escherichia coli* isolated from clinically healthy swine and the role of plasmids and extracellular vesicles in the dissemination and reservoir of antimicrobial resistance genes**. Adviser: Denise Mara Soares Bazzolli. Co-advisers: Mateus Ferreira Santana and Hilário Cuquetto Mantovani.

Antibiotic resistance is a global public health problem, and its association with reservoirs of antimicrobial resistance (AMR) genes in non-human sources, such as farm animals, including pigs, has raised concerns and highlighted the urgent need for monitoring. Mobile genetic elements, including plasmids, carrying different AMR genes have been reported in bacteria from pigs, in both sick and healthy animals. Therefore, studies focusing on clinically healthy animals are essential, as they can provide important information that improve actions to control antimicrobial resistance. In addition, it is crucial to understand the mechanisms involved in the spread of AMR genes in food-production animals. In this context, the present study aimed to characterize bacterial isolates of the Enterobacteriaceae family from clinically healthy pigs; evaluate the profile of antimicrobial susceptibility and AMR genes; investigate plasmids associated with AMR resistance; and investigate the participation of extracellular vesicles produced by multidrug-resistant (MDR) bacterial isolates as reservoirs of AMR genes. From the bacterial collection of 28 isolates, 96.4% (n = 27) were identified as belonging to the *Escherichia coli* species. All isolates were considered multidrug-resistant with multiple AMR genes. Horizontal transfer of the *blaCTX-M* gene to the recipient strain *E. coli* J53 was confirmed for nine MDR- *E. coli* isolates by conjugation assay, using ceftiofur as the selective agent. The ß-lactam resistance profile was confirmed in the transconjugants, in addition to the presence of the gene. Furthermore, three representative isolates were subsequently selected for genome sequencing and *in silico* characterization. As a result, consistent with their phenotypic profiles, these strains harbored multiple AMR genes and plasmid replicons belonging to different incompatibility groups (Inc). *In silico* analyses identified two hypothetical plasmids carrying the *blaCTX-M* gene and another carrying the *blaTEM* gene, all were conjugative and carrying additional AMR genes. To characterize the extracellular vesicles (EVs) produced by the selected bacteria, these were obtained by ultracentrifugation from the culture of isolates with and without ceftiofur and tetracycline as stress agents. As a result, treatment with tetracycline

resulted in an increase in both the diameter and number of EVs compared to the control group. However, the EVs obtained from the control and ceftiofur groups carried a higher number of AMR genes, including the *blaCTX-M 2, blaTEM, tetA*, and *tetB* genes. These results demonstrate that EVs are capable of packaging and potentially spreading AMR genes even in the absence of antibiotics, suggesting that this mechanism may contribute to the spread of genes in the environment and in hosts. In conclusion, our results reveal that clinically healthy animals, regardless of the use of diets without antimicrobial additives, carry AMR genes of importance in animal and human clinical practice. They highlight the importance of investigating clinically healthy animals as reservoirs of AMR genes and reinforce the need for future studies focusing on EVs derived from MDR- *E. coli* of animal origin to better understand their contribution to the spread of antibiotic resistance in livestock.

Keywords: horizontal gene transfer; vesiculation; food-producing animals; multirresistance; one health

RESUMO

OLIVEIRA, Rúzivia Pimentel, D.Sc., Universidade Federal de Viçosa, setembro de 2025. **Caracterização fenotípica e genética de *Escherichia coli* isoladas de suínos clinicamente saudáveis e o papel de plasmídeos e vesículas extracelulares na disseminação e reservatório de genes de resistência a antimicrobianos**. Orientadora: Denise Mara Soares Bazzolli. Coorientadores: Mateus Ferreira Santana e Hilário Cuquetto Mantovani.

A resistência a antibióticos é um problema de saúde pública global e a associação a reservatórios de genes de resistência a antimicrobianos (AMR) em fontes não humanas, como animais de produção, como suínos, tem despertado preocupação e a necessidade urgente de monitoramento. Os elementos genéticos móveis, incluindo plasmídeos, carregando diferentes genes AMR têm sido relatados em bactérias provenientes de suínos, em ambos animais doentes e saudáveis. Portanto, estudos com ênfase em animais clinicamente saudáveis são essenciais, visto que podem fornecer informações importantes que nortearão ações para controle de resistência a antimicrobianos. Além disso, é crucial compreender os mecanismos envolvidos na disseminação de genes AMR em animais de produção. Nesse contexto, o presente estudo teve como objetivo caracterizar isolados bacterianos da família Enterobacteriaceae a partir de suínos clinicamente saudáveis; avaliar o perfil de susceptibilidade a antimicrobianos e de genes AMR; investigar os plasmídeos associados à resistência a AMR e investigar a participação de vesículas extracelulares produzidas por isolados bacterianos MDR como reservatórios de genes AMR. Os isolados clínicos investigados foram obtidos a partir de animais em fase terminal de produção sem sinais clínicos de qualquer doença, com dietas com e sem adição de promotor de crescimento, sendo um total de 28. Destes, 96,4% (n = 27) foram identificados como *Escherichia coli*. Todos os isolados (28) foram considerados multidroga resistente com vários genes AMR. A transferência horizontal do gene *blaCTX-M* para a linhagem receptora *E. coli* J53 foi confirmada para nove isolados de *E. coli* MDR por ensaio de conjugação, usando ceftiofur como agente seletivo. O perfil de resistência aos beta-lactâmicos foi confirmado nos transconjugantes, além da presença do gene. Além disso, três isolados representativos foram subsequentemente selecionados para sequenciamento do genoma e caracterização *in silico*. Como resultado, consistente com seus perfis fenotípicos, essas linhagens abrigavam vários genes AMR e plasmídeos pertencentes a diferentes grupos de incompatibilidade (Inc). As análises *in silico* permitiram identificar dois plasmídeos, um deles carregando o gene *blaCTX-M* e outro carregando o

gene *blaTEM*, os dois caracterizados como conjugativos, contendo região tra típica, além de carrear outros genes AMR relevantes e de interesse clínico. Para a caracterização das vesículas extracelulares (VEs) produzidas pelas bactérias selecionadas, estas foram obtidas por ultracentrifugação a partir do cultivo dos isolados com e sem ceftiofur e tetraciclina, como agentes estressores. Como resultado, o tratamento com tetraciclina resultou em um aumento tanto no diâmetro quanto no número de VEs em comparação com o grupo controle. No entanto, as VEs obtidas dos grupos controle e ceftiofur apresentaram os mesmos marcadores de genes AMR, incluindo os genes *blaCTX-M-2*, *blaTEM*, *tetA* e *tetB*. Esses resultados demonstram que as VEs são capazes de transportar e potencialmente disseminar genes AMR mesmo na ausência de antibiótico, sugerindo que esse mecanismo pode contribuir para a disseminação de genes no ambiente e em hospedeiros. Em conclusão, nossos resultados revelam que animais clinicamente saudáveis, independente do uso de dietas sem adição de aditivos antimicrobianos, carreiam genes AMR de importância na prática clínica animal e humana e destacam a importância de se investigar animais clinicamente saudáveis como reservatórios de genes AMR e reforçam a necessidade de estudos futuros com foco em VEs derivadas de *E. coli* MDR de origem animal para melhor compreensão da sua contribuição na disseminação da resistência aos antibióticos na pecuária

Palavras-chave: transferência horizontal de genes; vesiculação; animais de produção; multirresistência; saúde única

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1 INTRODUCTION

As the global population continues to grow, meat consumption is also rising. Current projections reported from Organisation for Economic Cooperation and Development (OECD) and the Food and Agriculture Organization (FAO) in 2025, indicate an 13% increase in meat production and a 15% increase in global consumption by 2034. According to the same report, pork meat consumption is projected to grow 5% of this projected growth. Brazil currently ranks fourth position among the world's largest meat producer and exporter. Additionally, in 2024, the pork meat consumption in Brazil was 18.6 kg per capita. These data underscore the relevance of pork meat and swine industry in Brazil.

Given the raising the demands of the global meat market have driven efforts to improve animal weight gain, including investments in infrastructure and the use of antibiotics. These agents have historically been incorporated into animal feed to promote growth. However, their use for this purpose has been banned for some antibiotic classes in several countries, including Brazil. Despite these restrictions, multiple classes of antibiotics remain allowed for use in therapy and prophylaxis in veterinary medicine, including those considered critical for human health, such as β-lactams, aminoglycosides, trimethoprim and tetracyclines.

The acquired antibiotic resistance to certain antibiotic classes commonly occurs through the acquisition of genes encoding specific mechanisms. In relation to β-lactams resistance is often mediated by the production of enzymes, such as extended-spectrum beta-lactamase (CTX-M and TEM), causing the antibiotic inactivation. To other classes, such as tetracyclines, acquired resistance involves genes encoding efflux pumps, such as TetA and TetB pumps, which causes the drug efflux from the bacteria cell, consequently reducing the concentration within the cell. Otherwise, these genetic determinants are commonly disseminated through their association with mobile genetic elements, particularly plasmids by horizontal gene transfer well described mechanisms, including conjugation, transformation and transduction.

Furthermore, in recent years, a new mechanism of horizontal gene transfer has been proposed, involving extracellular vesicles (EVs), denominated vesiduction. Extracellular vesicles are spherical nanoparticles composed by membrane cellular contents, which are commonly secreted from the cell membrane to the environment. The EVs cargo are diverse, enriched with outer membrane proteins, toxins, enzymes, and nucleic acids. As they are long-distance delivery system, their functions involve bacterial virulence, cell communication and delivery of bioactive compounds. In addition, it is well described that compounds within the EVs are show to be protect against nucleases degradation. Based on this, EVs become important

reservoirs of antibiotic resistance genes, and can plays a key role as vehicles for horizontal gene transfer (HGT).

Therefore, investigating the mechanisms underlying horizontal transfer of antimicrobial resistance genes (ARGs) through plasmids presents bacterial strains from clinically healthy food-producing animals is needed, given their substantial potential to harbored commensal bacteria that serve as important ARGs reservoirs. Furthermore, it is crucial to assess the contribution of EVs as mediators of HGT, as current knowledge on their involvement in food-producing animals remains limited.

Considering the gaps in the literature regarding horizontal genes transfer through plasmids and extracellular vesicles in MDR- *Escherichia coli* from clinically healthy pigs, this study aimed to characterize bacterial isolates of Enterobacteriaceae family from microbiota of clinically healthy pigs, evaluate the phenotype and genotype profile of AMR resistance, investigate *in silico* and *in vitro* the plasmids associated with AMR resistance and characterize the extracellular vesicles produced under different stress conditions by MDR-*E. coli* isolates from clinically healthy pigs. Furthermore, the study aimed to assess the dissemination of ARGs of public health relevance through both conjugation and vesiduction.

1 BIBLIOGRAPHIC REVIEW

1.1 The swine industry in Brazil

In 2024, according to Associação Brasileira de Proteína Animal (ABPA), the consumption of pork meat was 18.6 kg per capita, and the Brazilian production was 5.3 million tons, occupies the fourth position in the world (ABPA, 2025). In relation to exports, Brazil also ranked the fourth position in the world with an average of 137 million tons (ABPA, 2025).

The swine production is commonly divided into intensive or extensive system, depending on the objective of production (Nicolaiewsky et al., 1998; Maes et al., 2020). In extensive systems, the animals are raised under open-air conditions, requiring lower infrastructure costs, these systems are not primarily focused on productivity, but rather on subsistence and small-scale production (Honeyman, 2005; Hoar and Angelos, 2014).

In the intensive system, characterized by elevated biological and economic productivity, with bigger herds raised in confinement under closely monitored and controlled environmental, health and nutritional conditions (Nicolaiewsky et al., 1998; Maes et al., 2020). This system often presents several disadvantages, including high infrastructure costs and environmental impact, and the confined environment can become stressful, potentially reducing animal welfare and the increasing the incidence of respiratory and gastrointestinal diseases, triggering of the raise utilization of antimicrobials (Clark et al., 2019; Maes et al., 2020).

Antimicrobials are widely used in pig production for diverse purposes, despites to treatment, they are also used to prophylaxis and for improvement growth promotion (Jacela et al., 2010; Lekagul et al., 2020). However, the widespread utilization of antibiotics in pig production, particularly for growth production, have become a problem in the sector, as it may lead to the selection of resistant bacteria carrying antibiotic resistance genes (ARGs), which can potentially transfer to other susceptible and/ or pathogenic bacteria present in the pig microbiota (Chantziaras et al., 2014; Tang et al., 2017; Tawfick et al., 2022).

Therefore, despite the worldwide prohibition of the use of antibiotics for growth promotion, the widespread misuse and overuse of antibiotics remains present (Matheson et al., 2022). The main concern regarding this extensively use in food-producing animals is due that many antibiotic classes allowed for use in veterinary medicine are also critically important for treating human infections (Durso and Cook, 2014; Van et al., 2020; Rached et al., 2025). This might contribute significantly to the selection and dissemination of antibiotic-resistant bacteria and consequently, antibiotic resistance genes, posing a serious threat to both animal and human health.

Due to the emergence of antibiotic resistance in livestock worldwide, the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the World Organization for Animal Health (OIE) developed the Global Action Plan on Antimicrobial Resistance in 2015, aiming to ensure the prevention and treatment of infectious diseases and to improve awareness and understanding of antimicrobial resistance. In Brazil, the Ministry of Health implemented the Plano de Ação Nacional para Prevenção e Controle de Resistência aos Antimicrobianos – PAN-BR (MAPA, 2018), which one of the objectives is the development of surveillance and monitoring of resistance to antimicrobials in the field of agriculture (PAN-BR AGRO), including the food-producing animals. In addition, this plan also aims to implement a program to monitor the use of antimicrobials in food-producing animals and improve awareness and understanding of antimicrobial resistance (PAN-BR 2018).

1.2 Dissemination of antibiotic resistance genes in food-producing animals

As discussed above, antibiotic resistance in livestock is a significant issue that poses a concern for both animal and human health within the One health concept. This is particularly concern due to the potential exchange of microbiota resulting from direct or indirect contact between humans and animals, as well as through related environments, including soil, water and air, which facilitates the dissemination of antibiotic resistance genes (ARGs) (Manyi-Loh et al., 2018). These ARGs may be originally located on the bacterial genome, caused by spontaneous mutations within chromosomally located genes, which confers intrinsic resistance to certain antibiotics classes, independent of antibiotic pressure (Cox and Wright, 2013). However, they are commonly associated with mobile genetic elements (MGEs), which facilitates the horizontal gene transfer (HGT) and triggers the acquisition of resistance to multiple antibiotic classes, as these MGEs often carry additional ARGs (Partridge, 2015; Munita and Arias, 2016; Reygaert, 2018).

Horizontal gene transfer (HGT) refers to the movement of genetic material between bacterial cells, often facilitated by mobile genetic elements (MGEs). These MGEs, including plasmids, integrative and conjugative elements (ICEs), transposons and insertion sequences (IS), are DNA segments responsible for capturing and disseminating genes (Frost et al., 2005; Partridge et al., 2018). They promote both intracellular mobility (within genomes) and intercellular transfer (between bacterial cells) of antimicrobial resistance determinants (Frost et al., 2005; Partridge et al., 2018; Liu et al., 2022).

The mobility of genetic material between bacterial cells involves three well described mechanisms: conjugation, transformation and transduction (Burmeister, 2015) and more

recently a fourth mechanism recognized as vesiduction (Soler and Forterre, 2020). During transformation, bacteria must be in a specific physiological state known as competence to uptake exogenous DNA, which can occur naturally or be artificially induced (Frost et al., 2005; Liu et al., 2022). Transduction is mediated by bacteriophages, which, at a low frequency, can package segments of host DNA and inject them into a new bacterial cell, potentially leading to chromosomal recombination (Partridge et al., 2018). Conjugation requires physical contact between donor and recipient cells and requires the use of plasmids, as vehicles for the transfer of genetic material (Smillie et al., 2010; Rodríguez-Beltrán et al., 2021).

Plasmids are typically extrachromosomal DNA molecules with double-strand that can replicate independently of the bacterial chromosome and carrying non-essential genes to bacteria, such as antibiotic resistance genes, virulence genes and health metal genes, becoming important for bacterial adaptability and survival (Kopotsa et al., 2019). The plasmids are classified according their incompatibility replicon groups (Inc) based on the replicon factors expressed (Rozwandowicz et al., 2018). Within the Enterobacteriaceae family, at least 27 incompatibility groups – (Inc) have been associated with ARGs, and several plasmid replicon groups, such as IncF, IncN, IncX, among others, have been linked to the dissemination of relevant ARGs, including genes encoding resistance to aminoglycosides, β-lactams, tetracyclines and sulfonamides (Carattoli, 2009; Kopotsa et al., 2019).

Several studies have explored plasmids in association with ARGs in *Escherichia coli* presents in food-producing animals worldwide (Garcia et al., 2022; Binsker et al., 2023; Ma et al., 2023; Seo et al., 2023; Dos Santos Alves et al., 2023). Almost all these ARGs also demonstrated critical importance to human medicine, such as *bla*$_{CTX\text{-}M\text{-}type}$, *mcr-1, tetA, tetX, dfrA,* and others (Trongjit and Chuanchuen, 2021; Garcia et al., 2022; Seo et al., 2023), as Wang et al. (2021) isolated Enterobacteriaceae strains from a pig farm in China, and demonstrated that the tigecycline resistance was mediated by *tetA* and *tetX* genes located on plasmid or ICEs; Trongjit et al. (2022) showed *E. coli* isolates from clinically healthy and sick pigs resistant to colistin and to β-lactams, which harbored different variants of *mcr* genes, and also extended-spectrum β-lactamase (ESBL) genes, and they also reported the co-existence of these genes in some isolates originated from healthy pigs. On the other hand, Song and coauthors (2024) demonstrated that *E. coli* isolates collected from veterinary clinics and farms from South Korea, carrying several ARGs located on plasmids, mostly with different variants, such as CTX-M and APH variants. Finally, Oliveira et al. (2024) demonstrated multidrug-resistance *E. coli* isolated from clinically healthy pigs feed supplemented or not with growth promoter antibiotics, harboring several ARGs, including *bla*$_{CTX\text{-}M\text{-}type}$, and they demonstrated the transfer through

conjugation to recipient strain. Thus, the presence of plasmids carrying multiple ARGs is frequently reported in food-producing animals, including pigs. Investigating the mobilome and resistome in clinically healthy animals can contribute to the improvement of surveillance programs and mitigating the risk of food chain contamination through the transmission of ARGs from animal-based products.

1.3 Extracellular vesicles and their role in antibiotic resistance dissemination

Extracellular vesicles (EVs) are spherical nanoparticles composed by membrane cellular contents, including membrane-associated proteins, lipopolysaccharide (LPS), phospholipids, and periplasmic lipids (Toyofuku et al., 2023; Torabian et al., 2024). EVs are naturally produced by organisms across all domains of life and the diameter typically ranging from 20 to 250 nm (Schwechheimer and Kuehn, 2015; Toyofuku et al., 2023). EVs are involved in important biological processes, including bacterial virulence, modulation of the host immune response, intercellular communication, cargo and delivery of bioactive molecules, and horizontal DNA transfer (Dell'Annunziata et al., 2021; Toyofuku et al., 2023).

The lumen of EVs can encompass a wide range of cellular components, including periplasmic proteins, toxins cytosolic components and nucleic acids, and its composition may be modulated by ecological factors such as temperature, nutrient limitation, oxidative stress, growth conditions, and exposure to antibiotics (Qing et al., 2019; Dell'Annunziata et al., 2021; Zhao et al., 2025). In Gram-negative bacteria, the composition of EVs varies according to their putative biogenesis pathway. Firstly, the formation is due to the blebbing from the outer membrane (OM), triggering the formation of OMVs. It is possible the formation of other type of EVs from blebbing, that comprehends outer and inner membrane (OIMVs), results of the weaking of the peptidoglycan layer by enzymes.

In addition, there are EVs originated as result of the explosive cell lysis (E-type), triggered by stress responses, where endolysins acts depredating the peptidoglycan layer, resulting in the release of membrane fragments that originate vesicles with distinct molecular cytoplasmatic contents, including nucleic acids (Perez-Cruz et al., 2013; Toyofuku et al., 2023; Jiang et al., 2024). The predominant model proposes that their formation is driven by envelope stress, such as the accumulation of peptidoglycan fragments or denatured proteins in the periplasmic space, which disrupts membrane homeostasis and promotes the release of outer membrane vesicles (OMVs) (Gan et al., 2023; Toyofuku et al., 2023). The OMVs content reflects might the origin from the outer membrane, which can contain structural features of the bacterial surface, including lipopolysaccharides, phospholipids, and integral membrane

proteins. Cytoplasmatic contents, including nucleic acids, are thought to be absent in OMVs, only present in OIMVs-type (Schwechheimer and Kuehn, 2015; Toyofuku et al., 2023; Jiang et al., 2024). However, there are already many studies demonstrating the presence of nucleic acids in the lumen of OMVs, although the exactly mechanism of nucleic acids package remains unclear (Rumbo et al., 2011; Bielaszewska et al., 2020; Li et al., 2022; Chen et al., 2023; Tang et al., 2023; Jiang et al., 2024; Xu et al., 2024).

In last years, researchers have demonstrated that EVs can contribute to antibiotic resistance by the transfer ARGs to other bacteria (Rumbo et al., 2011; Bielaszewska et al., 2020; Li et al., 2022; Shen et al., 2024). As EVs are long-distance system and their membrane composition, it can serve as efficient pathway to material exchange between bacteria cell including DNA through horizontal gene transfer (Bielaszewska et al., 2020; Dell'Annunziata et al., 2021).

Similarly, EVs had been also associated with a new mechanism of horizontal gene transfer, designated as vesiduction, whereas the production of EVs is designated as vesiculation (Soler and Forterre, 2020). Recent study has demonstrated that EVs isolated from avian pathogenic *E. coli* can transfer $bla_{CTX\text{-}M\text{-}55}$ to a *E. coli* strain C600, with highest transfer frequency in EVs isolated in stress conditions, as in the presence of antibiotic (Li et al., 2022). Additionally, Xu et al. (2024), demonstrated that EVs isolated from various ESBL- producing *E. coli* strains from commercial laying hen farms, were capable of transferring the $bla_{CTX\text{-}M\text{-}55}$ gene, originating from different plasmid replicons, with varying transfer frequencies. These findings suggesting that the uptake of DNA in association with EVs depends on multiple factors, including the machinery of plasmid replicons, competence of the recipients and others (Xu et al., 2024).

Based on the recent studies discussed above, research on EVs production from animal isolates remains limited, particularly regarding the presence of antibiotics. Therefore, further investigations are required to characterize EVs from food-producing animals isolates under exposure to antibiotics commonly used in livestock, in order to better understand their potential to package ARGs and transfer them through horizontal gene transfer.

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CHAPTER 1

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Prevalence and Characteristics of ESBL-Producing *Escherichia coli* in Clinically Healthy Pigs: Implications for Antibiotic Resistance Spread in Livestock

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Abstract

Aim

This study aimed to compare and characterize the resistance profile and the presence of ESBL-related genes in *E. coli* isolated from healthy finishing pigs fed with or without antibiotics in their diets.

Methods and results

A total of 27 ceftiofur-resistant *E. coli* isolates were obtained from 96 healthy pigs. The antibiotic resistance profile was tested, and all 27 isolates were classified as multidrug resistant (MDR). A high proportion of isolates were resistant to cephalosporins, ampicillin, ciprofloxacin, and tetracyclines. The ESBL production was observed in 85 % of isolates by double-disc synergy test. The MDR-*E. coli* isolates harbored ESBL genes, such as bla_{TEM}, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, and $bla_{\text{CTX-M-8,25}}$. In addition, other ARGs were also detected, such as *sul2, ant(3")-I, tetA*, and *mcr-1*. The mobilization of the $bla_{\text{CTX-M}}$ gene was confirmed for nine *E. coli* isolates by conjugation assays. The presence of $bla_{\text{CTX-M}}$ on mobile genetic elements in these isolates was demonstrated by Southern blot hybridization and the resistance to cephalosporins was confirmed in the transconjugants. Our results indicate the prevalence of

CTX-M-producing *E. coli* strains harboring mobile genetic elements in the normal microbiota of healthy pigs.

Conclusions

These findings highlight the significance of ESBL genes as a global health concern in livestock and the potential spread of resistance to other members of the gastrointestinal tract microbiota.

Impact Statement

Our study underscores the urgent need for holistic strategies to mitigate the spread of resistance across antibiotic classes and illuminates the concerning prevalence of β-lactam resistance genes, notably $bla_{CTX\text{-}M}$ variants, among *E. coli* isolates from pigs reared in Brazilian farms, regardless of antibiotic exposure history. Our data also provides insights into the mechanisms facilitating the horizontal transfer of resistance genes within the swine microbiome, notably through conjugative plasmids harboring $bla_{CTX\text{-}M}$ genes.

Keywords: Antimicrobial resistance; Swine; PCR; CTX-M-type extended-spectrum β-lactamases; Conjugative plasmids.

2 INTRODUCTION

The increased demand for swine products worldwide is inducing changes in the production system towards intensive pig farming and the adoption of management practices that improve feed efficiency (Aarestrup, 2015; Lassaletta et al., 2019). In intensive production systems, antibiotics can be used for therapeutic, prophylactic, and metaphylactic purposes as well as to enhance animal performance (Aarestrup, 2015). Importantly, on a global scale, antimicrobial consumption per population correction unit (PCU) is higher for swine (172 $mg.PCU^{-1}$), compared to poultry (148 $mg.PCU^{-1}$) and cattle (45 $mg.PCU^{-1}$) (Van Boeckel et al., 2015).

However, the use of antibiotics for growth promotion has been a matter of much debate worldwide (Manyi-loh et al., 2018, Oliveira et al., 2020). In recent years, several countries have restricted the use of antibiotics in livestock as growth promoters (Manyi-Loh et al., 2018). For example, in Brazil, antibiotics such as chloramphenicol, penicillins, tetracyclines, sulfonamides, and colistin have been withdrawn as additives in animal feeds and growth promotion in the last 20 years and implementation of The National Action Plan on Antimicrobial Resistance in Agriculture in 2018 aims to promote health education, surveillance, and monitoring of antimicrobial use in livestock and companion animals (MAPA, 2018). However, other medically important antibiotics such as bacitracin remain an approved growth promoter for poultry, swine, and cattle (MAPA, 2016 and 2020).

The global concern associated with the use of antibiotics in food-producing animals is that many antibiotics of veterinary importance belong to the same class of antibiotics used in human medicine, such as β-lactams, which in the swine sector include cephalosporins and penicillins commonly used to treat gastrointestinal and respiratory diseases (OIE, 2018; Oliveira et al., 2020). A recent study compared the use of antimicrobials in 25 Brazilian swine herds between 2016 and 2020 (Dutra et al., 2021). The authors detect in the first assessment (2016) a positive correlation between antimicrobial consumption and animal exposure time. A 30% reduction in antimicrobial consumption was observed in 2020, as well as a 44.3% reduction in lifetime antibiotic exposure. Among the herds evaluated, ceftiofur (40%), amoxicillin (24%), and gentamicin (16%) were among the most frequently used drugs during the weaning and growth-termination phases.

One of the mechanisms of resistance to the β-lactams involves hydrolysis of the antibiotic molecule through enzymes known as β-lactamases. β-lactamases are the main cause of resistance against β-lactams among Gram-negative bacteria, and recent studies demonstrate the presence of different types of β-lactamases in the farm environment (Meletis and Bagkeri, 2014; Palmeira et al., 2020, Zhang et al. 2021). CTX-M enzymes belonging to the class A beta-lactamases commonly confer resistance to the 2nd, 3rd, and 4th generation cephalosporins (Ruppé et al., 2015). *Bla*$_{CTX-M}$ genes have been detected in food-producing animals (Balázs et al., 2021; Lee et al., 2021; Tsuka et al., 2021; Trongjit et al., 2022), and are normally associated with mobile genetic elements. Therefore, resistance to β-lactams could potentially be transferred to opportunistic pathogens or commensal bacteria in both animals and humans (Bush and Bradford, 2020).

Mobile genetic elements (MGE) refer to elements that promote intracellular and intercellular DNA mobility and are classified into plasmids, transposons (Tn), insertion sequences (IS), and integrative and conjugative elements (ICEs) (Frost et al., 2005; Partridge et al., 2018). Plasmids in particular are important for Gram-negative bacteria to carry acquired resistance genes and other MGE (Partridge et al., 2018). The propagation of the genes in a plasmid occurs both through vertical and horizontal transmission (Bethke et al., 2023). Recent reports have shown the presence of plasmids carrying ESBL genes in Enterobacteriaceae isolated from food-producing animals, such as diseased pigs (Silva et al., 2016; Lucas et al., 2018; Lee et al., 2021; Trongjit et al., 2022).

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 *Escherichia coli* from clinically healthy swine is a potential reservoir of the ESBL genes and the spreading of resistance is facilitated by conjugative plasmids. We, therefore, evaluated the presence of genetic markers of resistance associated with ESBL genes and antibiotic resistance profile of *Escherichia coli* isolated from the feces of finishing healthy swine raised with and without antibiotics in their diets.

3 METHODS

3.1 Ethical Approval Statement

All animal handling procedures were carried out according to guidelines approved by the Ethics Committee on the Use of Production Animals of the Universidade Federal de Viçosa (CEUAP/UFV) under Protocol n° 027/2021.

3.2 Animal sampling and isolation of resistant *Escherichia coli*

The sampling collection was carried out in the swine sector of the Animal Science Department at the Universidade Federal de Viçosa, Brazil. Fecal samples were obtained directly from the rectum using sterile swabs. All animals used in the current study were castrated male commercial hybrid pigs born on the same farm and obtained from DB Genética Suína, Patos de Minas, Brazil. The age, weight, and average feed intake of the animals were 120 days, 77 ± 8 kg at the time, and 2.9 ± 0.3 kg of feed/day on the day of sample collection, respectively. All pigs were allocated in pens housing four animals and were fed with a commercial diet (Nutron Cargill) added with 55 ppm of zinc bacitracin and 5 ppm of enramycin (T1 group, 48 animals) or fed without antibiotics (T2 group, 48 animals). The rectal swabs were immediately placed into tubes containing the Stuart transport medium (Kasvi, São Paulo, Brazil) and transported to the laboratory. The swabs were streaked directly on the surface of MacConkey agar (Kasvi, Conda Laboratories, Spain) supplemented with 8 $\mu g.mL^{-1}$ ceftiofur (Ceftiomax, Biogénesis Bagó, Buenos Aires, Argentina) to screen for cephalosporins-resistant bacteria according to Clinical and Laboratory Standards Institute Veterinary Antimicrobial Testing (CLSI VET01S, 2023). After sub-culturing, pure isolates resistant to ceftiofur were selected and transferred to Luria Bertani (LB) broth (Kasvi, São Paulo, Brazil) added with 20 % glycerol, and stored at -80 °C for further characterization.

3.3 Identification of isolates - Biochemical and molecular assays

The isolates were subjected to molecular identification by sequencing the 16S rRNA gene. The amplicons were generated by conventional PCR using specific primer pairs targeting

the 16S rDNA (Table S1). Total DNA was obtained according to Oliveira et al. (2002). The PCR reaction was carried out with approximately 50 ng of total DNA and the reaction mixture contained (final concentration): 1x of Super Taq Reaction Buffer, 100 μM dNTPs, 0.5 μM of each primer, 1.5 $U.\mu L^{-1}$ of Super Taq Pol, 2.5 mM of $MgCl_2$ (Cellco Biotec, São Carlos, Brazil). The PCR products were cleaned up using ExoSap-IT according to the manufacturer's instructions (Cellco Biotec, São Carlos, Brazil). The PCR products were subjected to Sanger sequencing. Subsequently, the sequences obtained were aligned using BLASTn to generate a consensus sequence and were analyzed using the Basic Local Alignment Search Tool (BLAST). Sequences were compared with 16S rRNA sequences of bacteria deposited in the GenBank sequence database (https://www.ncbi.nlm.nih.gov/) and the Ribosomal Database Project (https://rdp.cme.msu.edu/).

Additionally, the isolates were cultured in two selective and differential media, the Eosin Methylene Blue Agar (EMB) and Violet Red Bile Agar (VRB) (MERCK, Darmstadt, Germany). These selective and differential media were used to evaluate the phenotypic traits of the isolates and distinguish closely related species, such as *Escherichia coli* and *Shigella* spp, which could not be separated based on the alignment of the 16S rRNA gene sequences.

3.4 Genetic polymorphism by DNA fingerprinting

BOX-PCR was conducted using the primer BOXA1R (Table S1) to evaluate the genetic polymorphism of the bacterial isolates. The amplification conditions included initiation denaturation at 95 °C for 2 min, followed by 35 cycles, denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 68 °C for 5 min, and final extension at 68 °C for 8 min. The amplified fragments were separated in 1.5% agarose gel. From the gel, a matrix was generated considering the presence and absence of bands for all isolates. From this matrix, a dendrogram was generated using the PAST (Paleontological statistics) program (Hammer, Harper and Ryan, 2001), using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) grouping method and Jaccard index coefficient.

3.5 Molecular detection of resistance and virulence genes

All isolates were investigated for the presence of ESBL-encoding genes, $bla_{\mathrm{CTX\text{-}M}}$, $bla_{\mathrm{CTX\text{-}M\text{-}1}}$, $bla_{\mathrm{CTX\text{-}M\text{-}2}}$, $bla_{\mathrm{CTX\text{-}M\text{-}9}}$, $bla_{\mathrm{CTX\text{-}M\text{-}8,25}}$, bla_{CMY}, bla_{NDM}, bla_{IMP}, bla_{VIM}, $bla_{\mathrm{OXA\text{-}48}}$, $bla_{\mathrm{OXA\text{-}23}}$, bla_{KPC}, bla_{TEM} and bla_{SHV}. The primers used for PCR amplification of resistance and virulence genes are presented in Table S1.

In addition to the ESBL genes, the *E. coli* isolates were screened for the presence of resistance genes to aminoglycosides (*ant(3")-I*), colistin (*mcr-1*), fluoroquinolones and

quinolones (*gyrA, qnrS*), sulfonamides (*sul2*), tetracyclines (*tetA, tetB*) and trimethoprim (*dfra15*), and to the presence of integron class 1 gene (*int1*). The virulence genes that encode toxins, adhesins and hemolysin, namely *eltB* (heat-labile toxin), *stx1* (Shiga toxin), *estA* (heat-stable toxin), *eaeA* (intimin - intimate attachment), *aggR* (attachment and adherence), *csgA* (curli fimbriae), *fimH* (type I fimbriae) and *hlyA* (hemolysin) were also screened. The amplification reaction contained (final concentration): 50 ng of DNA template, 1x of Taq reaction buffer, 100 µM of dNTP, 10 µM of each primer, and 1.5 $U.\mu L^{-1}$ of Taq Pol (Cellco Biotec, São Carlos, Brazil).

3.6 Antibiotic susceptibility testing

Antibiotic susceptibility testing was conducted using the standard disk diffusion assay according to the Clinical and Laboratory Standards Institute (CLSI, 2020; CLSIVET01S, 2023). The following antibiotics were tested: amoxicillin-clavulanate (20 µg/10 µg), ampicillin (10 µg), aztreonam (30 µg), cefazolin (30 µg), cefepime (30 µg), cefotaxime (5 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefuroxime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg), tetracycline (30 µg) and trimethoprim (5 µg). The antibiotics for phenotypic testing were selected to represent different classes of antibiotics, mainly β-lactams, as well as other antimicrobials used in pig farming for both therapeutic and prophylactic purposes. The *E. coli* ATCC 25922 strain was used as reference control. The experiment was performed in triplicate. The isolates were classified into multidrug-resistant (MDR) when they were resistant to three or more antimicrobial classes, according to the criteria of Magiorakos et al. (2012).

To evaluate the production of ESBL, we conducted the double-disc synergy test according to CLSIVET01S (2023) and CLSI (2020), using cefotaxime (5 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), and amoxicillin-clavulanate (20 µg/10 µg). The disks were placed at a distance of 20 mm from each other and an amoxicillin-clavulanate disk was placed at the center of the plate. This method is based on measuring the specific zone diameters for the antibiotic disks, and the formation of a ghost zone between the cephalosporins and amoxicillin-clavulanate. *E. coli* ATCC 25922 served as the negative control.

3.7 Conjugation assay

To investigate the ability to mobilize genetic determinants of resistance through conjugation the *Escherichia coli* J53 Azi^R (Matsumura et al., 2018) was used as the recipient strain (Koo and Woo, 2011) with modifications. In brief, donor strains and the recipient were cultured overnight in 2 mL of LB broth at 37 °C with orbital shaking at 180 rpm. Cultures were

transferred to fresh LB broth and incubated with agitation for 2h at 37 °C until cultures reached an absorbance (600 nm) of 0.5 – 1.0. Then, for each mating experiment, 200 µL of the recipient strain was incubated with 600 µL of the donor (1:3 ratio) strain and incubated for 3 h at 37 °C. Finally, 100 µL of the mating suspension was spread on the surface of LB agar supplemented with 8 $mg.L^{-1}$ ceftiofur and sodium azide (100 $\mu g.mL^{-1}$) and incubated for up to 24 h at 37 °C.

Two random transconjugants of each experiment were transferred to LB agar supplemented with sodium azide (100 $ug.mL^{-1}$) and ceftiofur (8 $mg.L^{-1}$) and cultured overnight. Then, a colony gene-specific PCR was conducted to confirm the mobilization of $bla_{CTX\text{-}M}$. Additionally, an antibiotic susceptibility test was performed by the disk diffusion assay using cephalosporin antibiotics (ceftriaxone, ceftiofur, cefuroxime, and ceftriaxone). The J53 recipient strain was used as a negative control and the diameter of the inhibition zone was calculated. ESBL production assay was also performed with the transconjugants as described above.

3.8 Pulsed-field gel electrophoresis and Southern-blot hybridization

Pulsed-field gel electrophoresis (PFGE) was performed to confirm the transfer of mobile genetic elements compatible with conjugative plasmids and to evaluate the plasmid profile of the transconjugants. Localization of the $bla_{CTX\text{-}M}$ gene was determined by Southern blot hybridization using the recipient strain *Escherichia coli* J53 as negative control. All the PFGE steps were performed following the standardized procedure for Bacterial DNA in the CHEF-DR II system (Bio-rad Laboratories, Nazareth, Belgium) at 14 °C with 5 v/cm^2 for 20 h. The gel was dyed with ethidium bromide visualized using a gel imaging system (L-Pix Chemi, Cotia, São Paulo). The products were subsequently used to perform Southern blot hybridization using PCR DIG Probe Synthesis Kit (Roche, Germany), with a digoxigenin-labeled *CTX-M* DNA probe.

3.9 Statistical analysis

The difference between the antibiotic resistance profile between the two treatments and the difference in the diameters of the inhibition zones between donors and transconjugants were carried out using two-way ANOVA, and the p-value was corrected using Sidak's multiple comparisons test. All statistical analyses were performed in R studio 4.3.1. Test results were declared statistically significant when the associated p-value was less than 0.05.

4 RESULTS

4.1 Identification and genetic polymorphism of bacterial isolates

A total of twenty-eight ceftiofur-resistant isolates were obtained from 96 pigs, of which 15 isolates were from the T1 group and 13 isolates from the T2 group. 16S rDNA sequencing confirmed that all isolates belonged to the *Escherichia* genus, and those that fermented lactose and produced bright green colonies on EMB agar were identified as *Escherichia coli*. One isolate was confirmed as *Escherichia fergusonii* (T2.86) by PCR analysis of the EFER 1569 gene.

The genetic polymorphism of the *Escherichia* spp. isolates was investigated using BOX-PCR fingerprinting (Figure 1A). A dendrogram was generated using a similarity index ≥ 40 % for grouping the isolate in clusters. Results demonstrated that *Escherichia* spp. isolated from pigs confined in pens distant from each other and in different treatment groups had similar genetic profiles (Figure 1A). As evidenced in the dendrogram, isolates T1.217/T2.270, T1.119/T2.182, as well as T1.111/T1.137 were grouped with 100% similarity. Additionally, isolates T1.5/T1.107 showed identical BOX-PCR pattern and the animals were confined in the same pen (Figure 1B).

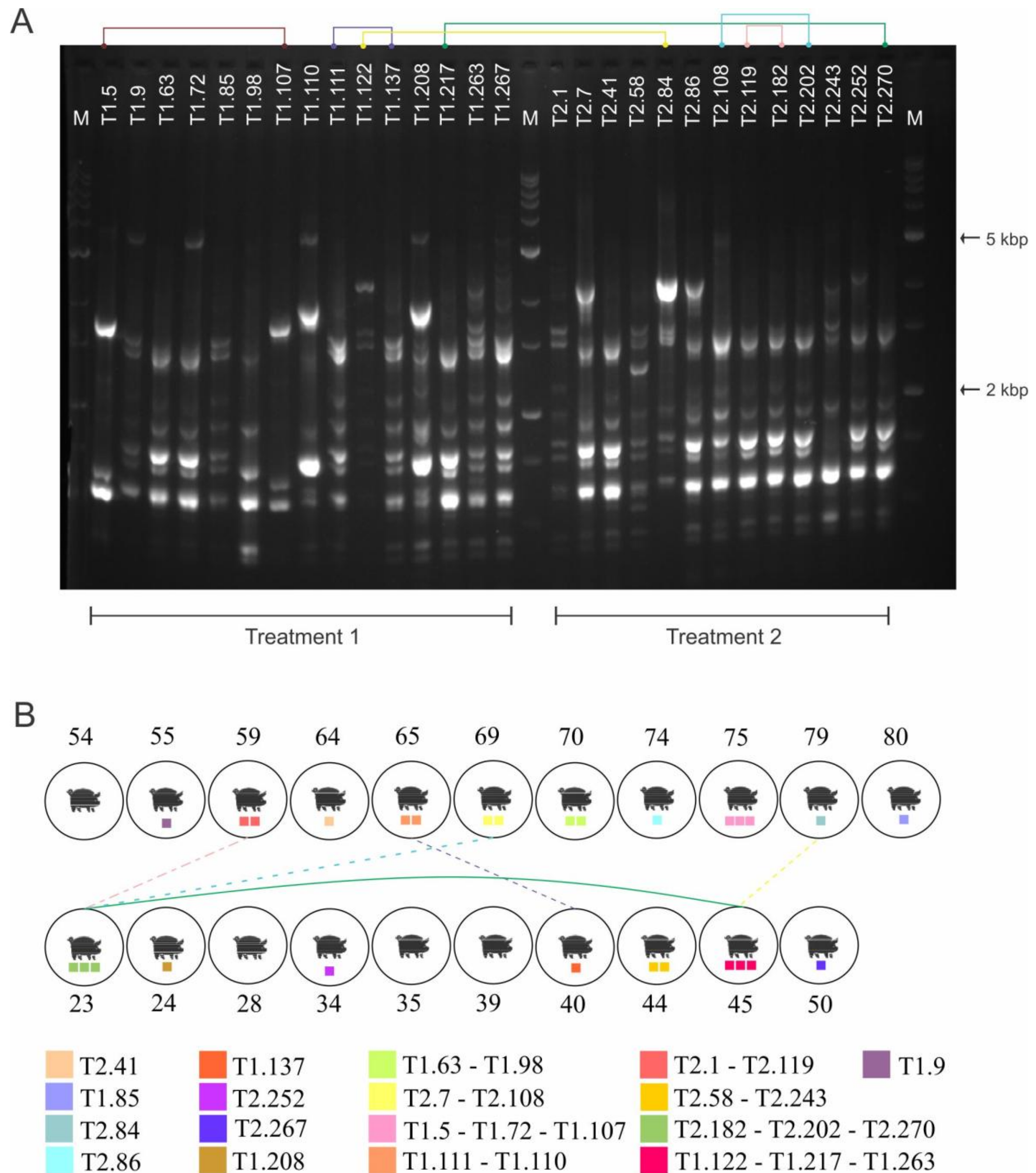

Figure 1. Origin of bacterial isolates according to animal treatment with (T1) and without (T2) growth promoter added in their diet and relatedness of *E. coli* strains. (A) BOX_PCR fingerprinting of all *Escherichia* spp. isolated from swine demonstrating genetic polymorphism. The brackets represent isolates that are grouped with ≥ 90% similarity. (B) Schematic representation of the swine pens (identified by the number above the circles) with bacterial isolates obtained from each animal in the pen. The 1000 bp marker was used as reference.

4.2 Screening of antibiotic resistance genes and virulence genes

The following experiments were carried out with all the 27 *E. coli* ceftiofur-resistant isolates. Antibiotic resistance genes were detected in isolates from both treatments and all *E. coli* isolates were positive for sulfonamide resistance gene (*sul2*). From these, 92.5% (n=25) were positive for aminoglycoside resistance gene (*ant(3")-I*) and carried a mutation in a gene

encoding a DNA gyrase associated with fluoroquinolone resistance (*gyrA*). Additionally, 74% (n=20) of the isolates carried a gene for trimethoprim resistance (*dfra15*) (Figure 2). The genes *tetA* and *tetB* were also present in 62.9% (n=17) and 85.1% (n=23) of the isolates, respectively. Genetic determinants of colistin resistance (*mcr-1*) and quinolones resistance (*qnrS*) were found among 37% (n=10) and 40.7% (n=11) of the isolates, respectively. Some virulence genes were detected in the majority of the isolates and were related primarily to biofilm formation (*csgA*, 92.5%; n=25) and adhesion phenotypes (*fimH*, 88.8%; n=24). One isolate from treatment 2 (T2.7) that carried multiple ARGs also had genes related to attachment (*eae*) and production of a heat-labile toxin (*eltB*).

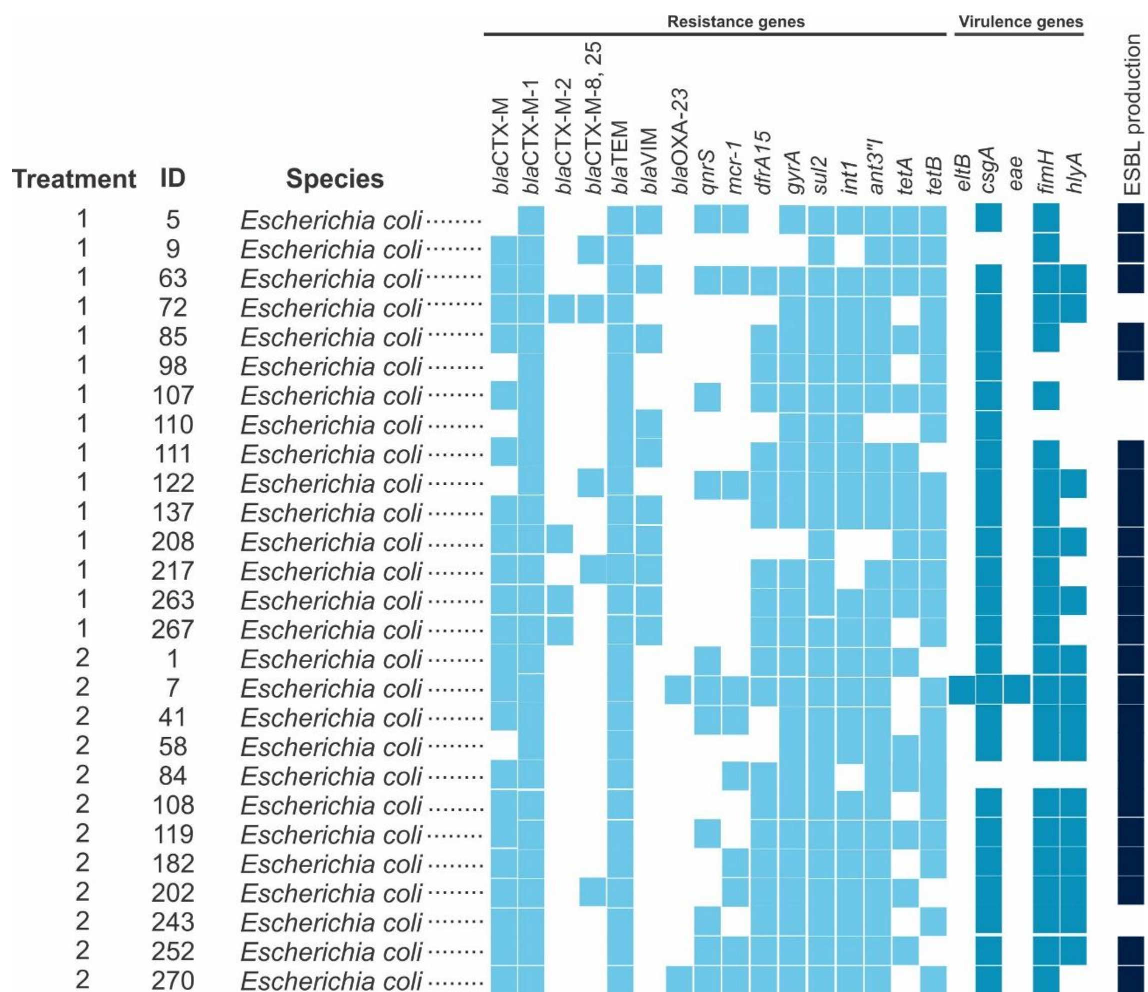

Figure 2. Genotypic profile of the *E. coli* isolates and production of ESBL. The detection of virulence genes and ARGs from different classes was done using PCR analysis (see text for details).

All *E. coli* isolates tested in the current study harbored beta-lactam resistance genes, including the $bla_{\text{CTX-M-1}}$ and bla_{TEM}. From these, 81.4% (n=22) isolates were also positive for $bla_{\text{CTX-M}}$ (Figure 2). Four isolates belonging to treatment 1 carried $bla_{\text{CTX-M-2}}$, and one of them (T1.72) also harbored the $bla_{\text{CTX-M-8-25}}$ gene. Additionally, three more isolates from treatment 1

(T1.9, T1.122, and T1.217) and one isolate from treatment 2 (T2.202) also harbored *bla*$_{CTX-M-8-25}$ gene. None of the isolates tested in this study harbored the *bla*$_{CTX-M-9}$ gene.

We also detected a genetic element (*intI1* gene) among the isolates (88.8%, n=24) that encodes a class 1 integrase. Regarding the carbapenemases genes, ten isolates carried *bla*$_{VIM}$ and two carried *bla*$_{OXA-23}$. None of the isolates harbored *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{IMP}$, *bla*$_{CMY-2}$, or *bla*$_{SHV}$. To confirm if these genetic markers were expressed by the bacterial isolates, phenotype *in vitro* assays were conducted.

4.3 Antibiotic resistance profiles and production of ESBL

Resistance phenotypes to 10 antibiotic classes were tested. With regards to the penicillin antibiotics, all isolates (100%, n=27) were resistant to ampicillin and 37% to amoxicillin-clavulanate. For cephalosporins, 100% of the isolates were resistant to cefazolin, cefotaxime, cefuroxime, and ceftriaxone (Figure 3). In addition, resistance to quinolones (ciprofloxacin), tetracyclines, and folates (trimethoprim) was equally distributed (96.2% of the total tested) among the isolates (Table 1). Furthermore, 85.1% of the isolates showed resistance to an aminoglycoside (gentamicin), 66.6% to ceftazidime, and 7.4% to cefepime. Resistance to imipenem and meropenem was not detected among any of the *E. coli* isolates investigated in the current study. Antibiotic resistance was distributed in both treatment groups, but significant differences ($p < 0.05$) were observed for some antibiotics (Figure S1). The production of ESBL was confirmed for 85.1% (n=23) of the *E. coli* isolates tested using the double-disc synergy test (Figure 2).

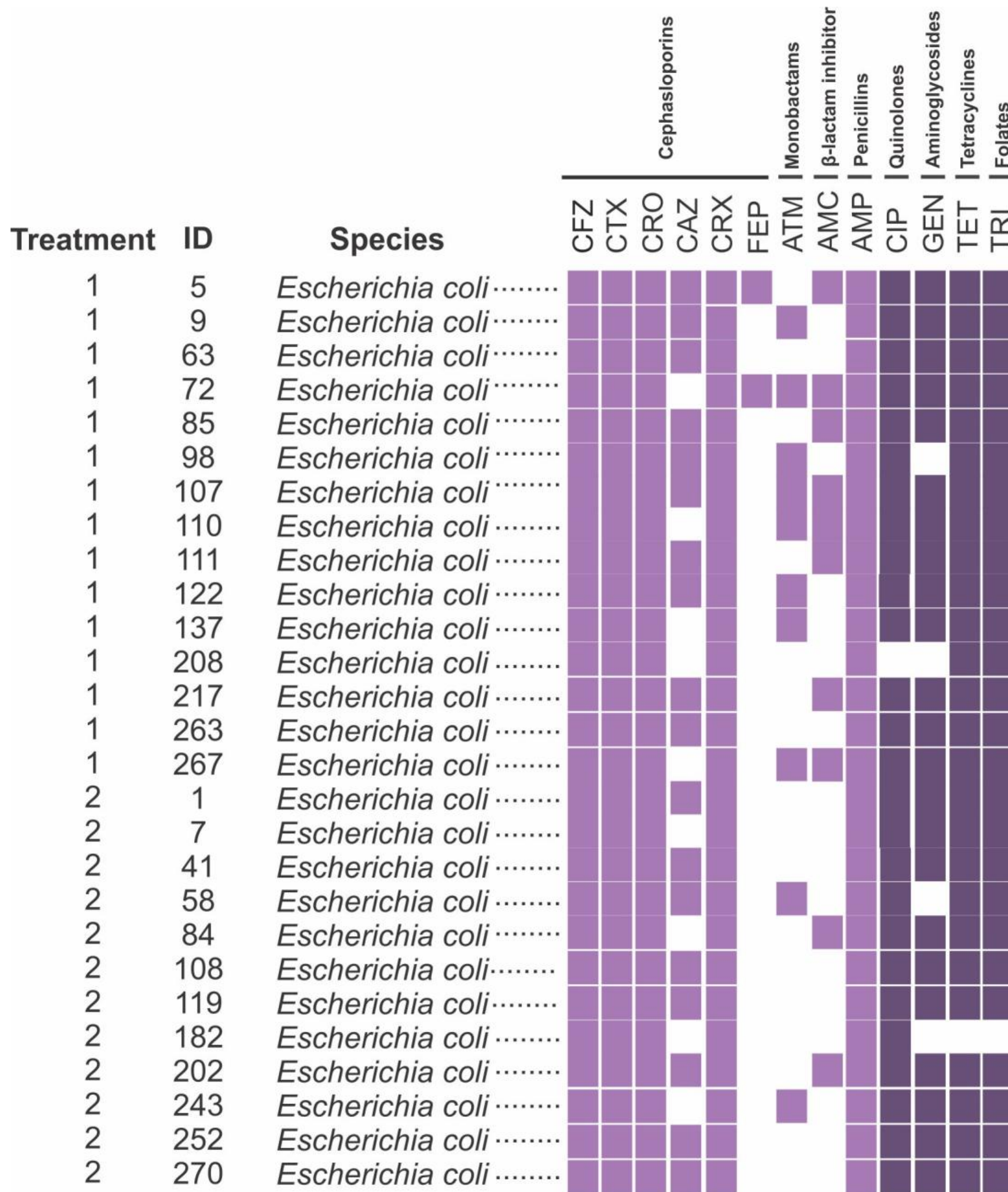

Figure 3. Phenotypic resistance profile of *E. coli* isolates from each treatment to different classes of antibiotics. AMC, amoxicillin/clavulanic acid; ATM, aztreonam; AMP, ampicillin; CFZ, cefazolin; CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; CRX, cefuroxime; FEP, cefepime; CIP, ciprofloxacin; GEN, gentamicin; TET, tetracycline; TRI, trimethoprim.

Table 1. Number and frequency of resistant *E. coli* isolates from both treatments. Disk diffusion assay was performed according to CLSI M100 (2020) and CLSIVET01S-Ed5 (2023). None of the isolates were resistant to meropenem and imipenem.

Antibiotic agent	**Antibiotic class**	**Zone diameter for resistance (mm)**	**Number and frequency of resistant *E. coli* isolates**	
			Treatment 1 (n=15)	*Treatment 2 (n=12)*

Amoxicillin clavulanate	β-lactam inhibitor	≤ 13	8 (53.3%)	2 (16.6%)
Ampicillin	Penicillins	≤ 13	15 (100%)	12 (100%)
Aztreonam	Monobactams	≤ 17	8 (53.3%)	2 (16.6%)
Cefazolin	Cephalosporins	≤ 19	15 (100%)	12 (100%)
Cefepime	Cephalosporins	≤ 18	2 (13.3%)	0 (0%)
Cefotaxime	Cephalosporins	≤ 22	15 (100%)	12 (100%)
Ceftazidime	Cephalosporins	≤ 17	10 (66.6 %)	8 (66.6 %)
Ceftriaxone	Cephalosporins	≤ 19	15 (100%)	12 (100%)
Cefuroxime	Cephalosporins	≤ 14	15 (100%)	12 (100%)
Ciprofloxacin	Fluoroquinolones	≤ 21	14 (93.3%)	12 (100%)
Gentamicin	Aminoglycosides	≤ 12	13 (86.6%)	10 (83.3%)
Tetracycline	Tetracyclines	≤ 11	15 (100%)	11 (91.6%)
Trimethoprim	Folates	≤ 10	15 (100%)	11 (91.6%)

Based on the multi-drug resistance profile of *E. coli* isolates, we investigated if these bacteria carried mobile genetic elements (MGE), such as conjugative plasmids and the *intI1* gene related to integron class 1. For that, the isolates were tested for their ability to horizontally transfer specific resistance markers through bacterial conjugation.

4.4 Conjugation assays

The horizontal transfer of resistance genes was investigated for all 27 *E. coli* isolates. Nine transconjugants was obtained (33.3%), which two isolates were from treatment 1 (T1.9 and T1.217) and seven from treatment 2 (T2.1, T2.7, T2.182, T2.202, T2.243, T2.252 and T2.270) and were selected from the mating experiments for further characterization. The transconjugants were selected on LB agar supplemented with ceftiofur (8 µL. mL^{-1}). PCR analysis was conducted in these transconjugants and the transfer of bla_{CTX-M} from the donor to the recipient strain was confirmed (Figure 4A). In addition, the transconjugants of isolates carrying the *intI1* gene were also tested, and Tc1, Tc7, Tc202, and Tc252 were positive for the bla_{CTX-M}. Furthermore, the $bla_{CTX-M-8}$ gene, which had been detected in T1.217, was also confirmed in the transconjugant (Tc217).

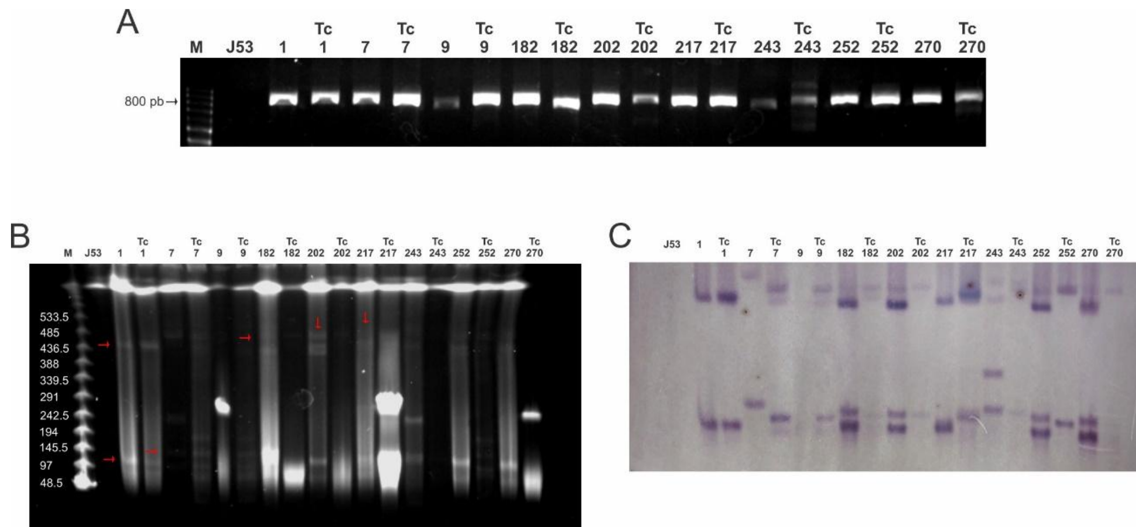

Figure 4. Profile characterization of donors, recipient, and transconjugants strains (Tc). (A) PCR of *bla*$_{\text{CTX-M}}$ gene. A marker of 100 bp was used. (B) Pulsed-field gel electrophoresis. (C) Southern blot hybridization using dig-labeled *CTX-M* DNA probe. Numbers 1-270 represent donor strains used as positive controls. Numbers Tc1-Tc270 represent transconjugants. The recipient strain (J53) is shown as negative control. Red arrows indicate plasmid bands that were similar in transconjugants.

To further confirm the transfer of cephalosporin resistance, antibiotic susceptibility tests were carried out using cefotaxime, ceftiofur, cefuroxime, and ceftriaxone (Figure S2). Results demonstrated that the transconjugant Tc182 was susceptible to ceftiofur but resistant to other cephalosporins tested. In addition, Tc270 was susceptible to three of the cephalosporins tested, but resistant to cefuroxime. Significant differences between the inhibition zones of donors and transconjugants were observed for some isolates (Table 2). However, the other transconjugants showed resistance to all four cephalosporins tested, and the absence of inhibition zones in the disk diffusion assays confirmed their high resistance to cefuroxime. The ESBL-production assays also confirmed the resistance phenotype of the transconjugants, with only Tc252 showing negative results for ESBL production (Figure S3).

Table 2: Antibiotic susceptibility profile of bacterial donors, transconjugants, and recipient strains.

Strains	**Diameter of resistance zone (mm)**			
	Cefotaxime (≤ 22)	*Ceftiofur* (≤ 17)	*Cefuroxime* (≤ 14)	*Ceftriaxone* (≤ 19)
T2.1	10.8	13.1	-	9.6
Tc1	11.6	14.6	-	13.1
p-value	ns	ns	ns	ns
T2.7	12.7	16.5	-	13.7
Tc7	13.1	13.5	-	13.8
p-value	ns	ns	ns	ns

T1.9	12	10.5	-	9.5
Tc9	15.6	15.4	-	13.2
p-value	ns	*	ns	ns
T2.182	9.3	9.9	-	8.1
Tc182	21.1	17.6	-	18.5
p-value	***	**	ns	**
T2.202	11.6	13.8	-	13.1
Tc202	18.2	16.9	-	16.8
p-value	**	ns	ns	ns
T1.217	13.5	14.3	-	13.8
Tc217	13.4	13.7	-	11.6
p-value	ns	ns	ns	ns
T2.243	13.4	12.8	-	11.8
Tc243	12.7	12.9	-	12
p-value	ns	ns	ns	ns
T2.252	14.1	13.5	-	11.9
Tc252	14.1	13.5	-	12.5
p-value	ns	ns	ns	ns
T2.270	14.9	13.7	-	11.7
Tc270	23.3	18.6	-	24.1
p-value	**	ns	ns	**
E. coli J53	32	31	34	34

One (*), two (**), three (***) asterisks represent, respectively, statistical differences at the significance level of 0.05, 0.01, and 0.001. Two-way ANOVA was applied and the p-value was corrected.

4.5 Pulsed-field gel electrophoresis and Southern blot

To analyze if bacterial isolates carried large plasmids that can transfer resistance genes to recipient cells, the transconjugants were subjected to both Pulsed-field gel electrophoresis (PFGE) and Southern blot analysis. The PFGE revealed different plasmid isoforms among the donor bacteria, but transconjugants showed an isoform similar to their respective donors (Figure 4B). Southern blot analysis confirmed the transfer of the conjugative plasmid containing the $bla_{CTX\text{-}M}$ gene to the recipient strain (Figure 4C).

5 DISCUSSION

Resistance to β-lactams, especially to third generation cephalosporins, has become a public health concern because of their wide use to treat microbial infections in clinical settings. In recent decades, antibiotics used to treat sick animals or enhance animal performance have been associated with the emergence of resistance to β-lactams in livestock (Balazs et al., 2021; Lee et al., 2021; Garcia et al., 2022). Additionally, swine farms have been recognized as an

important reservoir of β-lactams genes, particularly among members of the *Enterobacteriaceae* family (Abraham et al., 2018; Fournier et al., 2019; Faccone et al., 2019; Zelendova et al., 2019; Bacci et al., 2020; Misumi et al., 2021; Trongjit et al., 2022; Nguyet et al., 2022). In the present study, *E. coli* isolates recovered from clinically healthy pigs that were fed with or without antibiotics (zinc bacitracin and 5 enramycin) in the finishing stage demonstrated high levels of resistance to β-lactams agents, mainly cephalosporins. Importantly, these animals had no history of exposure to cephalosporins or other classes of antibiotics.

Cephalosporins are generally used to treat respiratory diseases and gastrointestinal disorders in pigs (Callens et al., 2012). Although we cannot fully explain the origin of cephalosporin-resistant isolates in pigs without antibiotic treatment, the presence of ESBL-producing bacteria in healthy swine could be associated with direct transmission of microbiota from sow to piglet during birth or inoculation from environmental sources (e.g., water, feeds, air, human contact, etc.). Moreover, β-lactams are heavily used in livestock production, which can in turn increase the presence of antibiotic residues in the environment and the prevalence of bacterial strains carrying resistance determinants. Importantly, it cannot be ignored that the use of other antibiotics in animal production systems might impose selective pressure to maintain certain resistance genes. This is relevant because ESBL genes are commonly co-localized with other resistance genes on the same plasmid.

All *E. coli* isolates carried bla_{TEM}, and most isolates carried the $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M}$ genes. These results are similar to previous reports demonstrating the distribution of these genes in *E. coli* isolates from swine in South Korea (Lee et al., 2021), Portugal (Fournier et al., 2019) and Czech Republic (Zelendova et al., 2020). Hayer and co-authors (2020) also reported that *E. coli* isolates recovered from diseased pigs were resistant to ceftiofur and carried the $bla_{CTX\text{-}M}$ gene on plasmids. Bastidas-Caldes and co-authors (2022) showed that CTX-M enzymes were the most prevalent ESBL-enzymes in animal sources, while in Brazil $bla_{CTX\text{-}M\text{-}2}$ was reported as the most frequent $bla_{CTX\text{-}M}$ variant gene in animal sources. In the current study, $bla_{CTX\text{-}M\text{-}8,25}$ and $bla_{CTX\text{-}M\text{-}2}$ were also detected in swine but at low frequencies. A previous study reported similar results in Argentina (Faccone et al., 2019), in which *E. coli* recovered from swine harbored $bla_{CTX\text{-}M\text{-}8,25}$ and $bla_{CTX\text{-}M\text{-}2}$. These observations highlight the distribution of β-lactams genes in bacterial isolates recovered from healthy and diseased swine and the emergence of resistance to cephalosporins in the pig farm environment.

Genes conferring resistance to different classes of antibiotics were also found among the *E. coli* isolates, such as sulfonamides, aminoglycosides, tetracyclines, and colistin. Importantly, these resistance genes have been frequently reported in bacteria isolated from

food-producing animals in different geographical regions, despite eventual differences in antimicrobials approved for animal use (Sadek et al., 2021; Liu et al., 2022). For example, Yu and colleagues (2021) reported that *E. coli* from piglets in China harbored antibiotic resistance determinants to several classes, such as aminoglycosides, polypeptides, and quinolones, which were confirmed with phenotypic profiling of antibiotic resistance in their isolates indicating high levels of resistance. These observations are similar to findings from the current work, which indicated that pigs carry resistance genes that confer high levels of resistance to tetracyclines, aminoglycosides, and quinolones.

Few reports describe the presence of carbapenemase genes in bacteria from swine. In the current study, we detected the gene for carbapenemase (bla_{VIM}) in *E. coli* isolates obtained from swine feces, similar results have been reported by Han et al. (2016), who also found a low frequency of bla_{VIM} among *E. coli* isolated from pigs in Korea. In addition, the bla_{OXA-23} gene was detected in two isolates belonging to the T2 group. In an unrelated study with piglets in India, similar findings were reported, and three *E. coli* isolates were found to harbor the gene bla_{OXA-48} (Nirupama et al., 2018). These carbapenemase genes encode enzymes that can hydrolyze most beta-lactam agents, such as carbapenems.

The VIM-enzyme belongs to class B β-lactamases and shows widespread distribution among Gram-negative bacteria (Nordmann et al., 2011; Kazmierczak et al., 2016), while the OXA-enzyme belongs to class D β-lactamase and is mainly encoded in plasmids (Munita and Arias, 2016). Most carbapenemase genes are located in mobile genetics elements and are commonly reported in clinical and wastewater samples. However, the presence of these genes in animal samples has become a public health concern, since β-lactams are also widely used in human medicine (Ma et al., 2020; Lee et al., 2021). The current study demonstrates the presence of these genes in *E. coli* from pigs, however, none of the isolates were resistant to carbapenems in disk diffusion assays. Since gene expression was not evaluated in the current study, it is possible that these genes are non-functional or that the enzymes encoded have a low hydrolytic activity against carbapenems.

Horizontal transfer of bla_{CTX-M} gene was verified in nine *E. coli* isolates, whereas genes for the class I integron (IntI1) were found in 4 of these isolates. A previous study demonstrated that bla_{CTX-M} genes can be associated with class I integrons (IntI1) in *E. coli* isolates from piglets (Xu et al., 2015). Integrons are important genetic elements that carry and spread antibiotic resistance genes, such as those encoding β-lactamases (Kaushik et al., 2018). The presence of integrons in these conjugative plasmids might explain the mobility, insertion, and spread of bla_{CTX-M} gene among commensal bacteria in livestock. Furthermore, once transferred to

plasmid or integron, the *bla*$_{CTX-M}$ gene has extensive opportunities for spreading among different Gram-negative bacteria. Therefore, such plasmids can become key elements defining the distribution of resistant genes in complex microbial communities (Singh et al., 2018).

Pulsed-field gel electrophoresis demonstrated that the plasmid profile of transconjugants was similar to the donor bacteria, and Southern blot hybridization confirmed that the conjugative plasmid carrying the *bla*$_{CTX-M}$ gene was transferred to the recipient strain (Figure 4C). Furthermore, results suggested that different plasmids from *E. coli* potentially encode *bla*$_{CTX-M}$, but further analysis will be needed to investigate the diversity and properties of these mobile elements.

Conjugative plasmids carrying ESBL genes have been widely reported in food-producing animals, such as pigs and cattle (Palmeira et al., 2020; Hayer et al., 2020; Lee et al., 2021; Peng et al., 2022). Horizontal gene transfer plays an important role in the dissemination of *bla*$_{CTX-M}$; however, the presence of clonal isolates could also be important in this dissemination. A report from Schmithausen and colleagues (2015) in which rectal swine samples were collected in the abattoir demonstrated the presence of ESBL-producing *E. coli* in different growth phases. The authors concluded that selective pressure exerted by antibiotics promotes horizontal gene transfer within the *E. coli* population in the intestinal microbiota, which might be influenced by the environment and hygiene. In the current work, three bacterial isolates that harbored conjugative plasmids (T2.182, T2.202, and T2.270) were isolated from the same pen (Figure 1B), suggesting that bacteria carrying ARGs in plasmids can spread from one pig to another. Thus, further studies are needed to investigate the prevalence of *E. coli* carrying the *bla*$_{CTX-M}$ gene in commercial farms in Brazil and to characterize the properties of conjugative plasmid harboring ESBL genes.

BOX-fingerprinting revealed that *E. coli* isolates obtained from pigs that were fed diets with and without antibiotics grouped in the same clade with similarity >90 %. Because the pigs were housed in pens distant from each other, these findings suggest that isolates carrying ESBL genes could circulate in the farm environment and become predominant. However, further analysis will be needed to confirm clonality.

In conclusion, this is the first study reporting plasmids carrying the *bla*$_{CTX-M}$ gene in healthy swine in Brazil. Our study demonstrates a high prevalence of ESBL-positive *E. coli* in healthy swine from Brazilian pig farms, which was supported by a high prevalence of β-lactamase and integron genes among these isolates. Therefore, resistance genes and conjugative plasmids were prevalent even in animals fed diets without antibiotics, emphasizing the wide distribution of these antibiotic resistance genes in livestock. The *bla*$_{CTX-M}$ were horizontally

transferred from nine *E. coli* isolates to a receptor strain and were confirmed in the transconjugants by Southern blot hybridization. These results highlight the significance of mobile genetic elements in spreading resistant determinants. These results also emphasize the role of commensal *E. coli* as an important reservoir of ESBL genes in pig farming. Further studies focused on whole genome sequencing of the isolates will help understand the diversity of ESBL genes circulating in the swine commensal microbiota.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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AUTHORS CONTRIBUTION STATEMENT

RO, DB, and HM conceived the project. RO, JS, JR, and GS performed the data analysis, data visualization, and interpretation of results. RO, JS, GS, JR, DB, and HM wrote the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

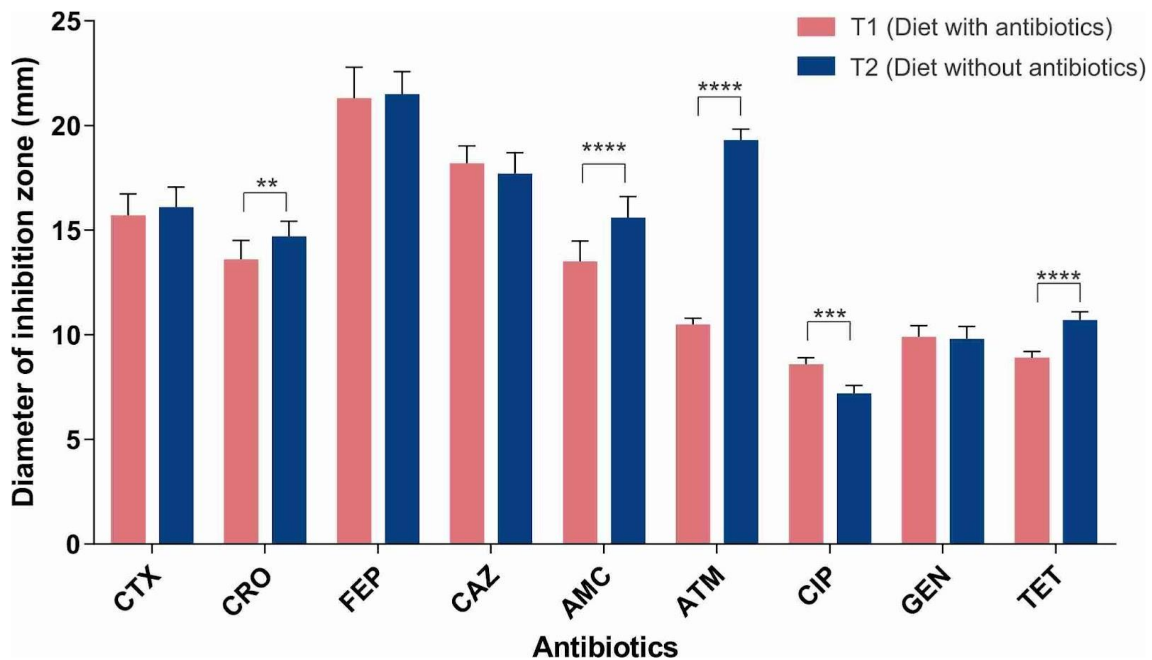
Supplementary Table 1. Primers used in this study.

Gene target	Primer name	Sequence (5' to 3')	Size (bp)	Reference
Genes for bacterial identification				
16S rRNA	27-F 1492-R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	1500	Lane, 1991

BOX1AR	Box1AR	(5' CTACGGCAAGGCGACGCTGACG 3')	-	Louws et al., 1994
EFER_1569	EFER-F EFER-R	GCAATATACAGGACACAGTGTCG CTATGAAGGGAAGGGTAGGAGC	432	Simmons et al., 2014
Resistance genes				
*bla*VIM	VIM-F VIM-R	GTTTGGTCGCATATCGCAAC AATGCGCAGCACCAGGATAG	389	Doyle et al., 2012
*bla*IMP	IMP-F IMP-R	GAAGGCGTTTATGTTCATAC GTACGTTTCAAGAGTGATGC	587	Doyle et al., 2012
*bla*NDM-1	NDM-F NDM-R	TGCATTGATGCTGAGCGGGTG ATCACGATCATGCTGGCCTTG	621	Hou et al., 2015
*bla*KPC	KPC-F KPC-R	TGTCACTGTATCGCCGTC CTCAGTGCTCTACAGAAAACC	900	Doyle et al., 2012
*bla*OXA-48	OXA-48-F OXA-48-R	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438	Nordmann et al., 2011
*bla*OXA-23	OXA-23-F OXA-23-R	AGGTCATTTACCGCTTGG TCCATCTGGCTGCTCAAC	396	Sun et al., 2019
*bla*CTX-M	CTX-M-F CTX-M-R	ATGGTTAAAAAATCACTGCGYCAGTTC TCACAAACCGTYGGTGACGATTTTAGCCGC	876	Hou et al., 2015
*bla*CTX-M(G1)	CTX-MG1-F CTX-MG1-R	TTAGGAARTGTGCCGCTGYA CGATAATCGTTGGTGGTRCCAT	688	Dallenne et al., 2010
*bla*CTX-M(G2)	CTX-MG2-F CTX-MG2-R	CGTTAACGGCACGATGAT CGATAATCGTTGGTGGTRCCAT	404	Dallenne et al., 2010
*bla*CTX-M(G9)	CTX-MG9-F CTX-MG9-R	TCAAGCCTGCCGATCTGGT TGATTCTCGCCGCTGAAG	561	Dallenne et al., 2010

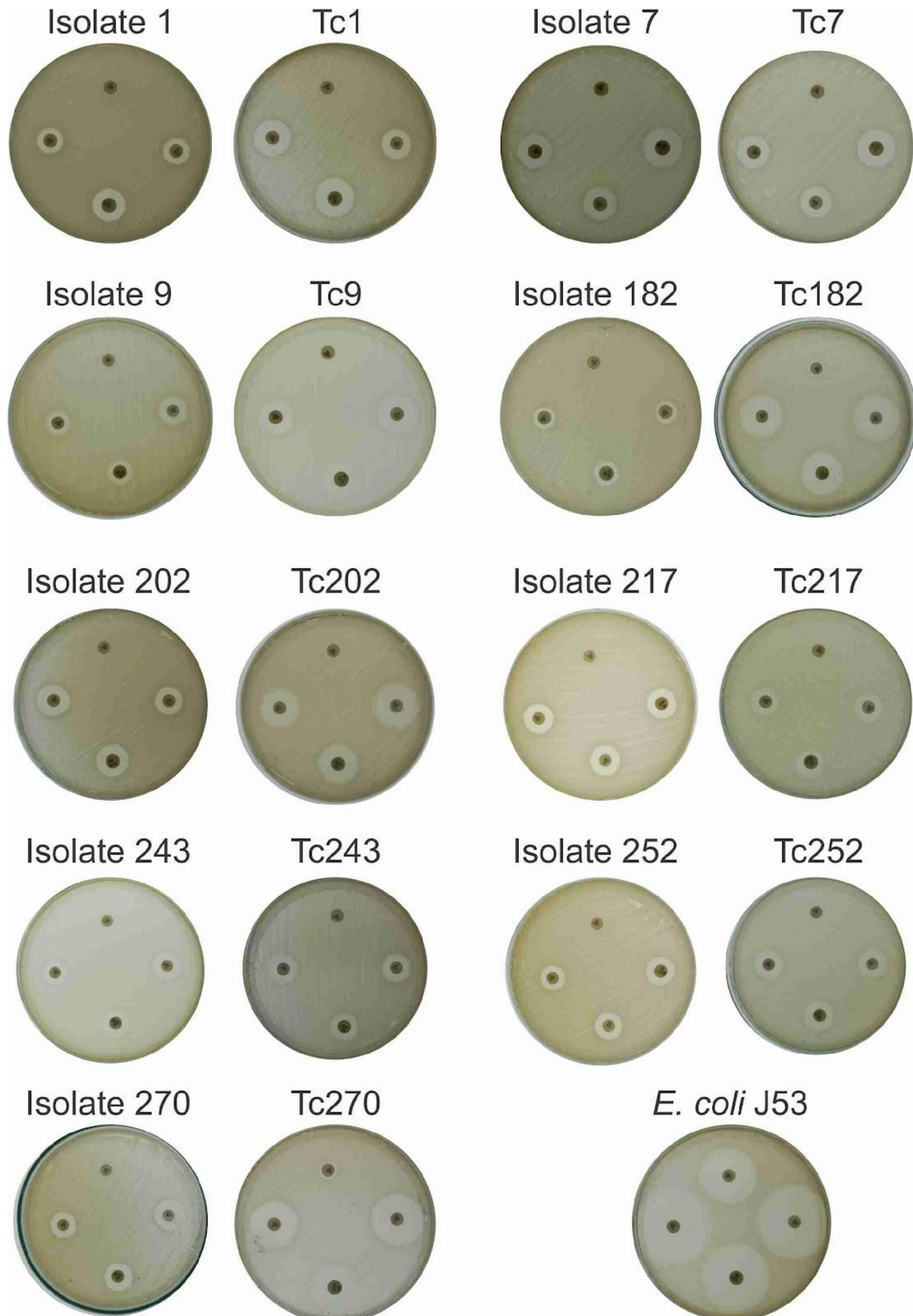
*bla*CTX-M(G8/25)	CTX-M8/25-F CTX-M8/25-R	AACRCRCAGACGCTCTAC TCGAGCCGGAASGTGTYAT	326	Dallenne et al., 2010
*bla*CMY-2	CMY2-F CMY2-R	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	Pérez-Pérez et al., 2002
*bla*TEM	TEM-F TEM-R	CAGCGGTAAGATCCTTGAGA ACTCCCCGTCGTGTAGATAA	643	Winokur et al., 2000
*bla*SHV	SHV-F SHV-R	TTCGCCTGTGTATTATCTCC TTTGCTGATTTCGCTCGG	807	Winokur et al., 2000
ant(3")-I	aadA-F aadA-R	CATCATGAGGGAAGCGGTG GACTACCTTGGTGATCTCG	787	Hu et al., 2013
mcr-1	MCR-1-F MCR-1-R	GGGTGTGCTACCAAGTTTGC CATTGGCGTGATGCCAGTTT	1147	Hatrongjit et al., 2018
gyrA	GYRA-F GYRA-R	GCGATGTCGGTCATTGTTGG CCGAACTGGTCACGGATCAG	1000	Hou et al., 2015
qnrS	QNRS-F QNRS-R	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	417	Robicsek et al., 2006
dfra15	DFRA15-F DFRA15-R	GTGAAACTATCACTAATGG CCCTTTTGCCAGATTTGG	417	Guerra et al., 2000
sul2	SUL2-F SUL2-R	CGGCATCGTCAACATAACCT TGTGCGGATGAAGTCAGCTC	721	Lanz et al., 2003
tetA	TETA-F TETA-R	GTAATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	1000	Saénz et al., 2004
tetB	TETB-F TETB-R	CTCAGTATTCCAAGCCTTTG CTAAGCACTTGTCTCCTGTT	414	Saénz et al., 2004
Genes associated with mobile genetic elements				
IntI1	IntI1-F IntI1-R	ATCATCGTCGTAGAGACGTCGG GTCAAGGTTCTGGACCAGTTGC	890	Rosser and Kav-Young, 1999

Virulence genes				
eltB	ELTB-F ELTB-R	TCTCTATGTGCATACGGAGC CCATACTGATTGCCGCAAT	322	Tamanai-Shacoori et al., 1994
estA	ESTA-F ESTA-R	GCTAAACCAGTAGAGGTCTTCAAAA CCCGGTACAGAGCAGGATTACAACA	147	Hornes et al., 1991
stx1	STX1-F STX1-R	ATAAATCGCCATTCGTTGACTAC GAACGCCCACTGAGATCATC	180	Paton and Paton, 1998
hlyA	HLYA-F HLYA-R	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	533	Paton and Paton, 1998
eaeA	EAE-F EAE-R	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	384	Paton and Paton, 1998
fimH	FIMH-F FIMH-R	GTGCCAATTCCTCTTACCGTT TGGAATAATCGTACCGTTGCG	164	Hojati et al., 2015
csgA	CSG-F CSG-R	ACTCTGACTTGACTATTACC AGATGCAGTCTGGTCAAC	200	Sepehri et al., 2011
aggR	AGG-F AGG-R	CGATACATTAAGACGCCTAAAG CTGATACATTAAATTCATCTGC	339	Andrade et al., 2014

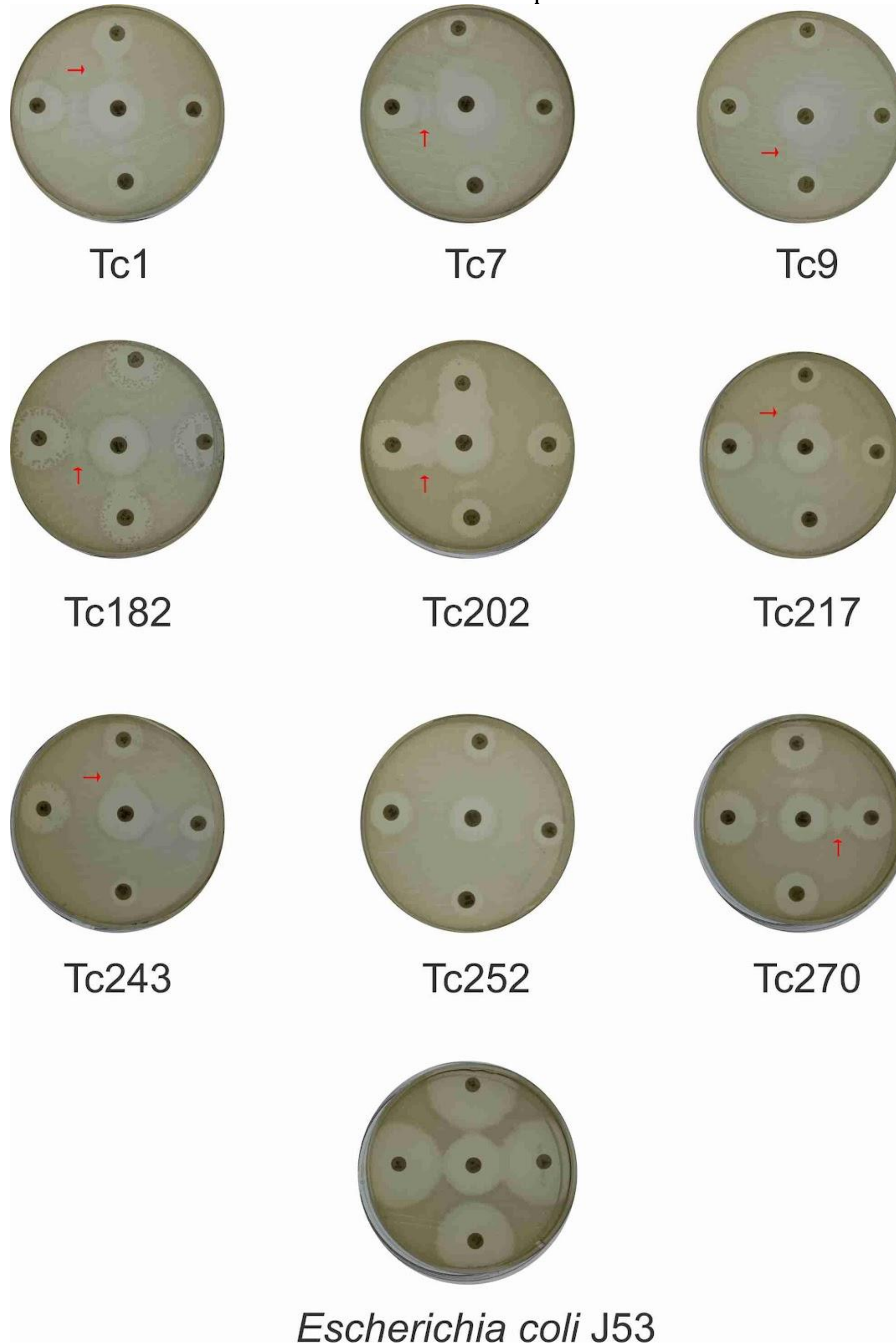


Supplementary Figure 1. Diameter of inhibition zone of the antibiotics against *E. coli* isolates. Some antibiotics did not produce an inhibition zone against the isolates (resistant phenotype) and were not included in the figure. The antibiotic susceptibility profile was assessed by disk

diffusion assay. ** $p < 0.0050$, *** $p = 0.0001$ and **** $p < 0.0001$. Two-way ANOVA was applied, and the p-value was corrected using Sidak's multiple comparisons test. The bars indicate the mean and the standard deviation of the inhibition zone diameter for each antibiotic between the treatments. Abbreviations and zone diameter for resistance (mm): AMC, amoxicillin/clavulanic acid (≤ 13); AMP, ampicillin (≤ 13); ATM, aztreonam (≤ 17); CFZ, cefazolin (≤ 19); FEP, cefepime (≤ 18); CTX, cefotaxime (≤ 22); CAZ, ceftazidime (≤ 17); CRO, ceftriaxone (≤ 19); CRX, cefuroxime (≤ 14); CIP, ciprofloxacin (≤ 21); GEN, gentamicin (≤ 12); TET, tetracycline (≤ 11); TRI, trimethoprim (≤ 10).



Supplementary Figure 2. Susceptibility profile of donor isolates, recipient strain, and transconjugants resulting from the conjugation assay. Disk diffusion assay according to CLSI M100 (2020) and CLSIVET01S (2023) using ceftiofur, cefotaxime, ceftriaxone and cefuroxime confirmed the antibiotic resistance transfer to the recipient strain.



Supplementary Figure 3. ESBL production assay of the isolates and transconjugants. Disk diffusion assay according to the CLSI using ceftriaxone, cefepime, cefotaxime, ceftazidime, and amoxicillin-clavulanate placed at the center of the plate. Red arrows indicate the expected results to confirm ESBL production.

CHAPTER 2

Genomic insights into multidrug-resistant *Escherichia coli* from clinically healthy pigs: reservoirs of conjugative ESBL plasmids in livestock

(Manuscript written according to the guidelines of Applied Microbiology - an Open Access Journal from Oxford University Press)

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Abstract

Aim: This study aimed to perform the genomic characterization of three MDR-*E. coli* isolates from clinically healthy pigs and their conjugative plasmids associated with $bla_{CTX\text{-}M\text{-}type}$ and bla_{TEM} genes.

Methods and results: DNA of MDR - *E. coli* isolates were extracted and sequenced using an Illumina NovaSeq platform. Genome assembly were performed using SPAdes. The contigs were annotated using Prokka, and further in silico analyses were performed. Genome sizes ranged from 4.9 to 5.2 Mb and Multilocus Sequence Typing (MLST) revealed that isolates UFV_ECT1-72, UFV_ECT1-217 and UFV_ECT2-7 belonged to sequence types of 101, 5229 and 1653, respectively. Manual genome annotation identified partial sequences of two plasmids carrying $bla_{CTX\text{-}M}$ variants, both classified within the IncF incompatibility group and exhibiting variable sizes. Additionally, both *in vitro* and *in silico* analysis revealed the presence of a conjugative plasmid harboring bla_{TEM} gene. Conjugation assays demonstrated successful plasmid transfer to a recipient strain, which acquired a β-lactam resistance phenotype.

Conclusions: The genomic characterization of MDR-*E. coli* isolates from clinically healthy pigs highlights the potential role of livestock as reservoir of antimicrobial resistance genes. The presence and mobilization of resistance determinants via conjugative plasmids, underscore the risk of AMR dissemination within animal populations and possibly across species barriers.

Impact Statement

Our study emphasizes the critical need for comprehensive genomic investigations of bacterial isolates from clinically healthy animals to explore their role as reservoirs of antimicrobial resistance genes. We provide evidence of the alarming prevalence of multidrug-resistant conjugative plasmids in *Escherichia coli* isolated from clinically healthy pigs in Brazilian farms, carrying resistance determinants against multiple antibiotic classes—most notably β-lactams, including *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-55}$, and *bla*$_{TEM}$ genes, regardless of prior antibiotic exposure. Our data further elucidate the horizontal gene transfer of resistance genes within pig intestinal microbiota, underscoring its essential yet often overlooked role in the dissemination of resistance among healthy animals in livestock settings.

Keywords: Food-producing animals; One Health; Horizontal gene transfer; CTX-M; *Bla*$_{TEM}$.

6 INTRODUCTION

Antimicrobial resistance (AMR) is a globally threat and infections due to it increased at alarming levels (WHO, 2024). In 2019, bacterial AMR was associated with 5 million deaths (Murray et al., 2022). Despite being commonly associated with healthcare-associated infections, in other environments, such as livestock, companion animals and wild animals, these levels of resistance have significantly increased (García et al., 2022; Zalewska et al., 2023; McCormick et al., 2023; Mediouni et al., 2025). The misuse and overuse of antibiotics increase the emergence of AMR in livestock (Andersson et al., 2020; Lee et al., 2022). Otherwise, resistance to veterinary clinically important antibiotics has been increasing in the Enterobacteriaceae family, including β-lactams, aminoglycosides and tetracyclines (WOAH, 2021).

Escherichia coli is a commensal member of gut microbiota in both humans and animals and is also commonly found in environmental settings such as water and soil (Hammer-Dedet et al., 2022; Song et al., 2022). In addition, it is one of the major causative agents of both intestinal and extraintestinal infections (Kaper et al., 2004). Due to its ubiquity and genome plasticity, commensal *E. coli* can serve as an important reservoir of antibiotic resistance genes (ARGs) across various environments, particularly within the gut microbiota in both animals and humans (Ramos et al., 2020; Tawfick et al., 2021). This genomic plasticity is largely attributed to the presence of mobile genetic elements (MGEs), including plasmids, transposons, and insertion sequences (Davies and Davies, 2010; Partridge et al., 2018). The MGEs play a key role in the dissemination of ARGs through horizontal gene transfer mechanisms, including conjugation (Arnold et al., 2022).

Extended-spectrum beta-lactamase genes (ESBL), including *bla*$_{CTX\text{-}M\text{-}type}$ and *bla*$_{TEM}$ genes, encoding enzymes that are responsible for the acquired resistance to β-lactams, which are commonly reported associated with MGEs (Peirano and Pitout, 2019). In recent decades, resistance to β-lactams antibiotics has increased among food-producing animals, becoming significant public health concerns due to the potential spread of these ESBL genes through animal products to humans and the environments (Palmeira et al., 2020; Peng et al., 2022; Seo et al., 2023; Song et al., 2024). However, the circulation of ESBL genes among clinically healthy animals, including pigs, requires further attention and investigation to better understand the transmission dynamics and emergence of these genes through MGEs, including plasmids.

Next generation sequencing is a valuable tool for the genomic characterization of antimicrobial resistance and for the epidemiological surveillance of multidrug plasmids involved in AMR. We previously isolated multidrug resistant (MDR) *E. coli* isolates harboring multiple ARGs from clinically healthy pigs (Oliveira et al., 2024). Here, we aimed to perform a genomic characterization of three MDR-*E. coli* isolates and characterize the plasmids associated with *bla*$_{CTX\text{-}M\text{-}type}$ genes by using *in silico* approach, confirming our previous *in vitro* results. We, therefore, characterized a multidrug-conjugative plasmid carrying *bla*$_{TEM}$ gene and other ARGs by *in silico* and *in vitro* analysis.

7 MATERIAL AND METHODS

7.1 Bacterial isolates

The isolates of this study were obtained from rectal samples from clinically healthy pigs in finishing phase from the swine sector of the Animal Science Department at the Universidade Federal de Viçosa, Brazil in 2019, according to guidelines approved by the Ethics Committee on the Use of Production Animals of the Universidade Federal de Viçosa (CEUAP/UFV) under Protocol no. 027/2021 (Oliveira et al., 2024). Briefly, the animals were received a diet supplemented with 55 ppm of zinc bacitracin and 5 ppm of enramycin (T1 group – 48 animals) or a diet without antibiotics (T2 group – 48 animals). The samples were obtained using sterile swabs and were streaked on MacConkey agar (Kasvi, Conda Laboratories, Spain) supplemented with 8 µg. mL^{-1} ceftiofur. The bacterial isolates were subjected to molecular identification by 16s rRNA gene sequencing and biochemistry assays, and were identified as *Escherichia coli*. The isolates were classified as multidrug-resistant and harbored diverse AMR genes. Thus, three isolates of MDR-*E. coli* (UFV_ECT1-72, UFV_ECT1-217 and UFV_ECT2-7) were selected for whole genome sequencing, according to their phenotype and genotype characteristics described in Table 1.

Table 1. Phenotype and genotype characteristics of isolates used in this study.

Isolate ID*	Treatment	Conjugation of ARGs	AMR genes	AMR profile	ESBL production
UFV_ECT1-72	1	bla_{TEM}	$bla_{CTX\text{-}M\text{-}2}$, bla_{TEM}, *sul2, ant3"I, tetB*	AMC, AMP, ATM, CFZ, CTX, CRO, CRX, FEP, CIP, GEN, TET, TRI	-
UFV_ECT1-217	1	$bla_{CTX\text{-}M}$	$bla_{CTX\text{-}M\text{-}1}$, bla_{TEM}, *dfrA15, sul2, ant3'I, tetA, tetB*	AMC, AMP, CFZ, CTX, CRO, CRX, CAZ, CIP, GEN, TET, TRI	+
UFV_ECT2-7	2	$bla_{CTX\text{-}M}$	$bla_{CTX\text{-}M\text{-}1}$, bla_{TEM}, $bla_{OXA\text{-}23}$, *qnrS, mcr-1, dfrA15, sul2, ant3"I, tetB*	AMP, CFZ, CTX, CRO, CRX, CIP, GEN, TET, TRI	+

*Reference of the isolates: Oliveira et al., 2024. Legend: CFZ: cefazolin; CTX: cefotaxim; CRO: ceftriaxone; CRX: cefuroxime; FEP: cefepime; CAZ: ceftazidime; AMC: amoxicillin-clavulanate; ATM: aztreonam; AMP: ampicillin; CIP: ciprofloxacin; GEN: gentamicin; TET: tetracycline; TRI: trimethoprim.

7.2 Genome sequencing and assembly

Bacterial genomic DNA from the three isolates were extracted using the Wizard Genomic DNA Purification Kit following the manufacturer's instructions (Promega, Madison, USA). The purity and quality of DNA were determined by measuring the A260/280 and A260/230 ratios using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts, EUA). The integrity of DNA was evaluated by agarose gel electrophoresis 0.8 %. The bacterial genomes were sequenced using the Illumina system at Macrogen (Seoul, South Korea) with the NovaSeq platform and paired-end reads, using the TruSeq Nano DNA (350 bp) library preparation kit. Genome assembly was performed de novo method using SPAdes v. 3.15.5 (Bankevich et al., 2012). Assembly quality was assessed using QUAST (Gurevich et al., 2013). Species-level identification was conducted using Average Nucleotide Index (ANI) analysis.

The assembled genomes have been deposited in the NCBI database under BioProject PRJNA1127590 with accession numbers JBEUMT010000000, JBEUMU010000000 and JBEUMV010000000 corresponding to UFV_ECT1-72, UFV_ECT1-217 and UFV_ECT2-7, respectively.

7.3 *In silico* genome analysis

7.3.1 Genome annotation and molecular typing

Genome annotation of the MDR-*E. coli* genomes (UFV_ECT1-72, UFV_ECT1-217 and UFV_ECT2-7) was carried out using Prokka 1.14.6 (Seeman, 2014) and NCBI Prokaryotic Genome Annotation Pipeline v.3.2 (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). Additionally, analysis of pan-genome and core genome was performed using MicroScope (Vallenet et al., 2019).

Escherichia coli genotyping was performed using in silico multi-locus sequence typing (MLST) based on the Achtman scheme (Wirth et al., 2006), which considers allelic variation in seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA* and *recA*) to assign sequences types (STs). The analysis was performed using the PubMLST database (Jolley et al., 2018), with cutoffs of 90 % identity and a minimum length of 60 %. Additionally, *E. coli* phylogroups were assigned according to the Clermont Typing scheme (Beghain et al., 2018), and *fimH* allelic typing was conducted using FimTyper 1.0 (Roer et al., 2017).

7.3.2 Genomic comparative analysis

The MDR-*E. coli* genomes were mapped against the complete genome of *E. coli* str. K-12 substr. MG1655 (U00096.3), as reference strain, for visualization of the conserved genomic sequences and genomic organization by using Progressive Mauve (PM) (Darling et al., 2012).

The MDR-*E. coli* genomes were mapped with an MDR-*E. coli* from human and pig sources. The human source was MDR- *E. coli* strain EC121 (VYQD00000000.1) from bloodstream infection isolated in Brazil in 2007, which also belonged to ST 101 (Santos et al., 2020). In addition, the pig source was MDR-*E. coli* strain XD34 (JAEMYT000000000.1) isolated from pig farms in China, which also belonged to ST 5229 and had MDR profile (Peng et al., 2022). The strains were visualized in comparison with the *E. coli* str. K-12 substr. MG1655 by using Proksee (Grant et al., 2023).

7.3.3 Characterization of the resistome and virulome

To characterize the resistome and virulome of the MDR-*E. coli* genomes investigated, the ResFinder 4.5 (Camacho et al., 2009) and Comprehensive Antibiotic Resistance Database 3.2.9 (CARD) (Alcock et al., 2023) were used concurrently to predict antibiotic resistance genes (ARGs) and detect chromosomal point mutations in quinolone resistant genes. The predicted ARGs represent a combination of results from both databases based on 90 % identity threshold. VirulenceFinder 2.0 (Joensen et al., 2014) available at the Center for Genomic Epidemiology

(https://cge.food.dtu.dk/services/VirulenceFinder/) was used to predicted and annotate virulence factors (VFs) among the strains.

7.3.4 *In silico* identification of mobile genetic elements (MGEs)

To identify plasmids carrying ARGs, PlasmidFinder 2.1 with a 95% identity cutoff (Carattoli et al., 2014), oriTFinder (Li et al., 2018), MOB-suite 3.0.3 (Robertson and Nash, 2018), MOBScan (Garcillán-Barcia et al., 2020) and CONJScan (Cury et al., 2019) were used to characterize plasmids sequences and to predict relaxase families and conjugative regions of putative mobile elements. Additionally, ISFinder (Siguier et al., 2006) and IntegronFinder 2.0 (Néron et al., 2022) were used in conjunction with MGEFinder 1.1.2 (Johansson et al., 2020) to confirm the presence of insertion sequences (ISs) and transposons. Gene organization diagrams were drawn by SnapGene software (https://www.snapgene.com/).

7.4 Pulsed Field Gel Electrophoresis (PFGE)

Plasmid content and size of the three MDR-*E. coli* strains were assessed by pulsed field gel electrophoresis (PFGE) using the S1 restriction enzyme (Barton et al., 1995). PFGE was carried out following the standardized protocol for bacterial DNA using the CHEF-DR II system (Bio-Rad Laboratories, Nazareth, Belgium) at 14 °C, with a field strength of 5 V/cm² applied for 20 h. The gel was stained with ethidium bromide (0.5 µg. mL^{-1}) and visualized using a Gel Imaging System (L-Pix Chemi, Cotia, São Paulo). The *E. coli* J53 strain was used as a DNA genomic control.

7.5 In - depth characterization of pEC72A: a conjugative multidrug resistance plasmid

Conjugation was performed using a broth mating assay according to Koo and Woo (2011) with modifications, using *E. coli* J53 Azi^R (Yi et al., 2012) as the recipient strain and UFV_ECT1-72 as donor strain. Briefly, overnight cultures of both donor and recipient strains were adjusted to OD_{600} to 0.1 in fresh LB broth and were incubated at 37 ° C under agitation (180 rpm) until reached OD_{600} 0.7 - 1.0. For the mating experiment, recipient and donor strains were mixed at a 1:3 ratio and incubated for 4 h at 37° C. Following incubation, 100 µL of the mixture was plated on LB agar supplemented with 100 µg. mL^{-1} sodium azide and 100 µg. mL^{-1} ampicillin. In order to count number of donors in the mating experiment, the dilutions were also plated on LB agar supplemented with 100 µg. mL^{-1} ampicillin. The conjugation frequencies were calculated as the number of transconjugants per donor. The mating experiments were performed in triplicate. To confirm successful conjugation,

transconjugant colonies were subjected to PCR to detect the presence of *bla_{TEM}* gene (F: 5'-ACAGCGGTAAGATCCTTGAGAG-3'; R: 5'-GAAGCTAGAGTAAGTAGTTCG-3'; size: 461 bp).

The transconjugants were also evaluated to antimicrobial susceptibility through minimum inhibitory concentration (MIC) testing for ampicillin and disk-diffusion test for ceftiofur and ceftriaxone, according to the guidelines of CLSI (Clinical Laboratory Standards Institute) (2020) and CLSIVet (2023). The PFGE with S1 nuclease treatment was performed to confirm the transfer of conjugative plasmids and to evaluate the plasmid profile of the transconjugants. All the steps of the S1-PFGE were according to the description mentioned in the previous section 2.4. The stability of plasmid in the transconjugant was carried out by serial passage for 7 days in LB agar without antibiotics. After, bacterial numbers were counted by plating onto LB agar supplemented or not with ampicillin.

Plasmid DNA was extracted from the transconjugant strains using the PhoenIX™ Maxiprep Kit (MP Biomedicals, LLC, France) according to the manufacturer's instructions. The plasmid DNA was subjected to DNA Library Prep for Illumina platform and sequenced using the 300-bp paired-end Illumina Miseq i100 plus system. Quality control of the raw reads was performed using FastQC v0.12.1, and low-quality reads were trimmed using Trimmomatic v0.39 (Bolger et al., 2014). The trimmed reads were de novo assembled using Unicycler v0.5.1 (Wick et al., 2017), and genome annotation was performed with Prokka v1.14.6 (Seemann, 2014). In silico analysis was performed with the web-based tools described in previous section 2.3.3. The final plasmid map was visualized using Proksee (Grant et al., 2023).

8 RESULTS

8.1 Genomic characterization and comparative analysis of MDR - *Escherichia coli* strains

The genomes of *E. coli* strains UFV_ECT1-72, UFV_ECT1-217 and UFV_ECT2-7 were sequenced, and their phylotypes, serotypes, sequence types (ST), and accession numbers are presented in Table 2, along with genomic features including genome length, GC content, N50 value, number of coding sequences (CDs) and RNAs genes. The genome sizes ranged from 4.9 Mb to 5.2 Mb, with a GC content varying between 50.3 % and 50.4 %. Based on phylogenetic typing and MLST, all three strains were classified within phylogroup B1. The strains from treatment 1, UFV_ECT1-72 and UFV_ECT1-217 were classified as O8:H45-ST101-*fimH86* and O51:H51-ST5229-*fimH86*, respectively. Regarding the strain from

treatment 2, the strain UFV_ECT2-7 was assigned to the serotype O(uncertain):H11, belonged to ST1653, and carried the *fimH31* allele.

Table 2. Genome features and characterization of MDR-*E. coli* strains from clinically healthy pigs investigated in this study.

Genomic characteristics			
	UFV_ECT1-72	UFV_ECT1-217	UFV_ECT2-7
Accession number	JBEUMT010000000	JBEUMU010000000	JBEUMV010000000
Genome size (bp)	5.125.197	4.982.985	5.225.408
GC content (%)	50.3	50.4	50.4
N50	122.579	231.808	241.897
Genes (total)	5046	4890	5190
CDs* (total)	4958	4796	5097
CDs (with protein)	4730	4594	4885
Genes (RNA)	88	94	93
rRNAs (5S, 16S, 23S)	6	11	10
tRNAs	73	73	74
ncRNAs	9	10	9
Pseudogenes (total)	228	202	212
Molecular characteristics			
ST**	101	5229	1653
Clonal complex	ST101 Cplx	ST101 Cplx	-
Phylogroup	B1	B1	B1
fimH	*fimH86*	*fimH86*	*fimH31*
Serotype	O8:H45	O51:H51	O(uncertain):H11

*CDs. Coding sequences; **ST. Sequence type.

The functional analysis of Cluster Orthologous Groups (COG) of the three strains, illustrated that the genes were divided into 20 categories of COGs, with number of genes in each category, similar among the three strains (Figure 1). A high number of genes with function unknown or uncharacterized was also observed. Furthermore, genes encoding defense mechanisms present in the strains, which included regulatory proteins (*ampE)*, transport permease proteins, efflux pump (*acrB*), type I restriction modification enzymes (*hsdM*), transporters (*yadG*), and others. The functional categories of each strain genome were described in supplementary Appendix A.

A total of 12061 genes were detected the strains, which constitute the core-genome of the MDR-*E. coli* strains. Whereas, the variable-genome compromised a total of 2585 genes among the three strains. The UFV_ECT2-7 strain possessed the highest number of coding sequences (CDs) among the strains (n=5009), of which 19.7% CDs belonged to the variable genome and 14.7% were strain-specific CDS. The individual gene counts for each strain are presented in Table 3.

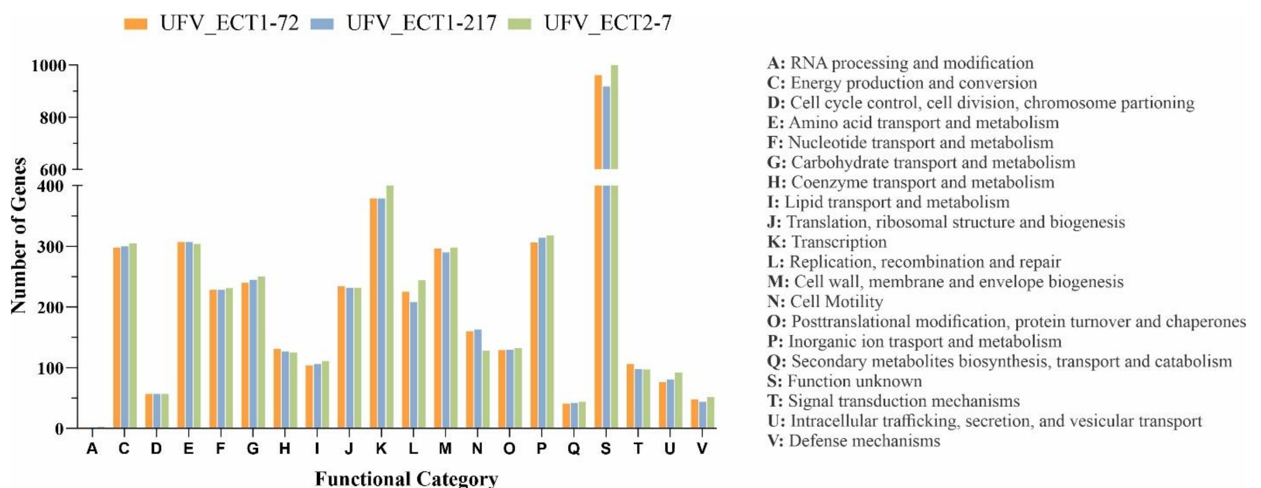

Figure 1. Distribution and comparison of COG functional categories in the three MDR-*E. coli* genomes from clinically healthy pigs. The COG categories are divided into 20 categories, and are shown on the X-axis as alphabets, with category names on the right.

Table 3. Number and frequency of gene counts for each MDR-*E. coli* genomes from clinically healthy pigs.

Strain ID	Pan CDs*	Core CDs (%)	Variable CDs (%)	Strain specifics CDs (%)

UFV_ECT1-72	4739	4027 (82.1)	871 (17.8)	510 (10.4)
UFV_ECT1-217	4898	4011 (84.6)	728 (15.3)	352 (7.4)
UFV_ECT2-7	5009	4023 (80.3)	986 (19.7)	740 (14.7)

*CDS: coding sequences.

In addition, the genomes of the three strains were compared with *E. coli* genomes from human and pig sources, and with reference strain *E. coli* K-12 (Figure 2).

As a result, the regions indicated by white blocks, which are absent in the sequenced strains but present in the reference genome (*E. coli* K-12), are primarily composed of metabolic gene clusters. These include the *ydj* gene cluster (involved in the catabolism of an uncharacterized carbohydrate) and the *ato* operon (associated with the degradation of acetoacetate and butyrate). Although present in *E. coli* K-12, these loci are not part of the species core genome and are variably distributed among *E. coli* lineages.

Furthermore, accessory genome of the strains (represented as gaps in each genome) almost included genes for integrases or transposases at the beginning of the region, in addition to genes involved in heavy metals resistance, siderophores, phages and others. The description of the strain-specific genes and variable genes of each genome were demonstrated in Appendix B and C. Some regions are shared between the strains UFV_ECT1-72 and UFV_ECT1-217, one of these regions harbored IS*406-cmlA1-ant1* genes. Interestingly, some genomic regions, such as the cryptic type II secretion system (*gsp* operon) and *fec* operon (Ferric Citrate Transport), were found exclusively in strain UFV_ECT2-7 and were absent in the other two strains.

In comparative analysis with *E. coli* genomes from human and pig sources, it several genomic regions were identified as being exclusive to strains from human source. Most of which correspond to gene clusters involved in metabolic functions. Conversely, other regions were present found exclusively in *E. coli* K-12, suggesting the presence of strain-specific genes (Figure 2). Notably, the *lsr* operon, responsible for regulating the expression of autoinducer-2 (AI-2), a molecule involved in quorum sensing mechanism, was observed only in *E. coli* from pig sources, including those sequenced in this study.

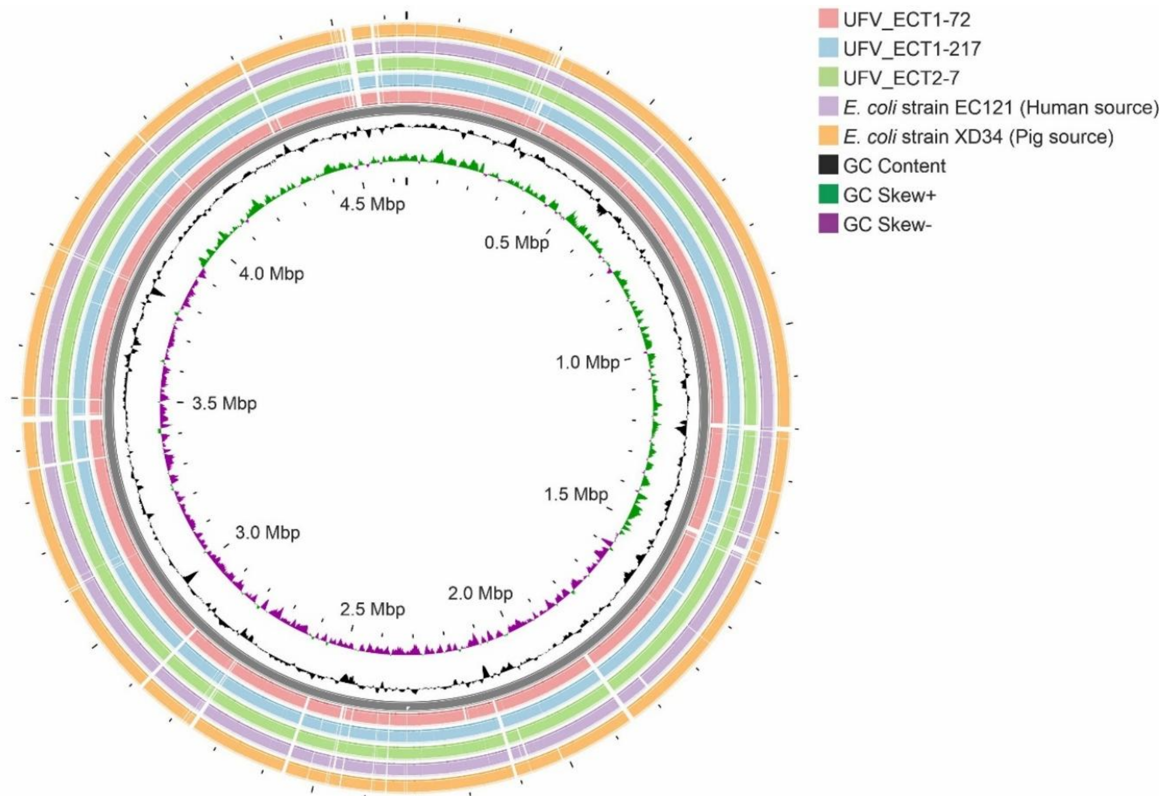

Figure 2. Genome comparative visualization of the three sequenced strains using *E. coli* K-12 as reference (U00096.3), *E. coli* EC121 (VYQD00000000.1) and *E. coli* XD34 (JAEMYT000000000.1) from human and pig source, respectively. From inside to outside, the first circle illustrates GC Skew, the second circle illustrates GC content, and the third to sixth circle signifies *E. coli* K-12 (gray circle), UFV_ECT1-72, UFV_ECT1-217, UFV_ECT2-7, *E. coli* XD34 and *E. coli* EC121, respectively. Some regions (white blocks) were absent in the strains sequenced and presented only in the reference genome, otherwise, there are regions that were absent in the three strains of this study and in the *E. coli* from pig source, but present in the human source and the reference genome, almost all involved in metabolic functions.

8.2 Antibiotic resistance and virulence genes in MDR - *E. coli* genomes

Consistent with their MDR phenotype (Oliveira et al., 2024), resistome analysis of the strain's genomes confirmed the resistance profiles previously observed. The resistome analysis revealed acquired resistance genes and chromosomal mutations that confer resistance to different antibiotic classes in the three strains genomes. The strains evidenced combinations of mechanisms of the antibiotic resistance, which included target alteration (*gyrA*, *parC, ugd*), antibiotic efflux pumps (*emrB, acrB, tetAB),* antibiotic inactivation (*bla* genes, *aadA, mphA*) and target replacement (*sul1, dfrA*). Aminoglycoside resistance genes were the most predominant in the three strains, for example, the *aadA1* gene was detected in the three strains (Figure 3A). Each strain harbored a different $bla_{CTX\text{-}M}$ variant, and the UFV_ECT2-7 simultaneously harbored the $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$ and $bla_{TEM\text{-}1B}$.

Additionally, chromosomal mutations mediating antibiotic resistance were also identified in the three strains, such as, mutations in quinolone resistance-determining region (QRDR) in *gyrA*, and *parC* genes. Mutations in *parE* gene were also detected (Table S1).

In relation to virulence genes identified belong to functional categories, including toxins, iron uptake and adhesion genes. Genes encoding toxins (*hlyE*) was detected in the three strains, whereas *astA* gene was detected only in strain UFV_ECT1-72 (Figure 3B). Otherwise, genes encoding adhesion were *fimH, lpfA,* and *fdeC* were also present. Virulence genes for protectins/serum resistance *traT, iss*, and outer membrane protein T (*ompT*) were detected in two strains (UFV_ECT1-72, UFV_ECT2-7). Aerobactin siderophore (*iuc* operon) genes were detected in UFV_ECT2-7 strain. Additionally, the *terC* gene, which encodes tellurite resistance protein, was detected in all strains.

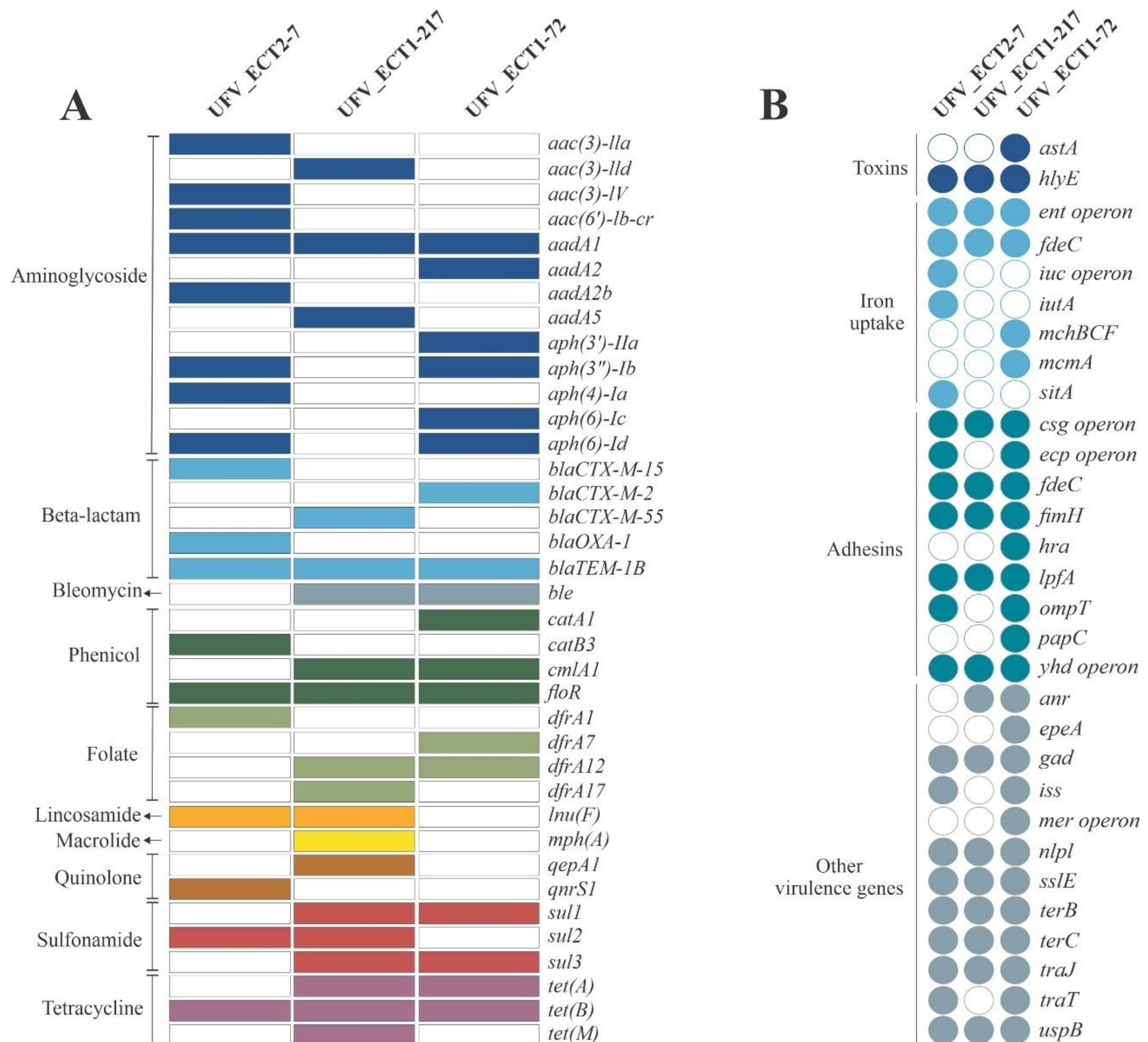

Figure 3. Genotype profile of acquired antibiotic resistance genes and virulence factors in the three MDR-*E. coli* genomes from clinically healthy pigs. (**A**) Confirmed the multirresistance

phenotype previously reported (Oliveira et al., 2024), whereas the colors blocks mean the presence of ARGs in each strain. (**B**) The color circles mean the presence of virulence genes in each strain. The operon which encodes the aerobactin siderophore was detected in strain UFV_ECT2-7.

8.3 Plasmids associated with *bla*$_{CTX\text{-}M\text{-}TYPE}$ in MDR - *E. coli*

In our previous study (Oliveira et al., 2024), the *bla*$_{CTX\text{-}M}$ gene was detected on plasmids of different sizes in strains UFV_ECT1-217 and UFV_ECT2-7 through in vitro analysis. In this study, S1-PFGE analysis was used to confirm the presence of the plasmids based on sizes from UFV_ECT1-217 (~48.5 kb, ~97 kb, and ~485 kb) and UFV_ECT2-7 (~97 kb, ~145.5kb, 242.5 kb, and 533.5 kb) strains (Figure S1). Nevertheless, in this previous work, we were unable to demonstrate conjugation in the strain UFV_ECT1-72 with the antibiotic used (Ceftiofur), despite the strain carrying the *bla*$_{CTX\text{-}M\text{-}2}$ gene. However, in this present study, we demonstrated that this strain harbored three plasmids (~97 kb, ~130 kb, and 242.5 kb).

In silico analysis from WGS data demonstrated that there were several incompatibility groups among the three strains, twelve different plasmid replicons were detected, and IncFIB, IncFII, and IncY were shared (Table S2). Interestingly, in strain UFV_ECT2-7, the IncFII was detected as two copies in different contigs. According to oriTfinder, all the three strains carried components of conjugative machinery, such as relaxase, the type IV coupling protein (T4CP) gene, type IV secretion system (T4SS)-related genes (Table S2).

As described above, in our previous study, we confirmed that the *bla*$_{CTX\text{-}M}$ gene was located on conjugative plasmids through mating assays, and this was further verified by PFGE analysis and Southern blot hybridization (Oliveira et al., 2024). Here, the genetic context of these putative plasmids harboring the *bla*$_{CTX\text{-}M}$ gene was analyzed.

According to MOB-recon analysis, in the strain UFV_ECT1-217, six regions were reconstructed with different sizes, these regions were characterized using web-based tools to identify, if present, their replicon type, conjugative machinery and ARGs. One of these regions, identified as AA941 with ~ 49 kb, the PlasmidFinder results indicated that it belonged to the IncFII group. Additionally, the region carrying eight ARGs, such as *bla*$_{CTX\text{-}M\text{-}55}$, *dfrA12, aadA5, sul1* and others (Figure 4). The fragment also carried the gene cluster *mphA-mrx-mphR* that is macrolide inactivation gene cluster. Additionally, genes related to type II toxin/antitoxin system (*pemK/pemI*), replication (*repA*) and transposases were identified in this region. However, BLASTn comparison was inconclusive in the identification of similar plasmids, as the region was divided in different contigs (11 contigs), thus may be a fragment of plasmid that was reassembled by the tool.

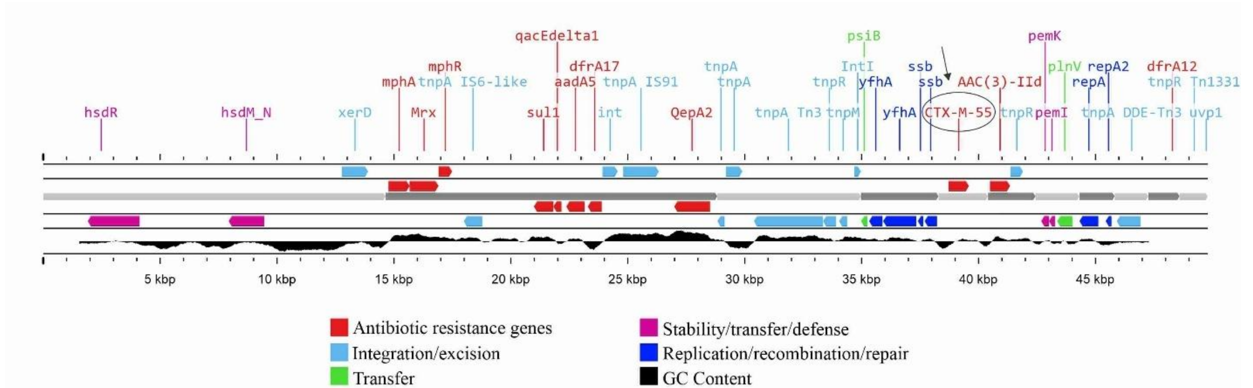

Figure 4. Genetic organization of putative plasmid from *E. coli* strain UFV_ECT1-217 harboring *bla*$_{CTX-M-55}$ gene. The organization illustrates the genomic context surrounding the *bla*$_{CTX-M-55}$ (black arrow) with additional ARGs in red, transfer and stability genes in pink, integration and excision in light blue.

Regarding the *E. coli* strain UFV_ECT2-7, one of the regions predicted by MOB-Recon tool, named region AA324 (~147 kb), harbored ESBL genes (*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$) and other ARGs related to resistance to tetracycline (*tetAB*) and aminoglycosides (*aac(6')lb-cr6, aac(3)-IIe*). PlasmidFinder results indicated that the putative plasmid belonged to the IncFIA group and, according to oriTFinder tool, harbored the complete conjugative machinery, suggesting that was a conjugative plasmid, confirmed the in vitro results obtained in our previous study for this strain (Oliveira et al., 2024). The BLASTn results indicated that plasmid AA324 exhibited 99% identity and > 85% of coverage with plasmid pJMK01 (CP180394.1) (accessed on June 25, 2025) from *Escherichia coli* strain JMK_KBN3987 isolated from blood from *Homo sapiens* in South Korea in 2015 (unpublished data). The plasmid pJMK01 was aligned against the plasmid AA324 in Proksee tool (Figure 5A). As a result, the plasmid pJMK01 displayed a *bla*$_{CTX-M-15}$ gene and other ARGs like plasmid AA324 from UFV_ECT2-7 strain.

Additionally, the *bla*$_{CTX-M-15}$ genetic context was characterized (Figure 5B), which was flanked by truncated IS*Ecp1* (948 bp) upstream and by *wbuC* gene and truncated *Tn3* transposon (1302 bp) downstream, demonstrated a conservated gene cluster of *bla*$_{CTX-M-15}$ gene.

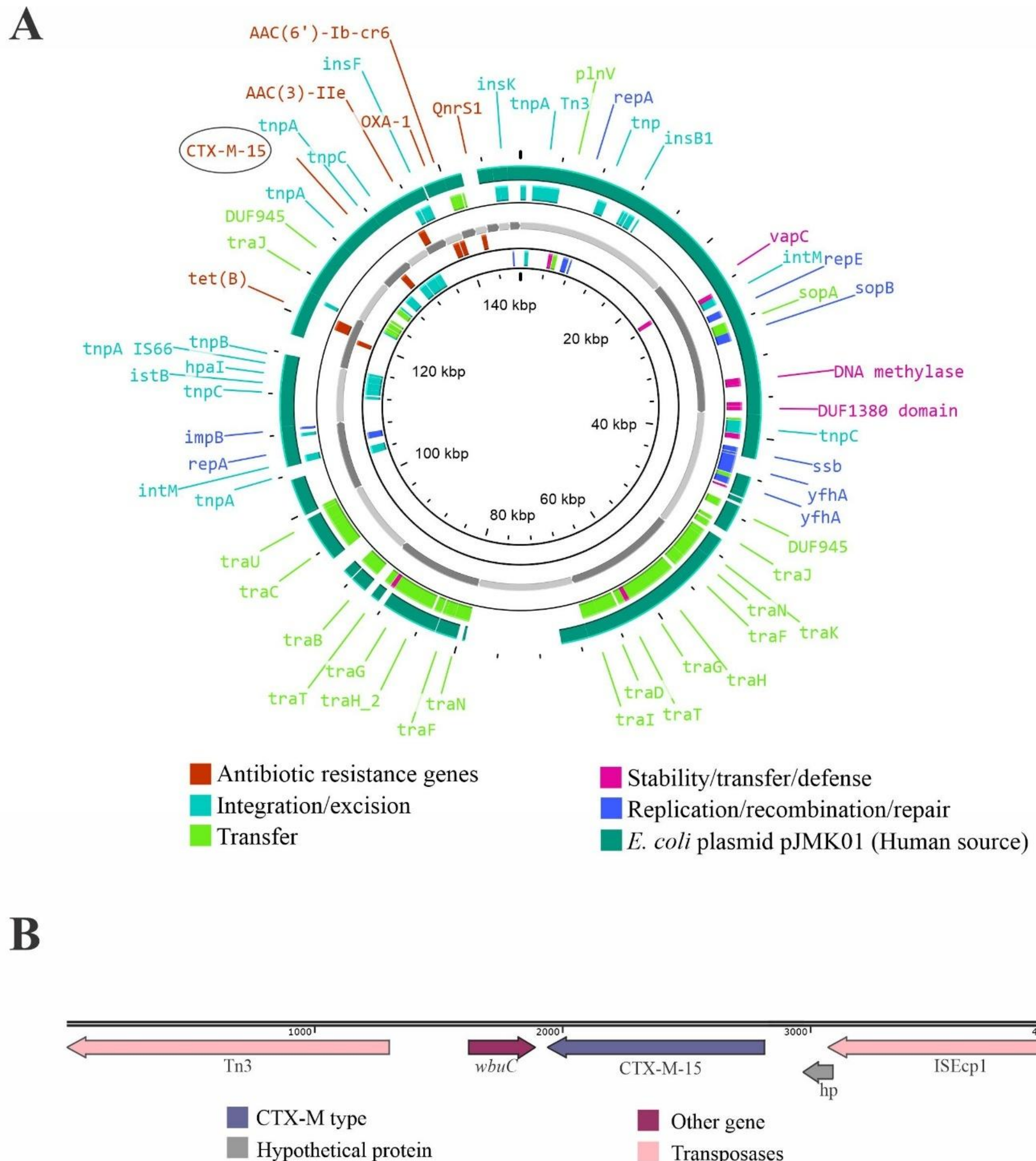

Figure 5. Genetic Organization of pECT2-7 with emphasis on the *bla*$_{CTX-M-15}$. (**A**) Circular comparison between the plasmid harboring *bla*$_{CTX-M-15}$ and the plasmid pJMK01 isolated from *E. coli* from human source in South Korea. Genes related to conjugative transfer, replication, integration, and plasmid stability were identified. (**B**) Genetic context of the *bla*$_{CTX-M-15}$ gene showing a conserved gene cluster typically associated with this ESBL determinant.

8.4 Characterization of an ESBL-encoding plasmid in UFV_ECT1-72 strain

Conjugation assays were performed using *E. coli* UFV_ECT1-72 as the donor strain and *E. coli* J53 as recipient strain. A total of 2.9 x 10^3 CFU.mL^{-1} transconjugants were obtained from plates containing LB supplemented with ampicillin and sodium azide (Figure 6A). The conjugation frequency was 1.16 x 10^{-5}. To confirm the transfer, a transconjugant named J53TC_72A was successfully isolated, characterized for plasmid stability, and exhibited resistance to β-lactams, including ceftiofur, ampicillin, and ceftriaxone. The MIC for ampicillin

was ≥ 256 µg. mL^{-1} for both the donor and transconjugants, while the recipient strain was susceptible, exhibiting an MIC of 8 µg. mL^{-1} (S ≤ 8 for Enterobacterales). The *bla_{TEM}* gene was detected in transconjugant strain (Figure 6B), and the PFGE analysis confirmed the transfer of the plasmid from the donor to recipient strain, showing the same size as that harbored by the donor (~ 120 kb) (Figure 6C). These results confirmed the transfer of the antibiotic resistance phenotype and genetic marker through the conjugative plasmid pEC72A.

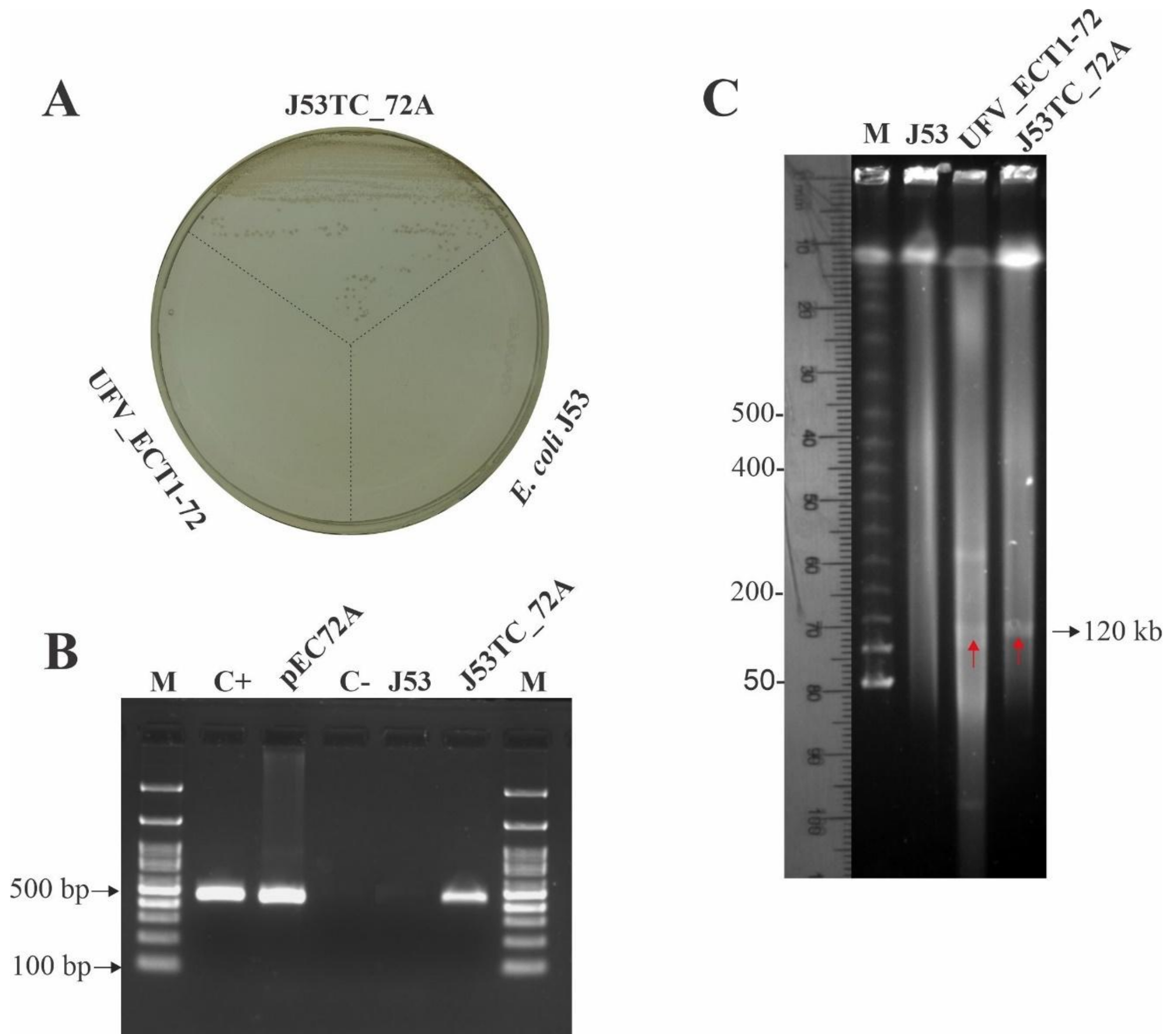

Figure 6. Phenotypic and genotypic characterization of transconjugant obtained through conjugation, with *E. coli* strain UFV_ECT1-72 and *E. coli* J53 as donor and recipient strains. (**A**) Transconjugant J53TC_72A in LB agar supplemented with 100 µg. mL^{-1} sodium azide and 100 µg. mL^{-1} ampicillin. (**B**) Detection of *bla_{TEM}* gene in transconjugant by PCR. Genomic DNA of donor and plasmid EC72A DNA were used as positive control, genomic DNA of *E. coli* J53 Az^{R} and sterile water were used as the negative control. Marker: Kasvi Ladder 100 bp. (**C**) Plasmid profile of *E. coli* strain UFV_ECT1-72 (donor), *E. coli* J53 (recipient), and transconjugant that acquired pEC72A by S1-PFGE analysis. Marker: ProMega-Markers® Lambda Ladders.

The plasmid DNA was successfully extracted from the transconjugant and was submitted for short-read sequencing using Illumina platform. The conjugative plasmid,

designated pEC72A, belonged to IncFIB group (99.2% identity), sequence type ST56, and harbored *blaTEM-1, sul2, aph(3')-IIa, aph(3")-Ib, aph(6)-Id* and *floR* genes, which encoding resistance to β-lactams, sulfonamides, aminoglycosides and phenicols.

The backbone of the plasmid harbored genes that is responsible for conjugative machinery, plasmid replication, stability, integration and recombination functions (Figure 7A). BLASTn searchers demonstrated similarity with 60% and 97.08% of coverage and identity, respectively, with plasmid pTL-1 from *E. coli* strain 800 (CP157959.1) isolated from calf with diarrhea in China in 2020, which also belonged to IncFIB group and carried the *blaTEM*, *aph(3")-lb* and *aph(6)-ld* genes (Wang et al., 2024) (accessed in July 28, 2025). The alignment of the plasmids was accessed through Progressive Mauve alignment, to identify conserved regions and genome rearrangements between them (Figure 7B). As a result, similar regions were identified, mainly encompassing plasmid backbone genes. However, rearrangements and gaps were also observed, which may have resulted from recombination events. These gaps appear to correspond to plasmid-specific regions.

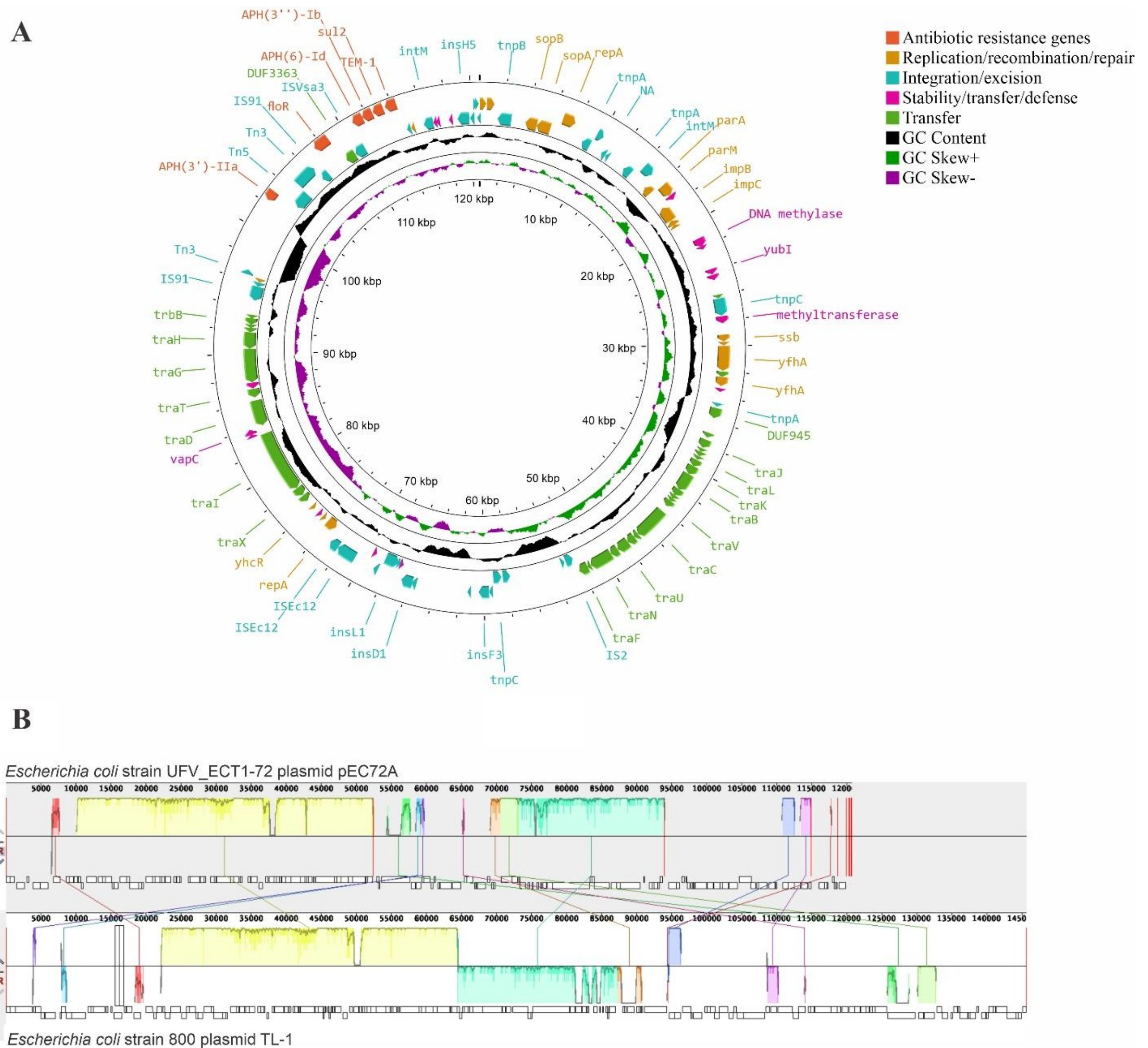

Figure 7. Conjugative plasmid pEC72A from the *E. coli* strain UFV_ECT1-72 harboring the *bla*$_{TEM}$ gene and plasmid alignment with *E. coli* plasmid pTL-1. (**A**) The conjugative plasmid with ~120 kb belonging to IncFIB group ST56 which harbored antibiotic resistance genes from beta-lactams, aminoglycoside, phenicol and sulfonamides, and the conjugative machinery system. Image created by Proksee. (**B**) Plasmid alignment of plasmid pTL-1, which demonstrated 60 % of coverage and 97.08 % of identity with plasmid sequenced in this study. Genome rearrangements were observed, although similar regions were conserved, which harbored backbone genes for plasmid conjugation and maintenance. Alignment performed by Progressive Mauve software.

11. DISCUSSION

The emergence of antibiotic resistance reported in healthy and sick food-producing animals has received attention in recent years (Dos Santos Alves et al., 2023; Mccormick et al., 2023; Oliveira et al., 2024; Rosa et al., 2025). Nevertheless, molecular studies are still needed to investigate the genetic reservoir of antibiotic resistance genes carried by these bacteria, in order to support the development of effective epidemiological surveillance and control strategies in the livestock sector. In our recent work, we isolated and characterized MDR-*E.*

coli from clinically healthy pigs in the finishing phase (Oliveira et al., 2024). Here, we performed the DNA sequencing for the in-silico characterization of three MDR-*E. coli* strains harboring multiple ARGs, including ESBL genes. In silico analysis revealed the presence of putative plasmids in the lineages carrying *bla*$_{\text{CTX-M}}$-type. Additionally, we sequenced and characterized a conjugative plasmid carrying *bla*$_{TEM}$ gene and confirmed its transferability for recipient strain.

MLST analysis revealed that the strain UFV_ECT1-72 belonged to sequence type ST101. This strain also harbored ESBL genes, other ARGs, and plasmids. These findings are consistent with previous studies reporting *E. coli* ST101 as an international pandemic clone frequently associated with *bla*$_{CTX\text{-}M}$ genes and the dissemination in hospitals and communities, is commonly associated with *E. coli* from clinical isolates from humans (Fernandes et al., 2016; Kalantar-Neyestanaki et al., 2020; Ramakrishnan et al., 2021; Medugu et al., 2022).

However, ST 101 has also been detected in *E. coli* isolates from food-producing animals, such as pigs, in the last years (Sadek et al., 2020; Zhang et al., 2021; Kromann et al., 2023; Dos Santos Alves et al., 2023). Recently, Seo and collaborators showed third-generation cephalosporin-resistant *E. coli* in pig farm in Korea, which harbored different CTX-M-type enzymes and ST101 was the second most common type in pig isolates. Additionally in this study, the most common ST detected among *E. coli* isolates from pigs was ST5229, which some isolates also carried *bla*$_{CTX\text{-}M\text{-}55}$ gene. Consistent with our results, the *E. coli* strain UFV_ECT1-217 also belonged to the ST5229 and harbored the same CTX-M variant. Interestingly, this ST has been reported in *E. coli* isolated from healthy pigs worldwide and has also been associated with ARGs, including ESBL genes (Carfora et al., 2022; Peng et al., 2022; Silva et al., 2024). However, to date, no studies have reported the presence of this ST in *E. coli* isolates from human, suggesting its prevalence in food-producing animals, mainly pigs, although, it might spread to human isolates.

The strain UFV_ECT2-7 belonged as ST1653 and carried 18 ARGs of different antimicrobial classes, in addition to virulence factors and mobile genetic elements. To date, there have been no reports of this clone in food-production animals. According to Enterobase database, only a single isolate with this ST has been deposited, originating from human source in the France in 2015. Therefore, this study represents the first report of MDR-*E. coli* ST1653 in Brazil and in clinically healthy pigs. The absence of information of this ST1653 in the literature suggests that may be an uncommon type, with limited circulation. The detection of this ST in food-producing animals for the first time highlights the importance of continued genomic surveillance to better understand its distribution, resistance mechanisms, and potential

risk to public health. In relation to *fimH,* the strain harboring the allele *fimH31¸* which has recently reported as a the most prevalent allele in *E. coli* isolated from poultry sector in Brazil (da Silva et al., 2022).

The sequencing of the genome of the strains was not enough to close the plasmids, as an Illumina short read sequencing was used. However, in silico analysis and manual characterization allowed us to identify the partial sequence of two plasmids, each strain carrying a *bla*$_{CTX-M}$ variant and other ARGs. Plasmid-encoded CTX-M genes have a potential transmission ability between different niches, which implies their transmission between animals and humans, and vice versa (Bevan et al., 2017). Interestingly, the IncFIB and IncFII plasmid groups were present in all three strains, supporting recent reports that these are the most frequently identified plasmid types in Brazil (Fuga et al., 2022).

In the present study, we detected the *bla*$_{CTX-M-55}$ gene in the strain UFV_ECT1-217 potentially located on an IncFII plasmid. This result is consistent with recent results from both human and animal sources in Brazil (dos Anjos Adur et al., 2022; de Jesus Bertani et al., 2023). These findings represent a public health concern and highlight the need for continuous monitoring of antibiotic resistance gene dissemination through conjugative plasmids among food-producing animals in Brazil.

The gene context of *bla*$_{CTX-M-15}$ cassette found in the UFV_ECT2-7 strain, reflected a highly conserved gene cluster and commonly associated with *bla*$_{CTX-M-15}$ (Zhao and Hu, 2013; Sartori et al., 2023). The insertion sequence IS*Ecp1* has been observed to be responsible for enhanced mobilization, expression and transposition of *bla*$_{CTX-M-15}$ from plasmids to chromosomes (Naseer and Sundsfjord, 2011; Zong et al., 2015). The Tn2 transposase, belonging to the Tn3 family, has also been reported as part of *bla*$_{CTX-M-15}$ cassette (Makhlouf et al., 2021; Yu et al., 2024). These results demonstrate the conserved gene cluster of *bla*$_{CTX-M-15}$ gene, which is widely disseminated worldwide. This supports the hypothesis that this gene cluster can transfer from the plasmid to the chromosome and may also be inserted in different plasmid replicon types, such as IncFII, IncHI2, IncI1 (Tadesse et al., 2018; Rozwandowicz et al., 2018; Cave et al., 2023; Yu et al., 2024).

In our study, we detected the *bla*$_{CTX-M-15}$ potentially located on an IncFIA plasmid, according to BLASTn comparison and PlasmidFinder results. This incompatibility group is commonly reported in Enterobacteriaceae family in both human and food-producing animals worldwide, associated with *bla*$_{CTX-M-15}$ gene (Founou et al., 2022; Yao et al., 2022; Fang et al., 2025).

We also characterized a conjugative plasmid from the *E. coli* strain UFV_ECT1-72, which was successfully transferred to recipient strain, including the resistance phenotype to β-

lactams. The beta-lactamase involved in the β-lactams in this plasmid was the *bla*$_{TEM}$ gene, as a ESBL type globally disseminated among Enterobacteriaceae family (Effendi et al., 2022; Widodo et al., 2023; Agustin et al., 2024). The plasmid, designated pEC72A, was sequenced and *in silico* characterization confirmed the presence of the *bla*$_{TEM}$ gene, in addition it carries a multidrug region composed of genes that encoding resistance to aminoglycosides (*aph3"-Ib, aph(6)-Id, aph(3)-Ia*), sulfonamides (*sul2*) and phenicol (*floR*). All antibiotic classes that are commonly used in swine farms, as therapeutic proposes (WOAH, 2025). These *in vitro* and *in silico* results reinforce concerns about the dissemination of antibiotic resistance through MDR conjugative plasmids in clinically healthy animals.

In conclusion, our study provides insights into the MGEs associated with *bla*$_{\text{CTX-M-type}}$ and *bla*$_{TEM}$ genes in MDR-*E. coli* strains isolated from clinically healthy pigs. These reports will contribute to comparative analyses of MDR-*E. coli* strains in other food-producing animals. We also emphasize the importance of further genomic investigations in commensal bacteria from healthy animals, as they are reservoirs for ARGs with relevant importance, to better characterize their dissemination through conjugative plasmids. Such efforts are essential in the One Health context, not only to prevent the dissemination of multidrug-resistant plasmids but also to improve our understanding of the evolution of antibiotic resistance in livestock.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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SUPPLEMENTARY MATERIAL

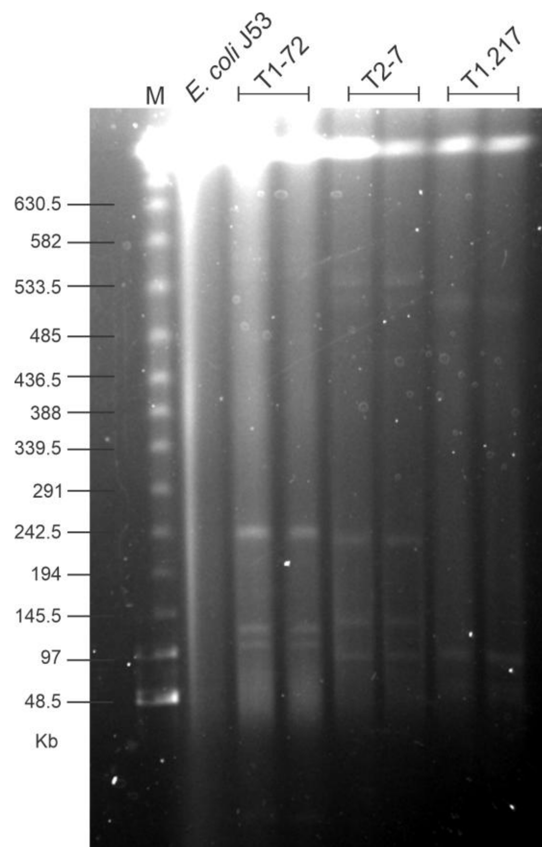

Figure S1. S1-PFGE of three MDR-*E. coli* strains from clinically healthy pigs. *E. coli* J53 was used as negative control for the presence of plasmids. Marker: Lamba PFGE Ladder.

Table S1. Chromosomal mutations conferring antibiotic resistance detected among MDR-*E. coli* genomes from clinically healthy pigs.

Strain ID	Chromosomal mutations
UFV_ECT1-72	*gyrA* (S83L), *gyrB, parC, parE, folP, rpoB, ampC-promoter*
UFV_ECT1-217	*gyrA* (S83L, D87N), *gyrB*, *parC* (S80I), *parE*, *pmrA, pmrB, folP, rpoB*
UFV_ECT2-7	*gyrA* (S83L, D87N), *gyrB, parC* (S80I), *parE* (S458A), *pmrA, pmrB, folP, 23S, rpoB, ampC-promoter*

Table S2. *In silico* characteristics of plasmids replicons, conjugation systems and relaxase families among MDR- *E. coli* strains.

Strain ID	Plasmid replicons	Conjugation system type (Conjscan)	Relaxase family (MobScan)
UFV_ECT1-72	IncFIA; IncFIB; IncFII; IncHI2; IncQ1 and IncY	T4SS_typeF	MOB_F, MOB_H
UFV_ECT1-217	IncFIB; IncFII; IncX1 and IncY	MOBP1, T4SS_typeT	MOB_P (two copies)
UFV_ECT2-7	IncFIA; IncFIB; IncFII; IncFII; IncR; IncY and pKPC-CAV1321	T4SS_typeF	MOB_H

CHAPTER 3

Antibiotic resistance gene reservoir in extracellular vesicles from MDR-*Escherichia coli* isolated from clinically healthy swine

(Manuscript written according to the guidelines of Pathogens and Disease – an Open Access Journal from Oxford University Press)

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Abstract

Over the past decade, extracellular vesicles (EVs) have been recognized as reservoirs and vehicles for transferring antibiotic resistance genes (ARGs). However, the characterization of EVs from MDR-*E. coli* isolated from clinically healthy pigs has not been described. In this study, we characterized EVs produced by the MDR-*E. coli* strain UFV_T1-72 and evaluated their potential to harbor ARGs. EVs were isolated by ultracentrifugation after culturing the strain with or without ceftiofur and tetracycline stress during the exponential growth phase. Morphological and cargo features were analyzed. Stress conditions enhanced vesiculation, increasing both EVs number and diameter. Tetracycline treatment induced the greatest rise in EVs production and size compared with other groups. EVs obtained under all conditions packaged clinically relevant ARGs, including $bla_{CTX\text{-}M\text{-}2}$, bla_{TEM}, and *tetA* genes, even in the absent of antibiotic pressure. These results demonstrate that antibiotic exposure modulates EVs production and morphologies, improving our understanding in the impact of antibiotic in vesiculation. Our data highlight the role of EVs in harboring diverse ARGs in MDR - *E. coli* from a clinically healthy pig. Antibiotic stress stimulates vesiculation and increases the number of EVs, thereby enhancing the packaging of ARGs, which reinforces the relevance of this process within a One Health perspective.

Keywords: Vesiculation; ESBL; Tetracycline resistance; Food-producing animals; One health; Multi-drug resistance.

9 INTRODUCTION

The multi-drug resistant bacteria (MDR) have emerged as significant public health concern, as bacterial isolates frequently also exhibit co-resistance to multiple classes of antimicrobials. Initially, considered a concern primarily associated with hospital settings and human infections, MDR has now been increasingly reported in other environments, including livestock, domestic and wild animals, whether healthy or not (Furlan et al., 2020; Janus et al., 2024; Tilahun et al., 2025; Lysitsas et al., 2025). This alarming advance underscore the need to better understand the pathways and mechanisms that drives the dissemination of resistance across these sectors.

In livestock settings, the antibiotic is used widely for therapeutic and prophylactic purposes (Aarestrup, 2015; Rigueira and Perecmanis, 2024). Many classes of antibiotic used, including β-lactams and tetracyclines are commonly used for these purposes. Regarding β-lactams, third-generation of cephalosporins, including ceftiofur and ceftriaxone, are used for treatment gastrointestinal and respiratory diseases in pigs (WOAH, 2025). In 2024, the World Health Organization (WHO) updated its Bacterial Priority Pathogens List, which includes bacterial pathogens of major public health concern. In this report, third-generation cephalosporin-resistant *Enterobacterales*, including *Escherichia coli,* were classified as critical priority group due to their capacity to transfer antibiotic resistance genes (ARGs). Meanwhile, third-generation cephalosporin resistant *E. coli* has been increasingly reported associated with food-producing animals over the last decades (Agerso et al., 2014; Faccone et al., 2019; Lee et al., 2021; Oliveira et al., 2024).

Tetracyclines are extremely important in veterinary medicine and are commonly used in pigs to treat diverse conditions, such as arthritis, cardiovascular and nervous systems, as well as respiratory and systematic infections (WOAH, 2025). During years, tetracycline was also used as growth promoter in livestock, including pigs, to enhance weight gain and improve herd health. However, in Brazil, the used of not only tetracycline, but also β-lactams as feed additives are prohibited since 2009, according to Technical Note No. 26/2009.

Acquired resistance to β-lactams and tetracyclines commonly occurs through the acquisition of genes encoding specific mechanisms. Regarding to β-lactams, resistance is often mediated by the production of enzymes, such as extended-spectrum beta-lactamase (ESBL), which causes the antibiotic inactivation (Munita and Arias, 2016; Bush and Bradford, 2019). While for tetracycline, acquired resistance involves genes encoding efflux pumps, such as TetA

and TetB pumps, which causes the drug efflux from the bacteria cell, thereby reducing its intracellular concentration (Nguyen et al., 2014). These resistance determinants (e.g., *bla*$_{CTX\text{-}M\text{-}type}$, *bla*$_{TEM}$ and *tet* genes) are commonly associated to mobile genetic elements (MGEs), including plasmids, that facilitates their dissemination among bacteria through horizontal gene transfer (HGT) mechanisms, including transformation, transduction, conjugation (Frost et al., 2005; Partridge et al., 2018). However, in recent years, a fourth mechanism has been included, designated vesiduction, involving extracellular vesicles (EVs) (Soler and Forterre, 2020).

Extracellular vesicles (EVs) are spherical nanoparticles composed by lipid membrane, secreted from the cell surface into environment. The EVs diameter commonly range between 20 – 400 nm, and their cargo are enriched with outer membrane proteins, hydrolytic enzymes, virulence factors and nucleic acids (Toyofuku et al., 2023; Potapova et al., 2024; Xiao et al., 2025). EVs plays important functions in bacterial virulence, host immune response, communication between cells, delivery of bioactive compounds, and has been emerging vehicle of DNA transfer (Dell' Annunziata et al., 2021). DNA carried by EVs includes plasmids, genomic fragments and genetic determinants. These compounds within the EVs are show to be protect against nucleases degradation, and can also be transport long distances, as well as, delivery them in recipient bacteria (Bielaszewska et al., 2020; Toyofuku et al., 2023; Tang et al., 2023; Xu et al., 2024).

In 2019, the MDR- *E. coli* strain UFV_T1-72 was isolated from a clinically healthy pig in the finishing phase. This strain exhibited a multidrug-resistant phenotype, with resistance to β-lactams, quinolones, aminoglycosides, folates, and tetracyclines (Oliveira et al., 2024). Additionally, it harbored 18 ARGs and two conjugative plasmids carrying genes encoding resistance to extended-spectrum β-lactams, tetracycline, sulfonamides, and trimethoprim. Based on these characteristics, the present study, aimed to investigate the extracellular vesicles production under cephalosporin and tetracycline stress conditions. In addition, we also evaluate the potential of EVs to serve as reservoirs of relevant ARGs under different stress conditions.

10 MATERIAL AND METHODS

10.1 Strain and growth conditions

The *Escherichia coli* strain UFV_ECT1-72 (accession number: JBEUMT010000000) isolated from clinically healthy pig from our previous study (Oliveira et al., 2024) was used for extracellular vesicles purification in this study. In summary, this strain was isolated from rectal of clinically healthy pig in finishing phase from the swine sector of the Animal Science Department at the Universidade Federal de Viçosa, Brazil in 2019, according to guidelines

approved by the Ethics Committee on the Use of Production Animals of the Universidade Federal de Viçosa (CEUAP/UFV) under Protocol no. 027/2021. The animal (T1-72) was received fed with diet supplemented with 55 ppm of zinc bacitracin and 5 ppm of enramycin. The sample was obtained using sterile swabs and streaked onto MacConkey agar (Kasvi, Spain) supplemented with 8 $\mu g \cdot mL^{-1}$ ceftiofur, and subsequently preserved in Luria-Bertani (LB) broth (Kasvi, Brazil) containing 20% glycerol at − 80 °C. The bacterial isolate was identified through 16S rRNA gene sequencing and biochemical assays, and confirmed as *Escherichia coli*. The strain was classified as multidrug-resistant, carrying diverse ARGs, including $bla_{CTX-M-2}$, bla_{TEM}, *tetAB, dfrA7, sul1, aph(3")-Ib*, among others. Furthermore, it harbored four plasmids, two of which were conjugative, both carrying ARGs such as bla_{TEM} and *tetA*.

The bacterial growth was monitored by measuring optical density at 600 nm (OD_{600}) at regular time intervals. The maximum growth rate and generation time were calculated from the natural logarithm (Ln) of the OD_{600} measurements over time. Briefly, overnight culture was adjusted of OD_{600} = 0.1 and was cultivated in LB broth supplemented or not with ceftiofur (CFT) (8 $\mu g.\ mL^{-1}$) or with tetracycline (TET) (16 $\mu g.\ mL^{-1}$) at 37 °C for 24 h with shaking (180 rpm). The antibiotics concentration applied were consistent with the breakpoint values established for each antibiotic, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020; CLSI VET01S, 2023). The growth curve was performed in triplicate with three biological duplicates.

10.2 EVs Characterization

10.2.1 Extracellular vesicles isolation and purification

To obtain the EVs, the strain UFV_ECT1-72 was pre-cultivated in 25 mL of LB broth at 37 °C overnight at 180 rpm. Thus, an aliquot of the adjusted cultures in OD600 = 0.1 were inoculated in 200 mL of LB broth supplemented with the conditions described above, and were cultivated at 37 °C at 180 rpm for 3 and 6 h. The bacterial cultures were centrifuged at 4 °C 10000 x *g* for 20 min, to remove bacterial cells. The supernatants were filtered through Millipore filters of 0.22 µm sterile (Merck Millipore, Germany). Then, the EVs were collected by ultracentrifugation at 4 °C 150000 x *g* for 2 h. The supernatants were removed and the pellets were resuspended in sterile PBS 1 X and ultracentrifuged for the second time to obtain the crude extract. The crude extract obtained was filtered by 0.22 µm Millipore microfiltration membrane and concentrated using a 100 kDa ultrafiltration tube Amicon (Millipore, USA). The EVs were recovered in PBS 1 X and stored at -20 ° C. An aliquot of the EVs was spread on a LB plate,

and cultured for 24 h to confirm that there were no bacterial cells. The EVs extraction was performed in duplicate.

10.2.2 Morphological characterization by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

To observe the cells of *E. coli* UFV_ECT1-72 strain and production of extracellular vesicles, we performed scanning electron microscopy (SEM). Briefly, after the incubation, the cells were centrifuged at 1000 x *g* for 15 min to pellet bacterial cells, washed with sterile PBS 1 X, loaded on glass coverslips and fixed with 2% (v/v) glutaraldehyde and formaldehyde overnight at 4 °C. Then, the cells were dehydrated through an ethanol gradient (30, 50, and 70% three times for 5 min and the 95% and 100% twice for 10 min), subjected to critical point drying in CO_2 and covered with gold sputtering. The samples were visualized by scanning electron microscope Jeol IT700 HRLA FEG located at the Microscopy and Microanalysis Center of the Universidade Federal de Viçosa.

Additionally, for transmission electron microscopy (TEM) analysis, 10 µL of EVs were placed onto a formvar-coated gold grid for 1 min and negatively stained with 2.5% uranyl acetate. After 1 min, the excess was removed with filter paper. The grid's visualization was performed in a Zeiss EM 109 Electron Transmission Microscope operating at 80 kV, located at the Microscopy and Microanalysis Center of the Universidade Federal de Viçosa.

10.2.3 Size measurement by Dynamic Light Scattering (DLS)

The measurement of size, polydispersity index and zeta potential of EVs were determined by Dynamic Light Scattering (DLS) analysis using a Litesizer DLS 500. The analysis was performed using Kalliope software v. 4.4.1. For this, 40 µL of EVs were transferred to cuvettes, which all the measurements were conducted according to the following parameters: refractive index: 1.3318; solvent viscosity: 0.0009041 at 25 °C, with three runs for each sample. In addition, the Z-potential was performed to measure the membrane charge of EVs. The results were reported as the average ± standard deviation of three independent replicates.

10.2.4 Quantification of proteins in EVs

The protein concentration of EVs obtained was determined by QuantiPro™ BCA Kit (Sigma-Aldrich, USA), with bicinchoninic acid (BCA), according to the manufacturer's instructions. The reactions were measured at the 562 nm by spectrophotometer and the analysis was performed in triplicates using a standard calibration curve of bovine serum albumin (BSA).

10.2.5 Flow Cytometry analysis of EVs

To measure the EVs concentration in the samples, estimate the population size distribution and the internal DNA amount in the EVs we conducted the flow cytometry analysis. Firstly, 200 µL of EVs, obtained as above-described, were treated with 20 µg. mL^{-1} RQ1 RNase-Free DNase (M6101, Promega, USA). Then, 120 µL of treated EVs were labeled with 20 µg. mL^{-1} of 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) dye, a lipid bilayer dye (Sigma-Aldrich, USA). For detection of internal DNA, 80 µL of treated EVs were labeled with 20 µg. mL^{-1} of propidium iodide (Life technologies, Thermo Fisher Scientific), and were incubated in the dark for 40 min at 37 °C. The samples were quantified by using BD Accuri C6 flow cytometer by using the filters FL-1 (green), FL-2 (short red), and FL-3 (medium red). The analysis was performed in biological duplicates with three replicates.

10.3 Detection of antibiotic resistance genes in EVs

To verify the presence of antibiotic resistance genes (ARGs) in EVs, PCR were performed using specify primers demonstrated in Table S1. Additionally, an aliquot of EVs were treated with RQ1 RNase-Free DNase (M6101, Promega, USA) according to manufacturer's instructions and were used as a template. Briefly, EVs were incubated with 100 ng. mL^{-1} DNase at 37 °C for 30 min, then followed by reagent of DNase inactivation at 65 °C for 10 min. The EVs untreated were also used as templates. The genomic DNA was used as positive control. The genes tested were followed: $bla_{CTX\text{-}M\text{-}2}$, bla_{TEM}, *tetA, tetB, dfrA17* and *sul1*. These ARGs were selected because the donor strain *E. coli* UFV_ECT1-72 harbored two conjugative plasmids, one of them with ~120 kb, which harbored bla_{TEM} gene, whereas the other conjugative plasmid (~220 kb) harbored the genes related to resistance to tetracycline, *tetA, tetB, sul1* and *dfrA17*. The information of the primers used in this study can be found in Table S1.

10.4 EVs-mediated transfer of ARGs to susceptible bacteria

To evaluate the ability of the EVs-mediated transfer, experiments were conducted as previously described by Rumbo et al. (2011) and Tang et al. (2023). The recipient strain was *E. coli* J53 Azi^R (Yi et al., 2012). Briefly, the recipient strain was grown in LB broth to an OD_{600} of 0.8, centrifuged and resuspended in LB broth. The suspension (100 µL) was mixed with 100 µL DNAse-treated OMVs isolated from all the conditions. The mixtures were incubated at 37 °C for 4 h stationary, then LB broth was added up to 1 mL and were incubated for 4 h at 220 rpm. Subsequently, LB broth was added to a final volume of 10 mL, and the mixture was incubated overnight at 37 °C with shaking (220 rpm). Bacteria cultures were centrifuged,

resuspended in 1 mL of LB broth, and serial dilutions were performed. Aliquots of 100 µL were spread onto LB agar supplemented with ceftiofur (8 µg. mL^{-1}) and sodium azide (100 µg. mL^{-1}), as well as onto LB agar containing sodium azide (100 µg. mL^{-1}), and incubated for 24 h. The transfer frequency was calculated as the number of transvesiculants ($CFU.mL^{-1}$ on LB agar supplemented with ceftiofur and sodium azide) divided by the total bacterial count ($CFU.mL^{-1}$ on LB agar supplemented with sodium azide). Plasmid pECT72A DNA (5 ng) extracted from the donor was incubated with *E. coli* J53 and cultured in the same method. Finally, PBS was incubated with *E. coli* J53 as the negative control.

10.5 Statistical analysis

Statistical analysis was carried out by using the R program version 4.5.1. For multiple comparisons, One-way analysis of variance (ANOVA) was used, followed by Tukey's multiple comparisons test. Additionally, the Mann-Whitney test was used to assess significant differences in log_{10} $CFU.mL^{-1}$ between the treatments and control groups. A significance level of $p < 0.05$ was considered.

11 RESULTS

11.1 Growth profile of *E. coli* strain UFV_ECT1-72 strain under stress conditions

To define time to analyze the vesiculation by *E. coli* strain UFV_ECT1-72 under stress conditions we performed the growth curve analysis based on determination of optical density (OD) and colony forming units (CFU) in LB medium in the presence or absence of the antibiotics: ceftiofur (8 µg. mL^{-1}) and tetracycline (16 µg. mL^{-1}) with initial $OD_{600} = 0.1$. The OD_{600} values over 24 h in each condition were demonstrated in Table S2. As a result, the log_{10} $CFU.mL^{-1}$ over 24 h indicates a similar profile between the condition without antibiotics and those with antibiotics added, with no significant difference in both treatments ($p > 0.05$) (Figure 1A-B). However, the growth kinetics revealed that, in the presence of tetracycline (16 µg. mL^{-1}), the strain UFV_ECT1-72 displayed a lower maximum growth rate ($\mu max = 0.55\ h^{-1}$) and a longer generation time (1.24 h), indicating a slower growth profile. In contrast, the growth profile observed in LB with ceftiofur (8 µg. mL^{-1}) was comparable to the control condition (LB) (Table 1), suggesting that the presence of the antibiotic did not impose a detectable fitness cost. The bacterial counts in $CFU.mL^{-1}$ and log_{10} $CFU.mL^{-1}$ were demonstrated in Table S3.

Based on these results, we decide to perform the extraction of EVs at the point of 3 and 6 h of growth in the three conditions.

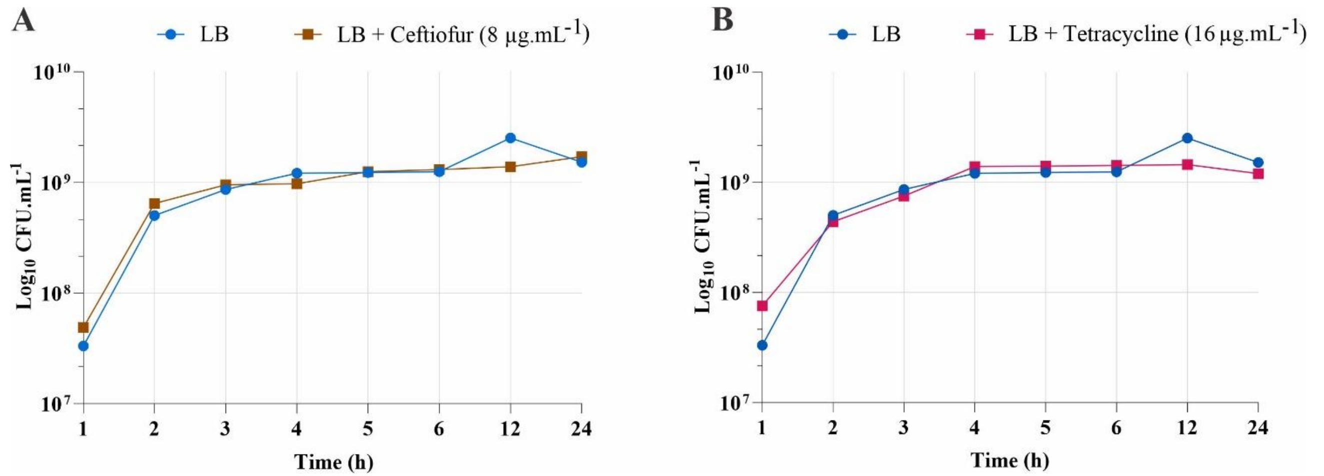

Figure 1. Growth curve of the strain *E. coli* strain UFV_ECT1-72 expressed as log_{10} CFU.mL^{-1} with and without antibiotic exposure. **(A)** Comparison of growth of *E. coli* UFV_ECT1-72 in control media (LB) and in LB supplemented with Ceftiofur (8 µg. mL^{-1}) over time in log_{10} CFU.mL^{-1}. **(B)** Comparison of growth of *E. coli* UFV_ECT1-72 in control media (LB) and in LB supplemented with Tetracycline (16 µg. mL^{-1}) over time in log_{10} CFU.mL^{-1}.

Table 1. Specific Growth Rate of *E. coli* strain UFV_ECT1-72 in different conditions.

Growth conditions	Growth rate µ (h^{-1})	Generation time (h)
LB (control)	0.76	0.90
LB + Ceftiofur (8 µg. mL^{-1})	0.77	0.90
LB + Tetracycline (16 µg. mL^{-1})	0.55	1.24

11.2 Morphological characterization of EVs produced by *E. coli* strain UFV_ECT1-72 under stress conditions

The EVs were extracted from *E. coli* strain UFV_ECT1-72 by ultracentrifugation and were examined using SEM, TEM and DLS and flow cytometry. The morphology and the production of extracellular vesicles of *E. coli* strain UFV_ECT1-72 were accessed through SEM analysis. As a result, SEM visualization showed typical bacilli morphology in *E. coli* cells and the presence of extracellular matrix around the cells. Additionally, it was possible to observe the presence of multiple spherical structures blebbing from the membrane of *E. coli* strain UFV_ECT1-72, consistent with extracellular vesicles production in all the growth conditions (Figure 2).

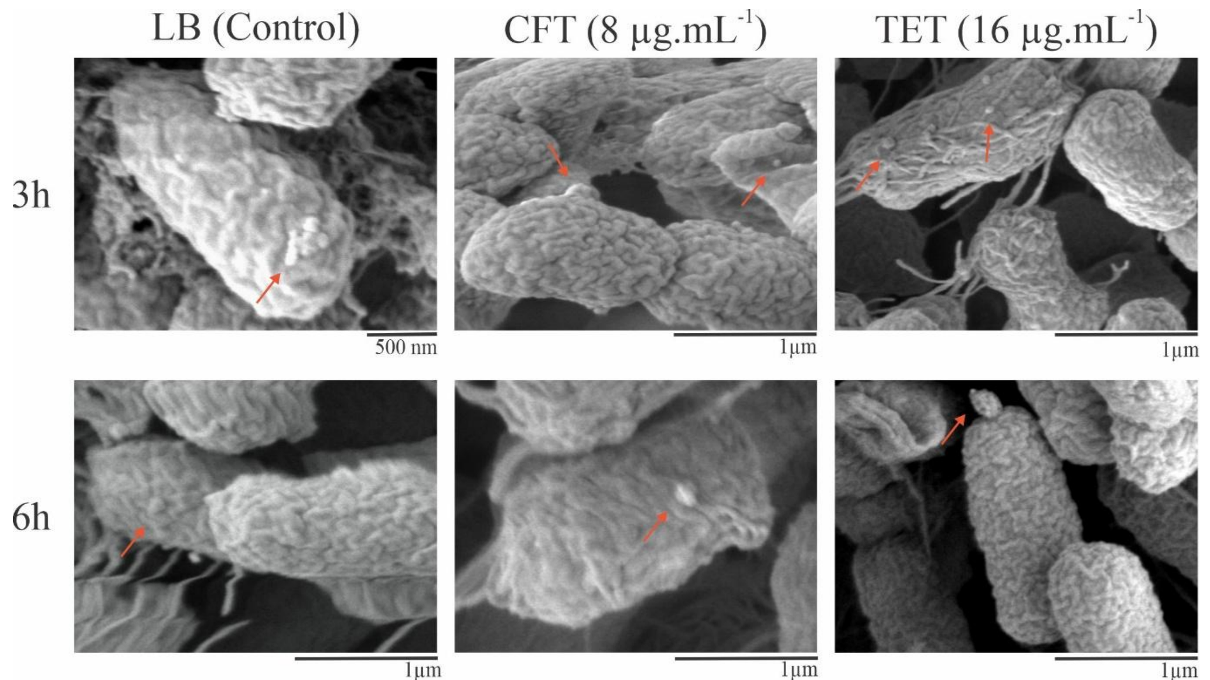

Figure 2. Scanning electron microscopy of *E. coli* strain UFV_ECT1-72 cells and the production of extracellular vesicles under different conditions. Orange arrows mean extracellular vesicles on the cell surface or near to them. Specific scale bar in each image.

The TEM images showed that the membrane structure of EVs was intact and present a bilayer membrane (internal and external) in almost all the conditions consist with the outer-inner membrane vesicles (OIMVs), although we also observed EVs only with outer membrane, consistent with outer membrane vesicles (OMVs) (Figure 3A-C). In general, the EVs had distinct morphologies and sizes, with electron-dense content among the growth conditions.

The TEM images and DLS measurements demonstrated that the average diameter of EVs produced by E. *coli* strain UFV_ECT1-72 was smaller under without stress conditions (LB condition), reaching 72.19 nm after 3 h of culture, compared to the control group (Figure 3A-B). However, an increase in the numbers and size of EVs produced by *E. coli* strain UFV_ECT1-72 was observed under exposure to 8 µg. mL^{-1} CFT and 16 µg. mL^{-1} TET after 3 h of culture, reaching average diameters of 108.21 nm and 119.17 nm, respectively (Figure 3B). Besides that, under TET exposure, EVs size increased by at least 1.65-fold and 1.44-fold after 3 h and 6 h of culture, respectively (up to 164 nm). On the other hand, under CFT exposure, the increases were 1.5-fold and 1.04-fold after 3 h and 6 h, respectively. As expected, after 6 h of culture, a notable increase in the number and size of EVs was detected under all conditions (Figure 3C-D). Notably, EVs from the LB condition at 6 h contained an excess of exopolysaccharides, which may have contributed to aggregation, as visualized by TEM (Figure 3C).

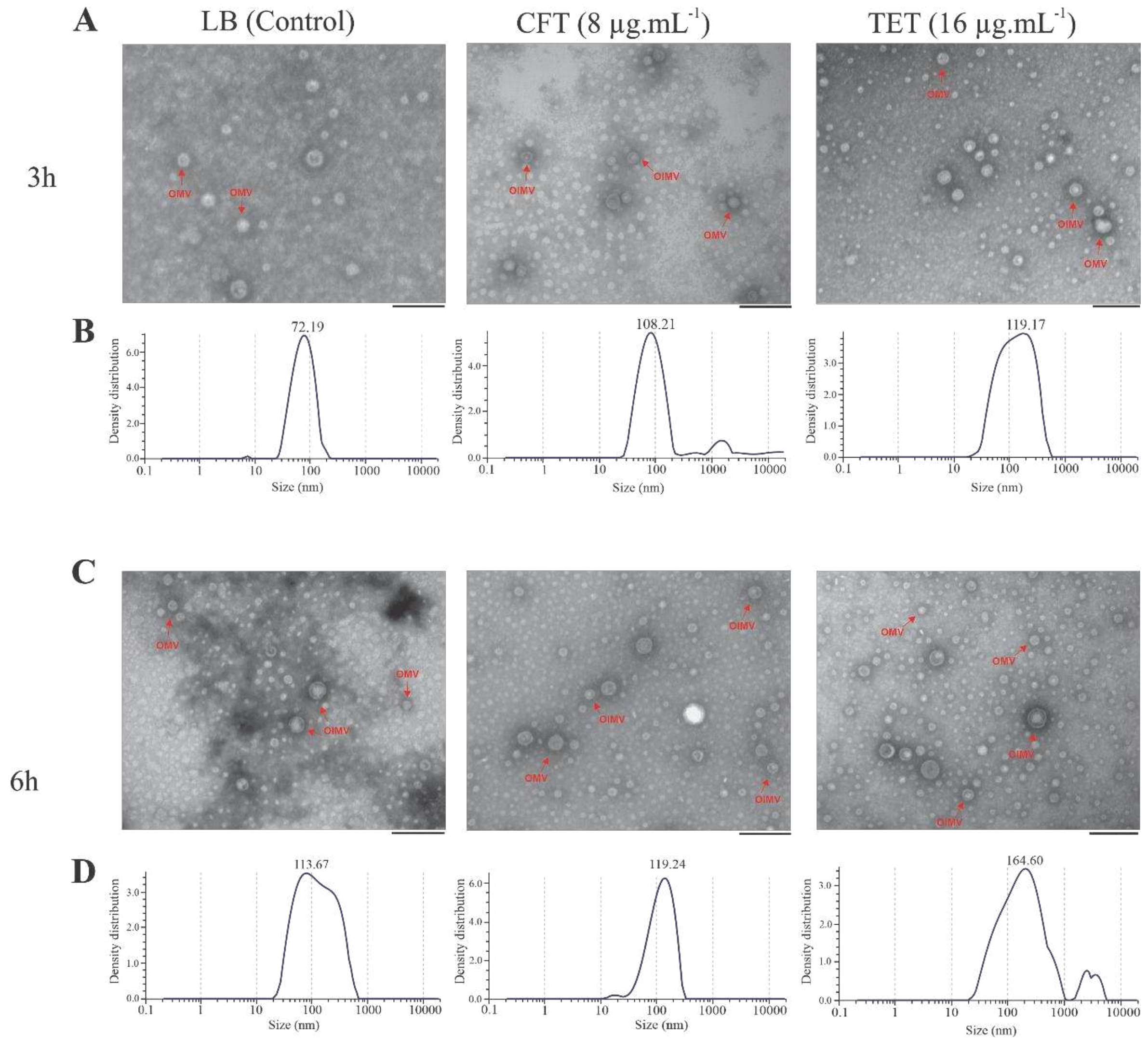

Figure 3. Physical characterization of extracellular vesicles (EVs) derived from *E. coli* strain UFV_ECT1-72 under different conditions. (**A-C)** Transmission electron microscopy images of EVs derived from UFV_T1-72 cells, after 3 h and 6 h of culture under different conditions, respectively. **(B-D)** Size distribution of EVs released by UFV_T1-72 cells assessed by dynamic light scattering (DLS). Red arrows indicate different morphologies of extracellular vesicles; OMV: Outer membrane vesicles; OIMV: Outer-inner membrane vesicles. Scale Bar: 200 nm.

The zeta potentials were variable among the conditions, whereas EVs from CFT 6 h and TET 6 h (-17.62 ± 6.15; -20.72 ± 1.15, respectively) were in a moderate stable state. As opposed to the EVs from other conditions with Z-potential < -10 mV, which indicated that were in an unstable state, which can increase the risk of the EVs aggregating (Table 2), consistent with TEM images from LB 6 h condition (Figure 3C).

Table 2: Zeta-potential of extracellular vesicles (EVs) from *E. coli* strain UFV_ECT1-72 under different conditions.

Time (h)	**LB (mV ± SD)**	**LB/CFT (mV ± SD)**	**LB/TET (mV ± SD)**

3 h	-7.57 ± 6.93	-1.08 ± 1.39	-1.32 ± 0.64
6 h	-7.85 ± 10.35	-17.62 ± 6.15	-20.72 ± 1.15

11.3 Quantification of proteins and DNA associated with EVs

BCA assay was used to quantify the protein concentration of the EVs. Our results showed varying protein concentrations in EVs obtained under antibiotic conditions compared to the control condition (Figure 4A–B). Under TET exposure, the protein yield increased by at least 2.9-fold (64.8 ± 3.56 µg. mL^{-1}) and 2.6-fold (73.5 ± 6.72 µg. mL^{-1}) after 3 h and 6 h of culture, respectively, compared to the LB condition (21.9 ± 8.63 µg. mL^{-1} and 28.2 ± 5.77 µg. mL^{-1}), with a significant difference ($p < 0.05$). In contrast, under CFT exposure, the protein yield increased by approximately 2.0- and 1.8-fold (45.5 ± 6.23 µg. mL^{-1} and 53.2 ± 5.88 µg. mL^{-1} after 3 h and 6 h, respectively) (Figure 4A–B). However, these differences were not statistically significant when compared to the LB condition.

The flow cytometry quantification of EVs using the dyes 3,3′-dioctadecyloxcarbocyanine perchlorate (DiO) and propidium iodide was accessed. In relation to the amounts of EVs dyed with DiO, the CFT condition demonstrated the higher amounts of EVs dyed (203.961 EVs per mL), followed by LB condition (177.217 EVs per mL), and TET condition was the condition with less amounts of EVs dyed (136.922 EVs per mL) after 3 h of culture (Figure 4C). Regarding the EVs obtained after 6 h of culture, all the conditions increased in relation to 3 h of culture. In CFT condition demonstrated an increased in EVs production based on a greater number of EVs dyed (290.572 EVs per mL) compared to LB (244.445 EVs per mL) and TET (190.917 EVs per mL), although no significant difference between the treatments was observed ($p > 0.05$) (Figure 4D). Similar results were observed with propidium iodide quantification, which dyes DNA located inside the EVs, since the EVs were pre-treated with DNAse I to remove external DNA associated to EVs. Despite no significant difference of the amount of DNA associated-EVs between the treatments, EVs produced from cells without stress conditions (LB condition) demonstrated higher amount of EVs dyed, suggesting the ability of packaging the DNA from the donor cell (Figure 4E-F), these results were confirmed below with detection of genetic markers into the EVs.

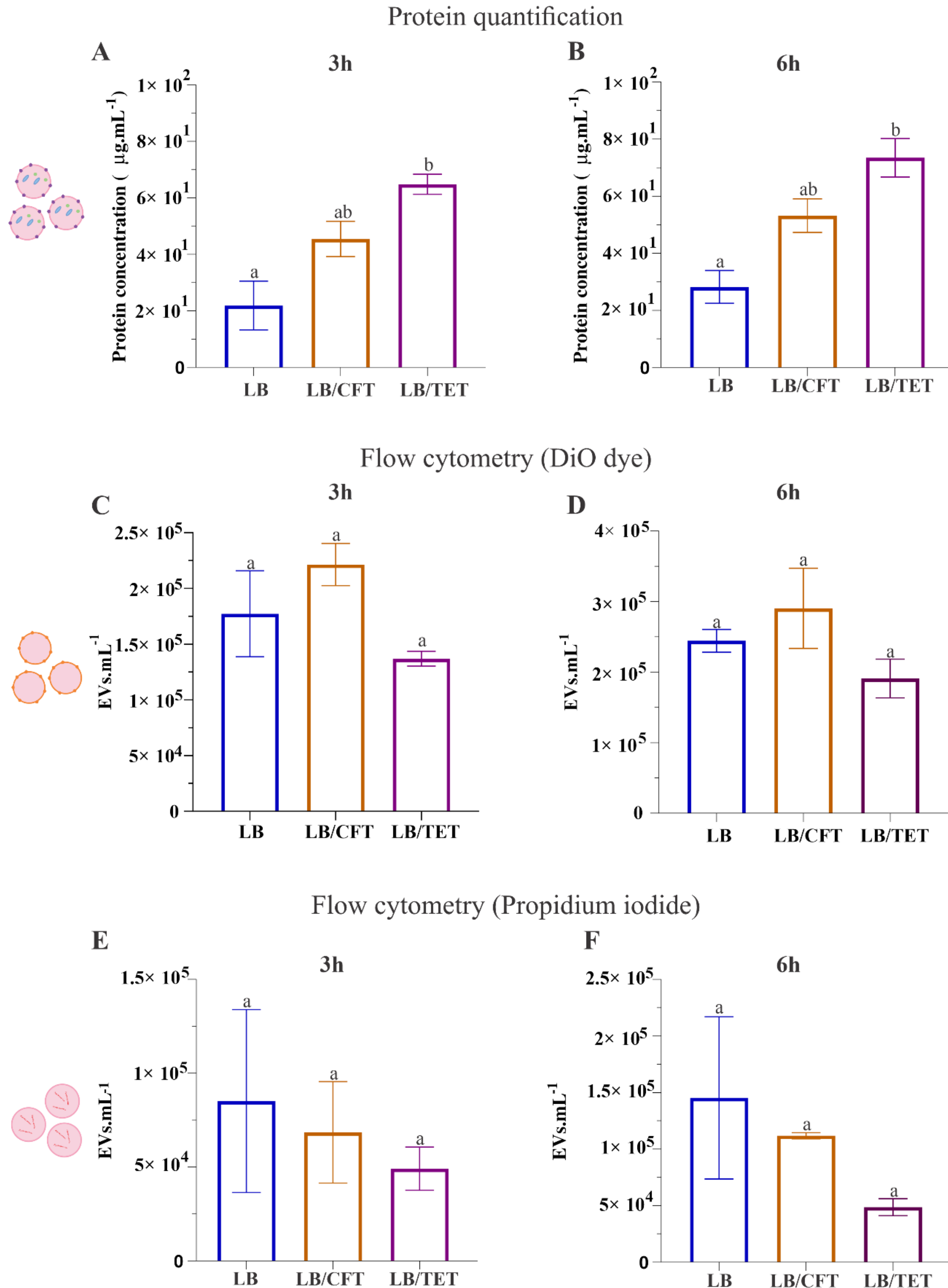

Figure 4. Protein and lipids quantifications, and DNA associated-EVs from *E. coli* strain UFV_ECT1-72 in different conditions. **(A)** and **(B)** Protein quantification demonstrating the amounts in production of EVs in LB, CFT and TET conditions after 3 h and 6 h of culture, respectively. **(C)** and **(D)** Quantification of EVs production by flow cytometry using lipophilic dye DiO after 3h and 6h of culture, respectively. **(E)** and **(F)** Quantification of internal DNA associated-EVs between the treatments, after 3h and 6h of culture, respectively. Significant differences between EVs production are indicated by different letters, whereas equal letters

indicate no significant difference between the conditions, as calculated by One-way ANOVA test with Tukey's multiple comparisons test ($p < 0.05$).

11.4 EVs produced under different conditions carried antibiotic resistance genes

To investigate whether genetic markers are packaged by EVs produced under different stress conditions, we used EVs as a template for performing the polymerase chain reaction (PCR) by using the primers described in Table S1. As a result, DNA associated with EVs from the *E. coli* strain UFV_ECT1-72 under different conditions demonstrated three or more ARGs, independent of the stress condition and hours of culture (Figure 5). PCR results showed that the $bla_{CTX-M-2}$ gene was absent only in DNAse-treated EVs from TET 3 h and 6 h conditions (Figure 5A), suggesting that $bla_{CTX-M-2}$ was associated on the EVs surface on these groups, whereas the bla_{TEM} gene was detected in all the EVs obtained from either the LB or antibiotics conditions, treated or not with DNAse (Figure 5B).

Interestingly, the *tetA* and *tetB* genes were absent in DNAse-treated EVs from TET 3 h - 6 h conditions (Figure 5C-D). However, they were detected in untreated EVs, suggesting that despite the stress condition with tetracycline, these genes were not packaged inside EVs, being located in the external surface of EVs.

Regarding the detection of non-antibiotic-specific ARGs, the *dfrA17* and *sul1* genes were identified only within EVs obtained under the LB 3 h condition, although they were associated with the EVs surface in other conditions (Figure 5E-F). Notably, EVs isolated from the LB 3 h, CFT 3 h and CFT 6 h conditions harbored almost all of the ARGs tested.

Finally, although these ARGs were detected inside the EVs, no successful horizontal gene transfer through vesiduction was observed, indicating that their presence in EVs does not necessarily result in gene dissemination.

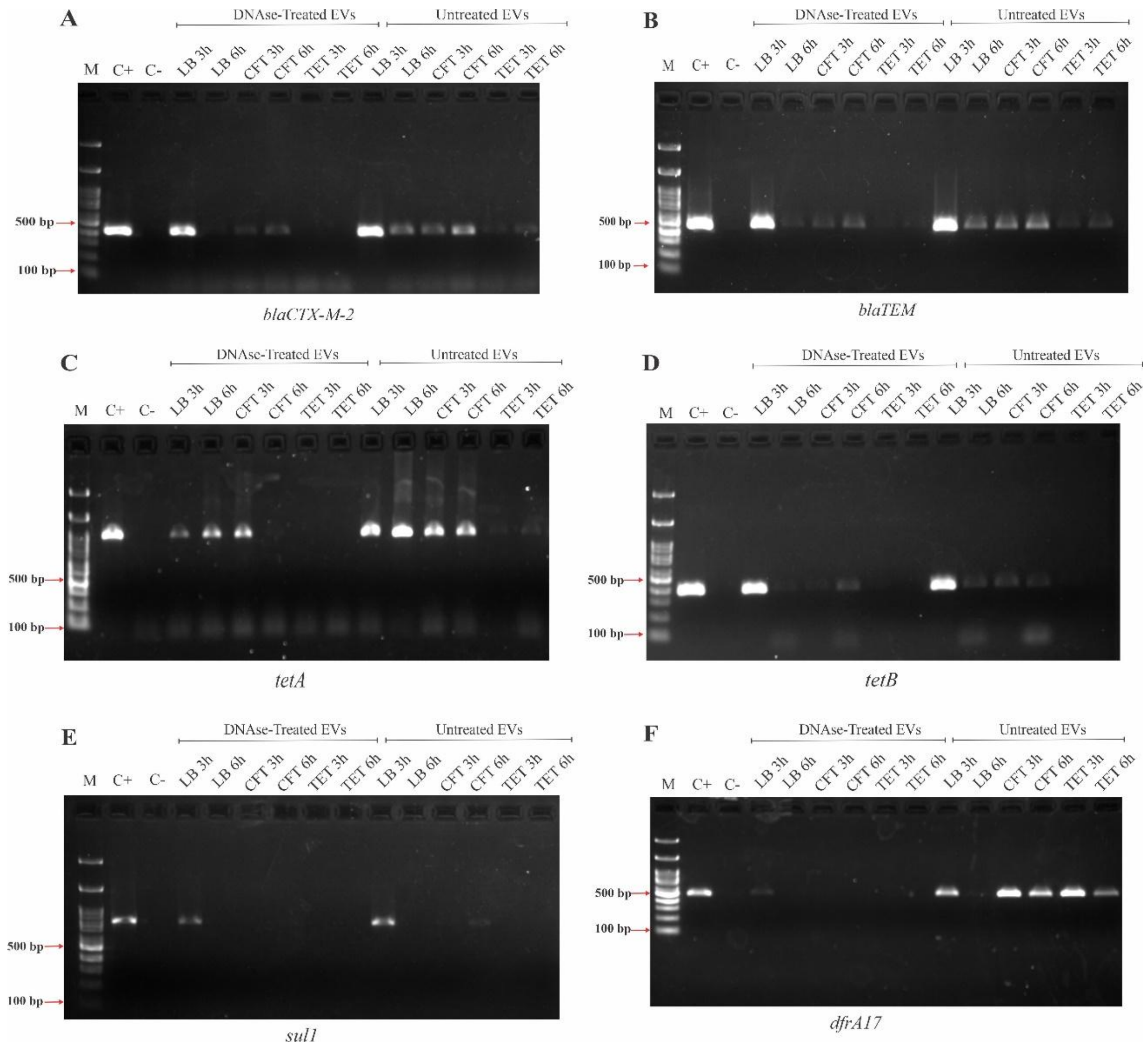

Figure 5. Detection of antibiotic resistance genes (ARGs) in EVs produced by *E. coli* strain UFV_ECT1-72 under different conditions by PCR. **(A)** Detection of $bla_{CTX\text{-}M\text{-}2}$ in EVs treated or not with DNAse, absent only in DNAse-treated EVs from the TET group. **(B)** The bla_{TEM} gene present in all EVs obtained. **(C)** and **(D)** Presence of the *tetA* and *tetB* genes, highlighting that were present only in EVs surface in EVs from TET group. **(E)** Detection of *sul1* gene in EVs, which was detected in less frequency inside the EVs. **(F)** Detection of *dfrA17* gene, demonstrated that it was associated on the EVs surface in almost all conditions. Genomic DNA of *E. coli* UFV_ECT1-72 strain was used as the positive control and sterile water was used as the negative control. Marker ladder 100 bp.

12 DISCUSSION

In the recent decades, antimicrobial resistance (AMR), particularly multidrug resistance, among members of the Enterobacteriaceae family has emerged as a major public health issue in both human and veterinary medicine (Li et al., 2022; Peng et al., 2022; Abdel Gawad et al., 2023; Basnet et al., 2024; Kerek et al., 2025). Notably, many antibiotic classes widely used in clinical settings, such as tetracycline, aminoglycosides, β-lactams, and trimethoprim, are also extensively employed in food-producing animals (Manyi-Loh et al., 2018; O'Neill et al., 2023). This overlap underscores the urgent need for prudent antibiotic use in animal farming, given

the frequent interactions between animals and humans, facilitating the potential transmission of resistant bacteria.

With the advance of antibiotic resistance in livestock, studies are essential to elucidate the pathways contributing to the accelerated dissemination of antibiotic resistance genes (ARGs) among bacteria associated with food-producing animals. Recently, a new horizontal gene transfer (HGT) mechanism has been proposed involving extracellular vesicles (EVs), referred to as vesiduction (Soler and Forterre, 2020). In recent years, studies have investigated the role of bacterial EVs in the transport and delivery of nucleic acids, and their contribution to the increase of antibiotic resistance (Tang et al., 2023; Xu et al., 2024).

However, the production and characteristics of EVs derived from *E. coli* isolated from clinically healthy pigs, and in exponential growth phases under antibiotic stress conditions were no reported yet. In this present study, we characterized the morphology, physical properties, and genetic content of EVs produced along the bacteria growth from the multidrug resistant *E. coli* strain UFV_ECT1-72 isolated from a clinically healthy pig (Oliveira et al., 2024). The EVs were analyzed under conditions with and without antibiotic stress, and after 3 and 6 h of culture. The antibiotics used in this study (Ceftiofur and Tetracycline) each target distinct bacterial processes, which may contribute to distinct impacts on EVs release.

Ceftiofur is a third-generation cephalosporin that is commonly used in the treatment of septicemias, respiratory infections in food-producing animals, such as pigs, while tetracycline is critical important class in the treatment of different bacterial infections (WOAH, 2025). The action mechanism of cephalosporins is the inhibition of the bacteria cell walls synthesis by inhibiting penicillin-binding proteins (PBPs), responsible for the cross-linking of cell wall (Batchelor et al., 2005; Bush and Bradford, 2016). Tetracycline inhibits the translation by binding in the 30S ribosomal subunit and can acquire genes that encode efflux pumps (Pioletti et al., 2001; Grossman, 2016). Studies demonstrated that the decreased number of bonds in outer membrane and peptidoglycan affects EVs production, increasing the vesiculation (Deatherage et al., 2009), corroborating with our results in the presence of ceftiofur, which we observed an increase in EVs formation and the presence of DNA into EVs after ceftiofur treatment at least 2-fold compared to control group (LB condition). Torabian and colleagues (2024) demonstrated similar results with cephalosporins, ceftriaxone and cefepime, also enhanced the release of EVs from *E. coli* at least 3- to 5-fold compared with the control group.

We observed that TET condition induced a significant increase in protein concentration, reaching 2.9- and 2.6-fold higher levels after 3 h and 6 h of culture, respectively, compared to the control group (LB condition). These results are consistent with the findings of Mao et al.

(2024), who reported that EVs production in *Klebsiella pneumoniae* increased 4.38-fold under tigecycline treatment compared to a 1.33-fold increase in the control group. As a bacteriostatic antibiotic that does not trigger spontaneous cell lysis, tetracycline allows bacteria to remain metabolically active under stress conditions, even at the CLSI breakpoint concentration of 16 µg. mL^{-1} (Thaker et al., 2009; CLSI, 2020). Importantly, the enhanced vesiculation observed under tetracycline stress is consistent with the slower growth kinetics of *E. coli* strain UFV_ECT1-72, which also carry a conjugative plasmid-encoded tetracycline resistance gene (*tetA, tetB*). This strain displayed a reduced maximum growth rate and prolonged generation time. This may suggest that the higher metabolic burden associated with plasmid-encoded tetracycline resistance mediated by efflux pumps, not only limits bacterial fitness but also stimulates vesiculation as an adaptive strategy. In contrast, ceftiofur exposure, despite the presence of extended-spectrum β-lactamase (ESBL) resistance genes (bla_{TEM} and $bla_{CTX\text{-}M\text{-}2}$), did not significantly alter protein yield or growth kinetics, likely due to the lower energetic cost of β-lactamase-mediated resistance (Grossman, 2016; Bush & Bradford, 2019; Kim et al., 2023). Recent studies demonstrated that the antibiotic resistance imposes a significant metabolic burden in bacteria, which may influence in cells mechanisms, including extracellular vesicles production. For example, Chen et al. (2023) observed that resistance to tetracycline mediated by *tetO* and *tetX* genes in *E. coli* triggers in the decrease of bacterial growth and increase in metabolic burden associated to the resistance. Furthermore, Dell'Annunziata and coauthors (2025) highlighted that the antibiotic exposure induces in morphological alterations in the EVs, including increase in the EVs production and enzymatic content with resistance-associated molecules.

Interestingly, all these morphological alterations might also reflect in EVs sizes, which EVs from TET condition were higher compared to the LB condition (~164 nm). Nevertheless, Huang and coauthors (2020), demonstrated that *Acinetobacter baumannii* under levofloxacin stress released elevated amounts of antibiotic-loaded vesicles parallel with high expression of efflux pump genes and EVs releases. These findings may explain our results in EVs produced under TET exposure. Further investigations for the relationships between the expression of these genes under the tetracycline stress and EVs production can help to understanding their role.

Another interesting point to highlight is the presence of nucleic acid in EVs from this condition, which was less detected within EVs compared to other conditions, as confirmed by flow cytometry with propidium iodide dye and PCR analysis. These findings suggest that the package of antibiotic resistance genes inside the EVs might depend on other factors, not only

antibiotic pressure, and that the tetracycline pressure might induce the package of other cell compounds. Further analysis it is necessary to explore the EVs cargo under TET exposure.

It is well described that the ESBL genes are a public health concern, since they contribute to the rise of β-lactams resistance worldwide in human and veterinary settings (Yang et al., 2019; Bush and Bradford, 2020; Nachimuthu et al., 2021; Balázs et al., 2021). Nevertheless, studies described that ESBL genes are commonly detected associated with ARGs from different antibiotic classes, including trimethoprim and sulfonamides, and almost all located in the genetic context of plasmids (Shawa et al., 2021; Trongjit et al., 2021; Da Silva et al., 2022). Here, we further detected two important ESBL genes, $bla_{CTX\text{-}M\text{-}2}$ and bla_{TEM} genes, within the EVs by PCR, not only under CFT exposure but also under LB conditions. Under TET exposure only bla_{TEM} was detected. Additionally, the *dfrA17* and *sul1* genes were exclusively detected within the EVs produced in LB conditions. These results suggest that without antibiotic stress, the EVs cargo might be diverse, with ARGs from different antibiotic classes that the donor strain harbored. On the other hand, in the presence of antibiotic pressure, the EVs cargo might be modulate, which might decrease the packaging of non-specifics ARGs. However, it is remains unclear the mechanisms of target specificity and package of these ARGs. Finally, these findings underscore the important role of EVs in the packaging ARGs of public health importance, even without any antibiotic pressure (Dell'Annunziata et al., 2025).

Although ARGs were detected in EVs, no successful horizontal gene transfer via vesiduction was observed in our assays. We hypothesized that the DNA packaged within EVs may be fragmented or degraded. In addition, with physical aspects such as low vesicle-to-cell ratios and numbers of EVs may also contributed to the absence of detectable horizontal gene transfer. These considerations highlight that, while vesiculation increases under antibiotic stress, the presence of ARGs within EVs does not necessarily implies in their dissemination, emphasizing the need for further studies and optimized experimental approaches to fully evaluate the potential role of EVs in horizontal gene transfer.

13 CONCLUSION

In conclusion, this study provides the first evidence of extracellular vesicles (EVs) derived from an MDR-*E. coli* strain from clinically healthy swine, produced under ceftiofur and tetracycline exposure, and reveals distinct morphological and physical characteristics among them.

We also emphasize two key findings regarding the relationship between antibiotic resistance genes (ARGs) and EVs: (1) EVs produced in the absence of antibiotic pressure were

fully capable of packaging relevant ARGs from the donor cell, even during the exponential growth phase; and (2) The incorporation of these ARGs does not appear to be strictly antibiotic-specific, as illustrated by the *tetAB* genes, which under tetracycline exposure were only detected outside the EVs. Nevertheless, further experiments are required to confirm the potential of horizontal gene transfer mediated by these EVs produced under different conditions.

Overall, our findings suggest that EVs produced under different stress conditions may act as reservoirs of ARGs of public health concern, such as ESBL genes, and could serve as vehicles for horizontal gene transfer to susceptible bacteria within the microbiota of clinically healthy animals.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPLEMENTARY MATERIAL

Table S1. Primers used in this study.

Primer identification	Sequence (5'-3')	Amplicon size (bp)	Tm (°C)
bla$_{CTX\text{-}M\text{-}2}$-F	CGTTAACGGCACGATGAC	404	55.7
bla$_{CTX\text{-}M\text{-}2}$-R	CGATATCGTTGGTGGTRCCAT		
bla$_{TEM}$-F	ACAGCGGTAAGATCCTTGAGAG	430	55
bla$_{TEM}$-R	GAAGCTAGAGTAAGTAGTTCG		
dfrA17-F	AAAATTTCATTGATTTCTGCA	471	50.8
dfrA17-R	TTAGCCTTTTTTCCAAATCT		
sul1-F	GTGACGGTGTTCGGCATTCT	779	59.6
sul1-R	TCCGAGAAGGTGATTGCGCT		
tetA-F	GTAATTCTGAGCACTGTCGC	957	59
tetA-R	CTGCCTGGACAACATTGCTT		
tetB-F	CTCAGTATTCCAAGCCTTTG	414	54.5
tetB-R	CTAAGCACTTGTCTCCTGTT		

Table S2. Mean optical density values (OD_{600} ± SD) of *E. coli* strain UFV_ECT1-72 measured at different incubation times and under different conditions.

Time (h)	Optical density (OD_{600} ± SD)		
	LB (control)	**LB/CFT**	**LB/TET**
0	0.12 ± 0.01	0.11 ± 0.009	0.12 ± 0.02
0.5	0.19 ± 0.005	0.18 ± 0.01	0.16 ± 0.02
1	0.43 ± 0.07	0.47 ± 0.02	0.36 ± 0.04

1.5	0.68 ± 0.02	0.75 ± 0.04	0.58 ± 0.02
2	0.99 ± 0.06	1.10 ± 0.07	0.74 ± 0.03
2.5	1.36 ± 0.02	1.49 ± 0.06	1.00 ± 0.04
3	1.48 ± 0.02	1.76 ± 0.03	1.33 ± 0.02
3.5	1.71 ± 0.03	1.83 ± 0.03	1.44 ± 0.05
4	1.86 ± 0.09	2.10 ± 0.08	1.62 ± 0.05
4.5	2.14 ± 0.15	2.56 ± 0.13	1.72 ± 0.05
5	2.28 ± 0.12	2.81 ± 0.02	1.83 ± 0.01
6	2.56 ± 0.06	3.03 ± 0.14	2.01 ± 0.05
7	2.79 ± 0.12	3.29 ± 0.12	2.49 ± 0.05
8	3.05 ± 0.02	3.54 ± 0.08	2.66 ± 0.05
9	3.31 ± 0.16	3.90 ± 0.15	2.90 ± 0.08
10	3.49 ± 0.14	3.98 ± 0.11	3.11 ± 0.12
11	3.65 ± 0.10	4.15 ± 0.14	3.29 ± 0.09
12	3.96 ± 0.13	4.37 ± 0.02	3.43 ± 0.05
24	4.7 ± 0.20	4.65 ± 0.11	4.17 ± 0.05

Table S3. Bacterial counts ($CFU.mL^{-1}$) and log_{10} $CFU.mL^{-1}$ of *E. coli* strain UFV_ECT1-72 measured at 1-6 h, 12 h and 24 h of incubation under different conditions. The initial DO_{600} was 0.1.

	$CFU.mL^{-1}$							
Growth conditions	**1 h**	**2 h**	**3 h**	**4 h**	**5 h**	**6 h**	**12 h**	**24 h**
LB	3.32×10^7	5.03×10^8	8.65×10^8	1.21×10^9	1.23×10^9	1.25×10^9	2.53×10^9	1.52×10^9

LB/CFT	4.88×10^7	6.45×10^8	9.53×10^8	9.72×10^8	1.25×10^9	1.31×10^9	1.39×10^9	1.71×10^9
LB/TET	7.5×10^7	4.4×10^8	7.5×10^8	1.39×10^9	1.41×10^9	1.43×10^9	1.45×10^9	1.20×10^9

Log_{10} CFU.mL^{-1}								
Growth conditions	**1 h**	**2 h**	**3 h**	**4 h**	**5 h**	**6 h**	**12 h**	**24 h**
LB	7.521	8.702	8.937	9.083	9.090	9.907	9.403	9.182
LB/CFT	7.688	8.81	8.979	8.988	9.097	9.117	9.143	9.233
LB/TET	7.879	8.643	8.877	9.145	9.149	9.155	9.162	9.081

14 CONCLUSIONS AND PERSPECTIVES

Based on our results, we can conclude that clinically healthy pigs are reservoir of *Escherichia coli* with multidrug-resistant (MDR) phenotype profile to a different antibiotic class, commonly used in both human and animal medicine. Furthermore, this MDR profile is reflected in the genotype, which harbors a wide range of antibiotic resistance genes, including extended-spectrum beta-lactamase (ESBL) genes (bla_{CTX-M}). We demonstrated that these genes were successfully transferred by horizontal gene transfer through conjugation to a susceptible bacterium (Chapter 1). Furthermore, the genomic characterization revealed that the strains UFV_ECT1-72, UFV_T1-217 and UFV_T2-7, belong not only to globally important STs (ST101), but also to rare STs, such as ST1653 and ST5229 (Chapter 2). We also characterized a novel multidrug conjugative plasmid designated as pEC72A, which were capable to transfer the resistance determinants, such as bla_{TEM}, *floR,* and *sul1* genes to recipient strain *E. coli* J53 Az^R.

Finally, we conclude that MDR-*E. coli* produces extracellular vesicles (EVs) in exponential growth phase, after 3h and 6h of culture. Furthermore, we conclude that antibiotic exposure can enhance the production of EVs and influences the morphological characteristics, including size and diameter. Tetracycline treatment stimulates the vesiculation rendering in large EVs compared to control group. Although, less DNA was packaging in this treatment. We also conclude that EVs are able to packaging ARGs of public health relevance, even in the absence of antibiotic pressure (Chapter 3). Here, we report for the first time the production and characterization of EVs derived from MDR- *E. coli* from clinically healthy pigs under ceftiofur and tetracycline exposure.

As perspectives, we intend to (1) explore the EVs production in other MDR-*E. coli* strains under antibiotic stress condition in different points of culture; (2) to investigate the factors involved in vesiduction mechanism; (3) to analyze the difference in EVs content from different conditions through omics technologies, such as DNA sequencing.