MARIANA SOFIA COSME DA FONSECA ARAÚJO

Bachelor of Science in Biology

ENTEROBACTERALES FROM THE PORK PRODUCTION CHAIN IN PORTUGAL ARE KEY RESERVOIRS OF ANTIBIOTIC RESISTANCE AND VIRULENCE GENES

MASTER IN MEDICAL MICROBIOLOGY
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Advisor: Dr. Maria Miragaia,

Auxiliar researcher, Laboratory of Bacterial Evolution and Molecular Epidemiology, ITQB-NOVA, Oeiras, Portugal

Co-advisor: Dr. Nuno A. Faria,

Doctoral Researcher, Laboratory of Bacterial Evolution and Molecular Epidemiology, ITQB NOVA, Oeiras, Portugal

Examination Committee:

Chair: Dr. José Paulo Sampaio,

Associate Professor with Aggregation, Department of Life Sciences FCT-NOVA, Almada, Portugal

Rapporteurs: Dr. Aida Duarte

Associate Professor with Aggregation, Pharmacy Faculty, UL, Lisbon, Portugal

Advisor: Dr. Maria Miragaia,

Auxiliar researcher, Laboratory of Bacterial Evolution and Molecular Epidemiology, ITQB-NOVA, Oeiras, Portugal

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ABSTRACT

Enterobacterales are a major public health threat due to their capacity to spread antibiotic resistance genes. Carbapenem resistant Enterobacterales (CRE) are important causes of infections in Portugal and worldwide. Food production animals have been described as reservoirs of CRE and resistance determinants. However, the extent of dissemination of CRE and their resistance genes between food production chain and hospitals is limited explored. We aimed to understand if the pork production chain in Portugal was a reservoir of clinically relevant antibiotic resistance genes and resistant Enterobacterales.

Enterobacterales collected from a slaughterhouse in Portugal, in 2019 were characterized for their antibiotic susceptibility profile by disk diffusion and microdilution and the whole genome of a representative collection of isolates was sequenced. Moreover, their content in antimicrobial resistance and virulence genes was determined by analysis of whole-genome sequencing data using bioinformatics tools. Enterobacterales genus/species were identified by 16S rRNA gene sequencing and confirmed by determining the average nucleotide identity (ANI). Clonal types were identified by *in silico* multilocus sequence typing.

We identified a wide variety of Enterobacterales, 79.5% of which were multidrug-resistant. Moreover, 45% showed resistance to colistin (MIC>8 mg/L). Genomic analysis showed that the great majority of Enterobacterales (74%) carried a diversity of class C β-lactamases, class A and class B - some previously associated to CRE causing infections in humans. Out of the 10 colistin-resistant isolates that were sequenced, three carried the *mcr-10* gene. A total of 264 different virulence factors were identified related to motility (41.7%), chemotaxis (3.8%), secretion system (22.3%), iron acquisition (10.6%), capsule (9.8%), toxins (7.5%), efflux pumps (2.75%) and porins (1.1%). Two isolates belonged to clonal types previously found causing infections in humans (*Escherichia coli* ST410 and *Klebsiella pneumoniae* ST60).

Our results suggest that Enterobacterales from the pig meat production chain constitute a reservoir of antibiotic resistance genes and resistant bacteria pathogenic to humans.

Keyword: Enterobacterales; Carbapenem-resistance; Pig production chain; Colistin

RESUMO

Enterobacterales representam muitas vezes uma ameaça à saúde pública devido à sua capacidade de adquirir e disseminar genes de resistência a antibióticos. Enterobacterales resistentes aos carbapenemos (CRE) são importantes causadores de infeções em Portugal e no mundo. Os animais utilizados para a produção de alimentos têm sido descritos como reservatórios de CRE e de determinantes de resistência aos antibióticos. No entanto, a extensão de disseminação de CRE e seus genes de resistência, entre a cadeia de produção de alimentos e o hospital, têm sido pouco explorados. Este estudo pretende compreender se a cadeia de produção de suínos constitui um reservatório de genes de resistência a antibióticos, bem como de Enterobacterales resistentes a antibióticos e clinicamente relevantes.

Para este estudo foram recolhidos isolados pertencentes à Ordem Enterobacterales num matadouro em Portugal, em 2019. Estes isolados foram caracterizados quanto ao seu perfil de suscetibilidade a antibióticos; e isolados representativos foram caracterizados através da sequenciação total do genoma. O seu conteúdo genómico foi determinado através da análise dos dados de sequenciação, utilizando ferramentas bioinformáticas. A identificação do género/espécie foi confirmada pela sequenciação do gene 16S rRNA e pelo método *Average Nucleotide Identity* (ANI). Os tipos clonais foram identificados pelo método de *multilocus sequence typing* (MLST) *in silico*.

Foi identificado uma grande variedade de Enterobacterales, sendo que 79,5% foram consideradas multirresistentes. Além disso, 45% apresentaram resistência à colistina (MIC> 8 mg/L). A análise genómica mostrou que a maioria dos Enterobacterales apresentava uma diversidade de β-lactamases da classe C, classe A e classe B, tendo sido algumas delas encontradas em CRE causadoras de infeções em humanos. Dos 10 isolados resistentes à colistina que foram sequenciados, três continham o gene *mcr-10*. Foram também descritos 264 fatores de virulência relacionados com motilidade (41,7%), quimiotaxia (3,8%), sistema de secreção (22,3%), aquisição de ferro (10,6%), cápsula (9,8%), toxinas (7,5%), bombas de efluxo (2,75%) e porinas (1,1%). Também identificamos dois isolados que pertencem a tipos clonais previamente associados a infeções em humanos (*Escherichia coli* ST410 e *Klebsiella pneumoniae* ST60).

No geral, os nossos resultados sugerem que Enterobacterales existentes na cadeia de produção de suínos constituem um reservatório de genes de resistência a antibióticos e de bactérias patogénicas para os humanos.

Palavras-chave: Enterobacterales; Resistência aos carbapenem; Cadeia de produção suína; Colistina

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABREVIATION LIST

AGP – Antibiotics as growth promoters

AMC – Amoxicillin + Clavulanic acid

ANI – Average Nucleotide Identity

ARG – Antimicrobial resistance genes

BLAST – Basic Local Alignment Search Tool

CAZ – Ceftazidime

cgMLST – Core genome multilocus sequence typing

CIP – Ciprofloxacin

CN – Gentamicin

COV – Gene coverage

CRE – Carbapenem resistance Enterobacterales

CTX – Cefotaxime

CZA – Ceftazidime + Avibactam

DDH – DNA-DNA Hybridization

DE – Delaware State

DNA – Deoxyribonucleic acid

dNTP – Deoxyribonucleotide triphosphate

EDTA – Ethylenediaminetetraacetic acid

EFSA – European Food Safety Authorities

EMA – European Medicines Agency

ERT – Ertapenem

ESBL – Extended spectrum β-lactamases

EU – European Union

EUCAST – European Committee on Antibiotic Susceptibility Testing

FAO – Food and Agriculture Organization

FDA – Food Drug Administration

FOX – Cefoxitin

GFN – Global Foodborne Infections Network

ICE – Integrative conjugative elements

ICU – Intensive care units

ID – Gene identity

IMI – Imipenem

KEC – *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp.

LB – Luria-Bertani

LPS – Lipopolysaccharide

MD – Maryland State

ME – Maine State

MER – Meropenem

MIC – Minimum inhibitory concentration

MLST – Multilocus sequence typing

NCBI – National Center for Biotechnology Information

OD – Optical density

OIE – World Organization for Animal Health

PBP – Penicillin binding protein

PBS – Phosphate Buffered Saline

PCR – Polymerase chain reaction

PCU – Population correction unit

PFGE – Pulse-field gel electrophoresis

RNA – Ribonucleic acid

rRNA – Ribosomal ribonucleic acid

SLV – Single locus variant

SNP – Single nucleotide polymorphisms

ST – Sequence type

SXT – Trimethoprim + Sulfamethoxazole

TAE – Tris-Acetate-EDTA

TEM – Temocillin

TIC – Ticarcillin

tRNA – transfer ribonucleic acid

TSB – Tryptic Soy Broth

TZP – Piperacillin + Tazobactam

UPGMA - Unweighted pair group method with arithmetic mean

USA – United States of America

UV – Ultraviolet

WGS – Whole Genome Sequencing

WHO – World Health Organization

WI – Wisconsin State

1. INTRODUCTION

1.1 The Enterobacterales Order

The Enterobacterales is a diverse Order of Gram-negative, rod-shaped, non-spore-forming and facultative anaerobic bacteria within the class Gammaproteobacteria. Within the Enterobacterales Order seven Families are described, such as Enterobacteriaceae, Erwiniaceae, Pectobacteriaceae, Yersiniaceae, Hafniaceae, Morganellaceae and Budviciaceae, which include in total 73 genera and more than 250 species (4).

1.1.1 The Enterobacteriaceae bacteria Family

The Enterobacteriaceae is the most diverse bacterial Family of the Enterobacterales Order. The creation of Enterobacteriaceae Family was first proposed by Rahn in 1936 and nowadays includes over 30 genera and 100 species, classified according to specific biochemical properties, antigenic composition and molecular analysis of their genomes. Among this Family, several genera are known to be associated with infections in humans, such as *Klebsiella* spp. *Escherichia* spp., *Citrobacter* spp., *Enterobacter* spp. (222).

The Enterobacteriaceae are facultative anaerobic, rod-shaped bacillus with moderate size, in average they measure 1-6 μm in length by 0.3-1.0 μm in width and are non-spore-forming. They have simple nutritional requirements and can rapidly grow, with a doubling time average between 20 and 30 minutes *in vitro*. They present the capacity to metabolize glucose, reduce nitrate to nitrite, are resistant to bile salts and produce catalase but do not produce oxidase. On the other hand, biochemical features, like the ability to metabolize lactose and urea and the capacity to produce indole, can be used to differentiate between some genus and species (see Table 1.1). Most Enterobacteriaceae species grow well at 37ºC; however, some species present optimal grow capacity between 25ºC and 30ºC, such as *Morganella morganii* that have optimum temperature for growth of 25ºC (108). The great majority of species are motile owning a peritrichous flagella. Moreover, they can also have fimbriae and/or conjugative pili, which are important in genetic transfer between bacteria, as they help in adherence to specific host cell and bacterial cell

receptors (147). Some species, like *Klebsiella pneumoniae* are characteristically mucoid, due to the production of a capsule – a factor that can be important for immune evasion in the host (50).

1.1.2 Other Enterobacterales Families

Other Families within the Enterobacterales Order that are as important as Enterobacteriaceae in terms of clinical impact, include Yersiniaceae and Morganellaceae. The Yersiniaceae Family comprises seven genera, including *Yersinia* spp. and *Serratia* spp. The Morganellaceae were originally members of the Enterobacteriaceae Family, and include some opportunistic human pathogens such as *Proteus* spp., *Providencia* spp. and *Morganella* spp.

Erwiniaceae and Hafniaceae are other two Families within Enterobacterales, which are typically plant and insect pathogens, but have also been associated to human infections (4), although in a lesser extent. These include genera such as *Erwinia* spp., *Pantoea* spp. (Erwiniaceae Family) and *Hafnia spp* (Hafniaceae Family) (4).

These different genera within Enterobacterales Order have distinctive biochemical characteristics that can be used for their identification (see Table 1.1).

Table 1.1 – Biochemical reactions used for the identification of genus within Enterobacterales and Aeromonadales. Adapted from (85, 100, 153).

Species	Lactose	Urease	Indole	Catalase	Oxidase
***Aeromonas* spp.**	Variable	-	+	+	+
***Citrobacter* spp.**	Variable	Variable	+	+	-
***Enterobacter* spp.**	Variable	Variable	-	+	-
***Escherichia* spp.**	+	-	+	+	-
***Hafnia* spp.**	-	-	-	+	-
***Klebsiella* spp.**	+	+	-	+	-
***Pantoea* spp.**	Variable	-	-	+	-
***Shigella* spp.**	-	-	Variable	+	-
***Serratia* spp.**	-	-	-	+	-

Legend: (+) – positive reaction; (-) – negative reaction; variable – a positive or negative reaction can happen

1.1.3 Enterobacterales spread in the environment

Enterobacterales are ubiquitous in nature, they can exist free in diverse ecological niches, including soil (sediments, agricultural environments) and aquatic habitats (wastewater, drinking water, natural waterways) or associated with animals (humans, companion animals, wildlife, food production animals, insects) and plants (140). Moreover, these bacteria are frequently transferred between the different actors in the environment.

One of the most important means of bacterial spread in the environment is through the food chain. Several studies have found that wild fauna can represent an important reservoir of bacteria, including Enterobacterales. Food consumption by animals, including birds, mammals and fish, can result in intestinal colonization by these bacteria. Nevertheless, there are other ways of bacterial transmission in the environment. These include, transmission through: infected manure, which contribute to the dissemination of bacteria in agriculture environments, including soil and plants (113); effluent discharges of hospital wastewater, wastewater treatment plants and urban wastewater into aquatic ecosystems; and through contaminated drinking water, especially in endemic areas. Furthermore, other hypothesis of environmental dispersal of bacteria may also be considered, which include flies as a vector, aerosol and dust, however with less success due to the lack of nutrients for survival (140).

1.1.4 Enterobacterales as human colonizers

Some Enterobacterales are part of the human microbiota, defined as the group of microorganisms that reside on the surface or within the human body, which are usually important for the development of human immune defenses, and for the production of a healthy hormonal and metabolic system (169). This is the case of *Escherichia coli*, *K. pneumoniae*, *Citrobacter* spp. and *Enterobacter* spp., which are part of the normal commensal flora of the lower gastrointestinal tract, frequently referred to as enteric bacteria (51). Other examples include *E. coli* that colonize the human urinary tract (181) and respiratory tract colonization by *K. pneumoniae*, *Enterobacter* spp. and *E. coli* in patients in clinical settings (178). Although microorganisms such as *Serratia* spp., *Hafnia* spp. and *Pantoea* spp. are not a common component of the human fecal flora, they are frequently identified in the digestive tract of neonates and in the respiratory and urinary tract of adults (109, 204). Most of their lifetime, these microorganisms are harmless commensals. However, when the immune system of the host is compromised, they can cause serious infections, being for that reason called opportunistic pathogens.

1.1.5 Enterobacterales, an important cause of human nosocomial and community infections

Members of the Enterobacteriaceae Family are responsible for a wide variety of human infections, such as urinary tract infections, pneumonia, lower-gastrointestinal infections, and bacteremia, among others (188). In the hospital setting they are especially associated to neonates, immunocompromised patients, patients with underlying diseases, patients that performed invasive procedures, like surgery or that are under the assistance of mechanical ventilation (8, 203). The most common species of the Enterobacteriaceae Family causing hospital infections are *K. pneumoniae* and *E. coli* (54). Nevertheless, other medically important genera are also found associated to infections in hospitals, namely, *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., and *Hafnia* spp., among others.

In particular, *K. pneumoniae* has been mainly associated to urinary tract infections, lower respiratory tract infections, intra-abdominal infections and bloodstream infections (54). They are also the cause of wound and soft tissue infections, meningitis and are one of the most frequent causes of sepsis in neonates in neonatal intensive care units (89, 93). Of particular concern is the emergence of multidrug resistance in *K. pneumoniae*, including resistance to last resort-antibiotics such as carbapenems, due to the reduced number of effective treatments (18). Actually, carbapenem resistant *K. pneumoniae* have been increasingly reported as a nosocomial pathogen worldwide. Only in Europe, in the year 2015, more than 16.000 infections were caused by carbapenem resistant *K. pneumoniae* strains and more than 2.000 deaths were reported (216).

On the other hand, *E. coli* has been associated to gastroenteritis, urinary tract infections, meningitis and sepsis, being considered the most common cause of severe urinary tract and bloodstream infection in Europe (54).

Another emergent human pathogen is *Citrobacter* spp. Although considered for a long time as environmental contaminants and low-virulence human colonizers, they are currently recognized as important infectious agents. They have been collected from a variety of infection products collected from the urinary tract, liver, biliary tract, intestines, bone, respiratory tract, wound, soft tissue and bloodstream (123). Additionally, *Serratia* spp., *Enterobacter* spp. and *Hafnia* spp. have also been described as opportunistic pathogens, being frequently associated with wounds and respiratory, urinary, and soft tissues infections in hospitals (107, 173, 174).

1.2 The food production chain

1.2.1 The pig as a major farming animal and it's importance for the human food chain

Pork meat has played a major role in human feeding, being the most preferred meat in the EU (European Union) and the most consumed in the world for a long period of time. Only in the last decade it was surpassed by poultry, because this is considered healthier and cheaper than pork (165, 192).

The use of domestic pigs for human diets, culture, lifestyles and also beliefs, dates from the Neolithic period. Some archeologists have actually suggested that the pig was the earliest animal that people domesticated for food purposes (212). During the Neolithic Revolution, also known as the Agricultural Revolution, the humans started to depend on plants and seeds, and animals domestication was essential for additional nutrition and for making the intensive farming possible (6). However, the revolution also brought some consequences that prevail until present times, one of which was the emergence of zoonosis, like infectious diseases, contracted from domesticated animals (146).

Nowadays, although pork production and consumption are slowly decreasing in the world, it continues to grow in the EU. According to FAO (Food and Agriculture Organization) in 2020 was estimated a pig meat output of 109.200 million tons in the world, a smaller value when compared to the 2017 output of 119.766 million tons. In the EU the pork production increased from 23.686 million tons in 2017 to a total of 24.141 million tons in 2020 (66, 67). In Portugal, in 2020 a total of 380 thousand tons of pork meat was recorded, representing a decrease of 2.1% compared to 2019 (96). Nevertheless, as much as 460 thousand tons of pork meat were consumed, corresponding to a *per capita* consumption of 44.7 kg per person per year (81). This is much higher than the average pork meat consumption per capita in the world, which corresponds to 15,6 kg. In Europe, Portugal is in the top 15 consumers of pork meat and Poland is in the first position with an average consumption of 57,6 kg per capita (166).

1.2.2 The food chain as a source of Human zoonoses

Zoonoses are infectious diseases caused by a pathogen (such as bacteria, virus, parasite or prions) that can be transmitted, directly or indirectly, between animals and humans, representing a major public health problem around the world. Zoonoses have different transmission routes including

consumption of contaminated food or water, known as foodborne route, direct contact with animals, including domestic animals, food animals and wildlife and, finally, via the environment (59). Common foodborne diseases include salmonellosis (caused by *Salmonella typhi or Salmonella paratyphi*) or food poisoning (*E. coli*), among others.

Although there are a large number of possible transmission routes, the basic human need of food intake makes the foodborne route the most predominant way of contracting zoonotic diseases. The foodborne zoonotic diseases in 2019 affected 50.000 humans in EU (15). The severity of these diseases varies from mild symptoms, such as nausea or vomiting diarrhea, to life-threatening situations like meningitis (202).

The spread of foodborne zoonosis through the food production chain has been referred to as 'farm-to-fork' and is related to the different stages of food production chain, from the farm to slaughterhouse and the consumers (211). The contamination of the production animals can occur at the level of the farm if the animal feed is contaminated with bacteria (or other pathogen), or if the milk or animal skin is contaminated through contact with feces or dust. Farmers and veterinaries contact with contaminated animals may facilitate transmission of harmful bacteria to humans. The contamination of meat can also occur during the meat processing chain, if the meat enters in contact with previously contaminated surfaces or through the handling of meat by contaminated people. At this stage workers involved in the preparation of meat products are particularly exposed to harmful bacteria. Another possible transmission in the food chain is at home due to food manipulation. Some studies strongly suggest that many outbreaks of zoonotic pathogens originate in the consumers' homes, mainly related to improper handling, preparation, food consumption practices, or inadequate hygiene practices of the consumers, like insufficient hand washing, use of unhygienic utensils and materials, consumption of raw food, as well as cross contamination via used surfaces (139).

The combat of zoonotic foodborne diseases has been achieved by annual monitoring and risk analysis carried by different international organization at the European and worldwide levels. This is the case of World Health Organization's (WHO) Global Foodborne Infections Network (GFN) that comprises more than 130 countries and integrates a laboratory-based surveillance system that consists in the collaboration among human, veterinary and food sectors. At European level the foodborne zoonosis and zoonotic agents are monitored by the European Food Safety Authorities (EFSA) (65, 76).

1.2.3 Antibiotic use in farming animals

Antibiotics have been used in livestock since 1950 in order to keep animals healthy and increase productivity. They are an essential component of intensive farming, being commonly used for the treatment of clinical diseases, to prevent infections (prophylaxis), and for controlling the spread of infectious diseases in a large group of animals (metaphylaxis). Moreover, they are applied as growth promoters, considered a nontherapeutic use (116).

Worldwide it is estimated that 73% of all antibiotics sold are used in animals raised for food, and not in humans as a treatment. Additionally, several of these antibiotics are simultaneously used in animal production as well as in humans (199). Several studies have concluded that the use of antibiotics in animals results in a continuous positive selection of resistant bacterial clones, which can contaminate the environment and be disseminated to humans in contact with the animals, representing a major public health concern (193). To combat the extensive use of antibiotics in animals, several countries have abolished the use of antibiotics as growth promoters (AGP). The EU banned AGP use in 2006 and Brazil banned the use of colistin in animals in 2016 (171). Moreover, in 2015, the Food and Drug Administration (FDA), the organization regulating food safety in the United States, created a directive that prohibited the over-the-counter antimicrobial animal drug products. From that point on, antibiotics administration in animals was only possible when performed by a licensed veterinarian in the course of the veterinarian's professional practice (44). Other countries like those in the East Asian region have announced strategies to restrict the use of AGP but, unfortunately, either they have failed to put it into practice or lack the capacity to ensure their implementation. As a result, antibiotics continue to be used in these countries as animal growth promoters (80).

In 2007, the WHO, together with the World Organization for Animal Health (OIE) and FAO, developed a list that classified antibiotics, based on their importance to human medicine, as “critically important”, “highly important” and “important”. The purpose of this list was to be used as a reference for choosing the type of antimicrobials to be used in animals, in a tentative way to minimize antimicrobial resistance of medical importance (210). According to that list, the classes with the “highest priority” include cephalosporins, glycopeptides, macrolides, polymyxins and quinolones. While classes like carbapenem, aminoglycosides and penicillins are classified as “high priority” (see Table 1.2).

In spite of these recommendations, antibiotics continue to be highly used in the veterinary setting. Considering the antibiotic sales for farm animals in Europe, tetracycline is the antibiotic class most used (31.7 mg/population correction unit (PCU)) followed by penicillins (29.7 mg/PCU), sulfonamides (8.7 mg/PCU) and macrolides (8.0 mg/PCU) (60). The sales of 3rd and 4th generation

of cephalosporins was 0.2 mg/PCU and polymyxins was 1.6 mg/PCU, respectively. Regarding carbapenems, since its use is not allowed in any country worldwide for treating food-producing animals, this class does not appear in the consumption reports (see Figure 1.1).

Table 1.2 – Antimicrobials categorized as Critically Important for human medicine 6th revision by World Health Organization (210).

Critically Important Antimicrobials	
Highest Priority	Cephalosporins (3rd, 4th, 5th generation)
	Glycopeptides
	Macrolides and ketolides
	Polymyxins
	Quinolones
High Priority	Aminoglycosides
	Ansamycins
	Carbapenems and other penems
	Glycylcyclines
	Lipopeptides
	Monobactams
	Oxazolidinones
	Penicillins (antipseudomonal)
	Penicillins (aminopenicillins)
	Penicillins (aminopenicillins with β-lactamase inhibitor
	Phosphonic acid derivatives
	Drugs used to treat tuberculosis

Nevertheless, there have been an overall decrease of veterinary antimicrobial agents sales equivalent to 34.6% between 2011 (161.4 mg/PCU) and 2018 (105.6 mg/PCU). In particular, the sales of quinolones 74.4%, of polymyxins 69.8% , and 3rd and 4th generation of cephalosporins decreased 24.4% (60).

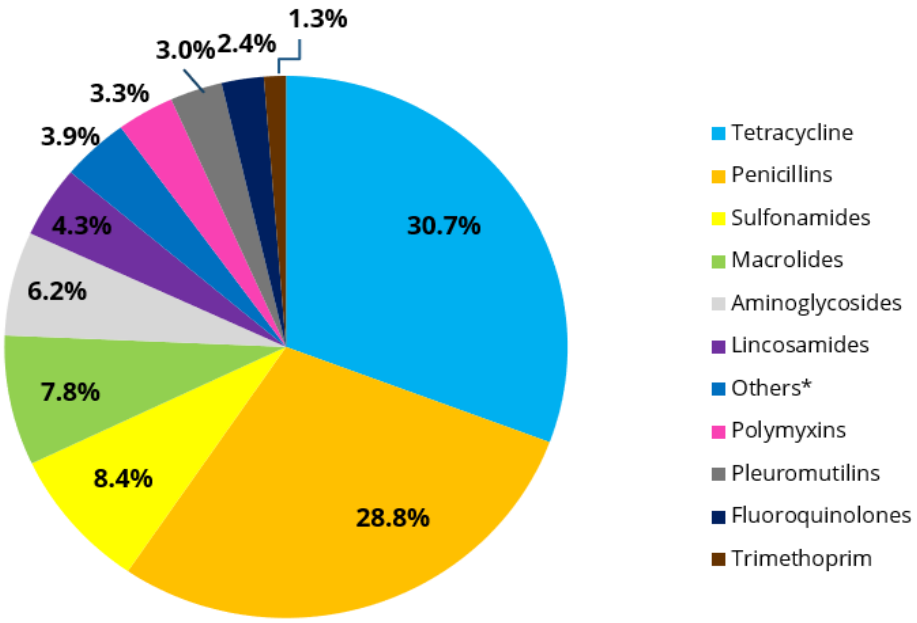

Figure 1.1 Proportion of different antibiotic classes used in food-producing animals in 31 European countries in 2018. Adapted from: Sales of veterinary antimicrobial agent in 31 European countries in 2018 (2020) European Medicines Agency (EMA) (60).

* Others – Amphenicols, cephalosporins, other quinolones and other antibacterial (classified in ATCvet system).

1.3 Antibiotic resistance in Enterobacteriaceae

The acquisition of antimicrobial resistance genes (ARG) has reduced the available treatment options for bacterial infections, which resulted in an increasing number of deaths due to treatment failure. Gram-negative bacteria from the Enterobacteriaceae Family have become a serious global public health problem because of strains resistant to the vast majority, if not all, of the antibiotics ever produced (97). In 2017, in order to prioritize the research and development of new antibiotics, the WHO published a list where they identified the most important resistant bacteria, at a global level, and for which new antimicrobial development is urgently needed. In this list, Enterobacteriaceae resistant to carbapenems and 3rd generation cephalosporins are part of the "critical priority" group (214).

Bacteria can be intrinsically and naturally resistant to certain classes of antibiotics, when resistance is provided by native and ubiquitous genes that are part of the bacteria species' genome and/or a consequence of the bacteria's structural or functional characteristics. However, bacteria can otherwise become resistant as a means of survival in the presence of an antibiotic stress. This may be achieved through the emergence of spontaneous mutations in chromosomal genes, or by

the acquisition of exogenous resistance genes through mechanisms of genetic transfer such as conjugation, transformation or transduction (14).

Antibiotic resistance genes are frequently transported in mobile genetic elements. The most relevant mobile elements, usually involved in the transfer of antibiotic resistance genes, are transposons, plasmids, insertion sequences as well as integrative conjugative elements (ICEs) (24). These mobile elements can carry more than one ARG, conferring resistance to different classes of antibiotics, which enables bacteria to become multidrug resistant in a single transfer event. Additionally, they can harbor other genetic determinants such as toxins and siderophores (189). The acquisition of elements carrying both resistance and virulence genes can lead to the emergence of pathogenic bacteria that are at the same time very difficult to treat.

Insertion sequences are the smallest transposable elements encoding only the enzymes necessary for their own transposition, using those for its insertion into another genome. Transposons carry two insertion sequences and other genes between them, for example antimicrobial resistance genes (43). Conjugative transposons, also classified as integrative conjugative elements (ICEs), encode all the necessary information for their own excision, conjugation and integration into a new host, being also capable of co-transferring other plasmids, transposons, and large chromosomal fragments between strains. ICEs are typically found integrated in the host bacterial chromosome and they are vertically transmitted during cell division (24).

Plasmids are the main mechanism responsible for the dissemination of AMR genes. Typically, plasmids are circular, double-stranded and self-replicating DNA molecules, existing as extra chromosomal elements and have the capacity to replicate separately from the host chromosome. The plasmids conferring multidrug resistance are usually large, self-conjugative and encode mechanisms to control copy number and regulate the rate of replication. The control of replication implies that two plasmids sharing the same replicon (composed of origin of replication, specific replication proteins and regulating factors) cannot stably be in the same cell line; this phenomenon is known as plasmid incompatibility and is used to classify plasmids. Classification of plasmids based on the replicon typing presents several limitations. This is particularly difficult to achieve in cases in which plasmids have multiple replicons. Moreover, there is limited information on the distribution and diversity of Inc types among microbial taxonomies, which difficult the identification of replicons (189).

There are more than 800 plasmids identified in Gammaproteobacteria however some plasmid families are more associated with the spread of antimicrobial resistance genes worldwide. The plasmids most frequently described carrying antibiotic resistance genes from human and animals are from the IncF type. This type of plasmids has been frequently identified in Enterobacteriaceae encoding ESBL (Extended spectrum β-lactamases), aminoglycoside resistance and

carbapenemases genes. Additionally, ESBL and AmpC (class C β-lactamases) genes have been described on IncI plasmid types (182). This type of plasmids was found predominantly in Europe associated with *E. coli* from poultry sources. Others plasmids in Enterobacterales that frequently carry resistance genes are IncA/C, IncN and ColE type (189).

1.3.1 β-lactamases resistance mechanisms

There are several mechanisms that allow bacteria to become resistant, being the most common the inactivation of the antimicrobial molecules and the modification of the antibiotic target site. The least common mechanism involves changes in the permeability of the bacterial cell envelope.

β-lactam antibiotics are one of the most effective and commonly used agents in the treatment of infectious diseases and the most important therapeutic option to treat Enterobacterales infections. The β-lactams, such as penicillins, cephalosporins, carbapenem and monobactams, represent 60% of the worldwide antibiotic usage. These antibiotics act by inhibiting the synthesis of the cell wall peptidoglycan. The β-lactam binds irreversibly to the active site of transpeptidase enzymes, known as penicillin binding proteins (PBPs), preventing the transpeptidation reaction and cell wall synthesis, which results in bacterial death. To circumvent the action of β-lactams, bacteria developed/acquired enzymes called β-lactamases that are able to inactivate the antibiotic through the hydrolysis of the amide bond of the four-membered β-lactam ring present in every β-lactam. An example are penicillinases, enzymes able to inactivate penicillin, that were discovered by Alexander Fleming in 1940, two years before the introduction of penicillin as a treatment in clinical practice (42).

β-lactamases are a very diverse group of enzymes and its nomenclature is based on two classification schemes. The first classification is based on amino-acid sequence similarity and was defined by Amber in 1980 (196). According to this scheme the β-lactamases are divided into four classes (A to D). Classes A, C (known as AmpC) and D (known as OXA) share a similar mechanism that involves the serine as the active center of the enzyme. The class B, also known as metallo-β-lactamases, are zinc metalloenzymes and are distinct from the serine β-lactamases in terms of sequence, fold and mechanism. They are broad spectrum and have the ability to hydrolyze all β-lactams existing to date, including carbapenems (see Table 1.3).

The second classification scheme is based on functional characteristics. In this classification the β-lactamases are divided in three major groups (Group 1-3), according to their substrate and inhibitor profiles (29).

Table 1.3 – Classification of β-lactamases by Bush-Jacoby-Medeiros (29) (Functional Classification), and by Amber (196) Molecular Classification. Adapted from (29).

Functional Classification	Molecular Classification	Distinctive Substrates	Principal localization	Examples
1	C	Cephalosporins,	Chromosome /Plasmid	CMY, FOX, MOX, ACT
2	A	Penicillins, extended-spectrum cephalosporins, monobactams, carbapenem	Plasmid	TEM, SHV, CARB, CTX, GES, KPC
	D			OXA
3	B	Carbapenem	Chromosome /Plasmid	IMP, VIM

1.3.1.1 Group 1 β-lactamases

The group 1 corresponds to the cephalosporinases AmpC group, which are mostly encoded on the chromosomes of many Enterobacterales and are usually resistant to inhibition by classical inhibitor like clavulanic acid. In addition to penicillins and cephalosporins they also confer resistance to oxyimino-cephalosporins, such as ceftazidime, cefotaxime and ceftriaxone, to monobactams and cephamycin, such as cefoxitin. (99).

In many organisms, including *Citrobacter freundii*, *Enterobacter cloacae* complex, *Hafnia alvei*, *Morganella morganii* and *Serratia marcescens*, inducible AmpC are encoded on the chromosomes (111). Usually, these bacteria have a low expression of AmpC, but this expression increases upon exposure to certain β-lactams such as amoxicillin, imipenem and clavulanic acid. In this case the expression of AmpC is under the control of AmpR, a transcriptional regulator. The repression and activation are linked to the process of cell wall peptidoglycan synthesis involving additional proteins such as AmpD and AmpG, which are AmpC inhibitors. If a mutation occurs in any of these genes a constitutive upregulation of AmpC can occur, which corresponds to the most common cause of constitutive high-level expression of AmpC in clinical isolates (98). In some species like *E. coli* and *Shigella* spp., however, the AmpC is noninducible, and its expression is regulated by the promoter and attenuator mechanisms. A higher expression of AmpC can occur by gene duplication or mutations in the promoter or attenuator regions (137).

When produced in large amounts AmpC can provide resistance to carbapenems, especially ertapenem. An alternative mechanism of carbapenem resistance is the decrease of β-lactam concentration in the periplasm, due to alterations on the permeability of the cell outer membrane.

These alterations are usually due either to variations in the porin channels or to the increase of efflux systems expression, increasing the efficiency of AmpC β-lactamases (98).

Some AmpC β-lactamases can be plasmid mediated like CMY, MOX and FOX (see Table 1.3). The expression of this enzymes is usually constitutive (137) and are frequently found in Enterobacteriaceae that lack or have low expression of chromosomally encoded AmpC, such as *Klebsiella* spp., *E. coli*, *Shigella* spp., *Proteus* spp. and *Salmonella* spp. (179). Infections caused by plasmid mediated AmpC producing Enterobacteriaceae have high therapy failure and high mortality rates, since they are often associated to multidrug resistant phenotype (179). Moreover, they are frequently involved in community acquired infections while the Enterobacterales carrying chromosomal AmpC are frequently involved in nosocomial infections (137).

1.3.1.2 Group 2 β-lactamases

The group 2 is divided in several subgroups, that are composed of penicillinases and broad-spectrum β-lactamases (class A and class D).

The OXA β-lactamases are, essentially, penicillinases with the ability to hydrolyze oxacillin and penicillin, but are less effective against 1st generation cephalosporins and are poorly inhibited by β-lactamase inhibitors, such as clavulanic acid. Some OXA β-lactamases can also exhibit the extended spectrum β-lactamases (ESBL) phenotype. This is the case of OXA-11 that can hydrolyze 3rd and 4th generation cephalosporins in addition to penicillin and cephalosporins. Furthermore, there is also a subgroup composed by OXA enzymes with carbapenem-hydrolyzing activities. An example are OXA-23 enzymes that are able to hydrolyze oxyimino cephalosporins, aminopenicillins, piperacillin, oxacillin and aztreonam in addition of carbapenems (61). Like ESBLs, OXA enzymes are usually carried within plasmids.

The ESBLs include β-lactamases such as TEM, SHV and CTX (see Table 1.3). ESBL are class A β-lactamases and biochemically are characterized by their ability to hydrolyze penicillins from the 1st, 2nd and 3rd generation cephalosporins and aztreonam (but not cephamycin or carbapenem), and generally are inhibited by clavulanate. (34). The majority of the group 2 enzymes are plasmid encoded. In particular, SHV β-lactamases, that were originally chromosome-encoded enzymes, are nowadays found in plasmids (34).

1.3.1.3 Group 3 β-lactamases

Finally, group 3 (class B), includes metallo-β-lactamases and is composed by three subgroups that hydrolyze penicillins, cephalosporins and carbapenems. They are not inhibited by almost all β-lactamases inhibitor, like clavulanic acid or tazobactam, however they are inhibited by metal ions chelators, such as EDTA (Ethylenediaminetetraacetic acid) Examples of these enzymes are IMP and VIM (see Table 1.3) (29). These β-lactamases can be found either in the chromosome or in plasmids.

1.3.2 Carbapenem resistance mechanisms

Carbapenems are last resort drugs for treatment of multidrug-resistance bacterial infections. The first carbapenem discovered was thienamycin in 1976, a natural product important for the imipenem synthesis. Imipenem was the first carbapenem available in 1985 (158). This antibiotic family is composed mostly of meropenem, doripenem, ertapenem and imipenem. Carbapenems are bactericidal β-lactam antibiotics structurally related to the penicillins, being defined by a carbapenem coupled to a β-lactam ring. As β-lactams, carbapenems have the capacity to penetrate the bacterial cell wall and bind to PBPs, leading to cell death. They are broad-spectrum and act against Gram-positive and Gram-negative bacteria, including anaerobes. It is mainly used in the treatment of infections caused by multidrug resistant Gram-negative bacteria. The development of resistance to carbapenem can occur due to several mechanisms including the production of β-lactamases, specially carbapenemases, efflux pumps, mutations that alter the expression and function of porins, or even a combination of these mechanisms. For example the presence of an AmpC or ESBL coupled with overexpression of efflux pumps, can result in carbapenem resistance (158).

There is a large variety of carbapenemases already identified in Enterobacterales and all belong to β-lactamase Amber classes A, B and D. The carbapenemases from class A actively hydrolyze carbapenems and are partially inhibited by clavulanic acid. They can be chromosomally encoded, such as SME or IMI, or can be plasmid encoded, such as KPCs. Among carbapenemases of class A, KPC is one of the most prevalent and have spread worldwide causing several outbreaks (167).

The majority of the carbapenemase enzymes belong to class B, they have the ability to hydrolyze carbapenem but are susceptible to inhibition by EDTA and divalent cations like Zn^{2+}. These carbapenemases are often located within a variety of integron structures that are associated with

transposons and can either insert on the bacterial chromosome or within plasmids. Some of the best-known plasmid mediated carbapenemases from this class are IMP, VIM and NDM (167).

Finally, the class D carbapenemases are composed by OXA enzymes with weak activity against carbapenems. Generally, they have hydrolytic activity against penicillins and broad spectrum carbapenems, but are poorly inhibited by β-lactamases inhibitors, with the exception of avibactam. The major concerns with OXA carbapenemases is their ability to rapidly mutate and expand their spectrum of activity and their low resistance level, which is difficult to detect in the routine laboratory. One example of this is OXA-48, a class D carbapenemase among the most difficult to be identified due to their low-level resistance to carbapenems (167).

1.3.3 Colistin resistance mechanisms

Colistin (also known as polymyxin E) is a cationic polypeptide antibiotic and belongs to the polymyxin family. It was first discovered in 1949 and was approved by FDA for treatment of infections caused by Gram-negative bacteria since 1959. However, due to its high rates of toxicity, the use of this antibiotic was discontinued, being replaced by newer, safer and more effective antibiotics like gentamicin. Recently, with the increase in the frequency of multidrug resistant bacteria and the lack of effective antibiotics, colistin was rescued again as last resort for the treatment of complicated infections with multidrug resistance Gram-negative bacteria (120).

Colistin interacts with the lipopolysaccharide (LPS) and phospholipids in the outer cell membrane of Gram-negative bacteria and competitively displaces divalent cations (Ca^{2+} and Mg^{2+}) from the phosphate groups of membrane lipids, which leads to disruption of the outer cell membrane. But the exact mechanisms by which colistin kill bacterial cells are still unclear (23). This process is independent of colistin entry into the cell and is inhibited in the presence of these bivalent cations.

The pathogens *Proteus mirabilis, S. marcescens, Morganella morganii* are some of the species that are naturally resistant to colistin, most of them because of alteration in the LPS. There is limited information on resistance mechanisms to colistin. In Enterobacteriaceae, the majority of the mechanisms are associated to modification of the LPS, which is the main target of colistin in bacterial cell. One of such mechanisms includes the reduction of the negative charge of the LPS, caused by changes in the lipid A, one of the domains of the LPS. One of the common resistance mechanisms associated to the modification of LPS, is the emergence of mutations in *mgr*B, known to negatively regulate the PhoPQ two-component system. This system is responsible for the activation of the transcription of the operon *pmr*HFIJKLM that encodes enzymes involved in the modification of the LPS through the addition of 4-amino-4-deoxy-$_L$-arabinose. This modification

creates a more positively charged LPS, which increases the resistance to polymyxins (22, 86). Other described resistance mechanisms include the modification of the outer membrane porins, overexpression of efflux pumps systems, and overproduction of capsule polysaccharide (77).

The first plasmid encoded colistin resistance gene, the *mcr-1* was isolated in 2015. Since then, ten different *mcr* genes were described with different variants and isolates carrying these genes have been reported worldwide in humans, animals and the environment (115, 125). Like the majority of the previously described colistin resistant mechanisms, the *mcr-1*-based colistin resistance is associated to LPS modification. In this case, the *mcr*-1 encodes an enzyme (phosphoethanolamine transferase) capable of modifying the lipid A structure through the addition of phosphoethanolamine, and consequently impacting the affinity to colistin (73).

Resistance to colistin in clinical isolates may be difficult to detect, when routine antibiograms are used, due to the fact that colistin resistance is frequently heteroresistant. This can be particularly relevant, because during treatment, the small fraction of the resistant subpopulation can be selected and dominate the infection, leading to therapeutic failure. However, the mechanism associated to heteroresistance is still unknown. Some studies suggested that the heterogeneity may be related to modifications in PmrAB system, a regulatory operon similar to PhoPQ, as well as to overexpression of efflux pumps or biofilm formation (122).

1.3.4 Resistance to other antibiotics

Resistance to other antibiotic classes is also increasing, as in the case of quinolones (nalidixic acid) and fluroquinolones (ciprofloxacin). This class of antibiotics are extensively used to treat a variety of infections caused by Gram-negative. They target gyrase and topoisomerase IV, disabling DNA replication. The classical mechanisms of resistance are chromosomal encoded, and include mutations in antibiotic binding sites, and overexpression of efflux pumps. The plasmid-mediated quinolone resistance is associated to efflux pumps (*oqxAB*), DNA gyrase and topoisomerase IV protecting enzymes (*qnr* genes), or by antibiotic inactivating enzymes (*aac(6')-Ib-cr*) (141).

Aminoglycosides bind to 16S ribosomal RNA (rRNA) that constitutes the 30S ribosomal subunit, leading to polypeptide synthesis inhibition and consequent cell death. Aminoglycoside resistance may occur through different mechanisms, including: changes in cell wall permeability; modification of the antibiotic binding site; modification or inactivation of the antibiotic by aminoglycoside acetyltransferases, nucleotidyltransferases or phosphotransferases; mutations and post-transcriptional modifications of the 16S rRNA (49).

Trimethoprim and sulphonamides are antibiotics usually used in combinations to target two steps in the folic acid synthesis pathway. The antibiotics act by binding to catalytic enzymes (dihydropteroate synthases) in the folic acid pathway, inhibiting its synthesis. Sulphonamide resistance in Enterobacterales is mainly associated with genes, *sul1*, *sul2* and *sul3*, that encode for dihydropteroate synthases with a low affinity for the antibiotic, which participate in the folic acid biosynthesis, when the other synthases are inactivated by the antibiotic. Trimethoprim resistance is mainly caused by modifications in the target enzyme dihydrofolate reductase encoded by *dfr* genes that impede antibiotic binding. Both *sul* and *dfr* genes, are usually associated with integrons, which facilitates their spread among different strains and species (53, 95).

Macrolides, like erythromycin and azithromycin, act by binding in a reversible manner to 50S ribosomal subunit, hindering protein synthesis. Macrolides resistance may result from a variety of mechanisms such as target site modification through methylation (*erm* genes), antibiotic inactivation by phosphotransferases (*mph*) and esterases (*ere*) or by antibiotic extrusion through efflux systems (78).

Tetracycline interacts with the conserved 16S rRNA in the 30S ribosomal subunit, interfering with the transfer RNA (tRNA) during elongation. Resistance can emerge by mutations that give rise to alterations in the 30S ribosomal subunit, but also through the production of ribosomal protection proteins (GTPases with structural similarity to elongation factors), by antibiotic extrusion by specific efflux pumps and finally by enzymatic inactivation that are encoded by *tet* genes (83). The ribosomal protection proteins compete with tetracycline for binding the ribosome. The proteins encoded by *tet*(M*)* and *tet*(O) are considered the most prevalent ribosomal protection proteins (208). The most common mechanism of tetracycline resistance is through efflux pumps, which are often encoded by *tet*(A) and *tet*(A)-1 genes (207).

1.3.5 Resistance to disinfectants

Besides antibiotics, other compounds that have been extensively used for infection control in a plethora of settings are disinfectants. These have been applied in health care settings, water treatment and distribution, food processing, agriculture industries and households.

To successfully clean a slaughterhouse, physical and chemical steps are performed consisting in mechanical cleaning followed by the application of concentrated detergents and disinfectants combined with high temperature. The disinfectants used in food industry usually are alcohols, chlorine-based compounds, quaternary ammonium compounds, oxidants (peracetic acid, hydrogen peroxide, ozone), persulfates, surfactants and iodophors (213). The disinfectants are

chosen according to the surfaces and type of process. Moreover, the iodophors are the compounds more active against Gram-positive and Gram-negative bacteria as well as yeasts; hydrogen peroxide is the more effective in removing biofilms (213).

Bacteria develop disinfectant tolerance through phenotypic adaptation, gene mutation and horizontal gene transfer. The genes related to the disinfectant resistance can be associated with antimicrobial resistance genes, outer membrane efflux pumps or porins (134). Disinfectants resistance genes can be classified in two groups: mediated by chromosomes, such as *emrE*, *sugE* and *mdfA*; or carried in plasmids such as *qac* genes (200).

1.3.6 Treatment options

Antibiotics are still the best therapeutic option to treat bacterial infections, but the emergence and accumulation of resistance to each and every antibiotic that was introduced into clinical practice has been decreasing the therapeutic options available (105).

Multidrug resistance in Enterobacteriaceae results frequently from the acquisition of plasmids, like IncI and IncF carrying genes encoding resistance to multiple antimicrobial agents, such as aminoglycosides, quinolones, trimethoprim, sulphonamides, tetracyclines and chloramphenicol (182).

To combat uncomplicated infections, the most common treatments include fluoroquinolones, trimethoprim, fosfomycin, gentamicin, amikacin or inhibitor combinations, depending, obviously, on microorganism susceptibility profile. In the case of complicated life threating infections, the therapeutics used are mainly restricted to carbapenem, colistin, temocillin and tigecycline. Combination therapies are also applied, including the combination of colistin or/and tigecycline combined with carbapenems, or combinations between β-lactam/β-lactamase inhibitor. In particular, ceftazidime-avibactam can act against class A and class D carbapenemases, meropenem-vaborbactam can inhibit class A carbapenemases, and imipenem-relebactam can be effective against carbapenem resistant Gram-negative bacteria (188).

There are several new drug trail strategies emerging, including new antibiotic-antibiotic potentiator combinations, like the use of eravacycline, plazomicin and cefiderocol, new drugs not trialed. Additionally, new therapeutics are also focused on reducing the hospital-stay periods to decrease the risk of further complications and improve treatment outcomes, by the development of oral antibiotics. Alternatively, nondrug therapies, like those that are immune-based, have been used in an attempt to control AMR burden. In a study in which monoclonal antibodies targeting the surface polysaccharide poly-(b-1,6)-N-acetyl-glucosamine were used in a mouse peritonitis

model, a significant protection against NDM producing Enterobacteriaceae was achieved (190). Another option that has been explored is the bacteriophage therapy to prevent burn wound infections and respiratory infections, but this is still being tested in the clinical trials (36, 163). Finally, restoration of a diverse gut microbiota, by fecal microbiota transplant following antibiotic treatment, is being used to restore colonization resistance against multidrug resistance pathogens (135).

1.4 Virulence factors in Enterobacteriaceae

Numerous virulence factors have been identified in the Enterobacteriaceae Family. They play a crucial role in disease development, by contributing to microbial growth, and by promoting adherence, penetration and establishment of the pathogen into their host, including immune evasion. Some factors are produced by specific virulent strains, others are common to all genera. Virulence factors may be encoded on chromosome or in mobile elements (51).

1.4.1 Biofilms as a virulence factor

Biofilms comprise aggregates of microorganism anchored to a surface and embedded in an extracellular polymeric substance. The biofilm formation usually involves five steps: initial and reversible attachment to the surface; irreversible attachment; development of the polymeric matrix; maturation of the biofilm and the last step is dispersal or detachment, in which single cells detached can adhere to another surface to start a new biofilm. Being encased within the biofilms, bacterial cells are protected against antibiotics and the immune system of the host, being particularly difficult to eradicate (205). Additionally, the cells within a biofilm are in close contact, being able to more easily exchange genetic material (191) and spread antimicrobial resistant determinants.

Biofilm formation in surfaces linked to hospital environments, like medical devices, increases the probability of causing nosocomial infections. Furthermore, the presence of biofilms in the surfaces of machines and equipment, such as the ones used in the food production chain, can result in the possibility of serious contaminations (food and workers) that directly or indirectly affect the human health, where foodborne diseases outbreaks are a clear example (1).

1.4.2 Other virulence mechanism

Bacterial virulence factors present a huge diversity of molecules, including those responsible for bacterial attachment to the host cells and motility (adhesins, flagella, chemotaxis), for inhibition of phagocytosis (polysaccharide capsules), invasion of tissues (endotoxins and exotoxins), interaction with the host (secretion systems) and iron acquisitions (siderophores) (136).

The first step to infection is the adherence of bacteria to a mucosal surface. To move into their colonization sites and to explore the best niches, bacteria use a combination of chemotaxis, which enable them to move according to chemical gradients, by using mobility elements like flagella, also recognized as type III secretion system. Colonization is then achieved by the adhesion of bacteria through flagella, pili, fimbriae and outer membrane proteins (2, 104).

In addition to the type III secretion system, there are other types of secretion system equally important in pathogenesis. They are responsible for delivering virulence factors into the extracellular environment or directly into host cells. Type I, type III, type IV and type VI secretion system use a single energy step to transport proteins across both inner and outer membranes. The type II and type V translocate substrates, first into the periplasm, and then to the cell host. The Type VII is restricted to Gram-positive bacteria, and finally, type VIII concerns the curli biogenesis pathways (45). Secretion systems are used by pathogens to manipulate the host and establish a replicative niche; they also serve to take advantage of an environmental niche by secreting proteins that help bacteria to defend from other microorganisms.

The polysaccharide capsule, covering the bacterial cell, mediates the interactions between the bacteria and the environment. The capsule performs several functions directly involved in the pathogenicity including resistance to desiccation, adherence, resistance to nonspecific host and specific host immunity. An example of specific resistance to immune response, is the structural similarity between the capsule of *E. coli* and the host, which result in a poor host immune response (194). On the other hand, resistance to nonspecific host immunity can be achieved by the induction of high levels of interleukin-10 by *K. pneumoniae* capsule, which inhibits the activation of macrophages (219).

Many members of the Enterobacteriaceae Family produce toxins capable of inducing host cells lysis. Bacterial toxins can be divided in groups regarding their nature and mode of actions. Endotoxins are protein complexes part of the lipopolysaccharide that are liberated when the bacteria die, on the other hand, exotoxins are produced inside the pathogen and are secreted into the surrounding medium. This last group includes enterotoxins, a type of exotoxin that act in the enterocytes; and hemolysins, capable of causing red blood cells lysis by cell membrane disruption (47, 147).

The capacity to acquire iron directly from the host is also an essential component of the infection process, since iron is involve in several biological process important for both pathogen and host, such as oxygen transport, respiration and DNA synthesis (7). The iron uptake by the pathogen weakens the host immune system, which can lead to consequences such as anemia or chronic inflammation (149). The two best-known mechanisms to obtain iron from the host are: i) the synthesis of siderophores, small iron chelators synthesized by bacteria to scavenge iron from the surroundings; and ii) direct iron uptake from host iron complexes, like heme and hemoglobin, through efflux pumps that specifically transfer iron into the bacterial cell. These siderophores and the iron specific pumps can be encoded in plasmids, and others mobile elements, allowing its dissemination between different strains and species (117).

1.5 Enterobacteriaceae molecular epidemiology

1.5.1 Enterobacteriaceae infections landscape in Portugal and Europe

The dissemination of carbapenem-resistant and ESBL producing Enterobacteriaceae, especially *K. pneumoniae* and *E. coli*, are provoking serious concerns in Europe. Surveillance studies have shown that more than half (57.1%) of the invasive *E. coli*, and more than a third (36.6 %) of the invasive *K. pneumoniae* reported in 2019, were resistant to at least one of the antimicrobial classes, including aminopenicillins, fluoroquinolones, 3rd generation cephalosporins, aminoglycosides and carbapenems. In the same surveillance study, a high mean frequency of combined resistance to fluroquinolones, 3rd generation cephalosporins and aminoglycosides in *K. pneumoniae* was observed in different regions in Europe, reaching 19.3%. On the other hand, the mean frequency of carbapenem resistance was 7.9% (see Figure 1.2) (55).

Regarding *E. coli*, the overall prevalence of resistance was lower than what was found for *K. pneumoniae*. In particular, the frequency of combined resistance to fluroquinolones, 3rd generation cephalosporins and aminoglycosides was 5.9%. In what concerns to resistance to 3rd generation cephalosporins, the mean frequency was 15.1% (see Figure 1.3). Carbapenem resistance in E. coli, in 2019 was rare (0.3%) (55).

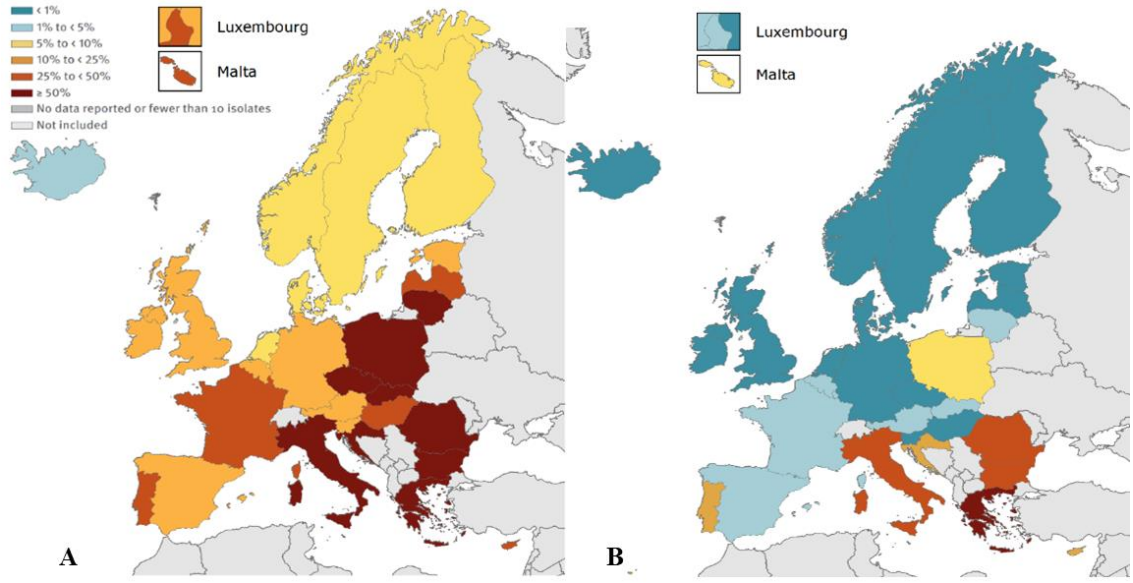

Figure 1.2 – Surveillance in Europe in 2019, of invasive isolates of *K. pneumoniae* by country **A** – Isolates with 3rd generation cephalosporins resistance; **B** – Isolates with carbapenems resistance (55).

The rise in resistance to 3rd generation cephalosporins and carbapenems is associated to the dissemination of antibiotic resistance determinants encoding extended spectrum β-lactamases (ESBLs) and carbapenemases among different strains and species as well as to the expansion of specific antibiotic resistant high-risk clones.

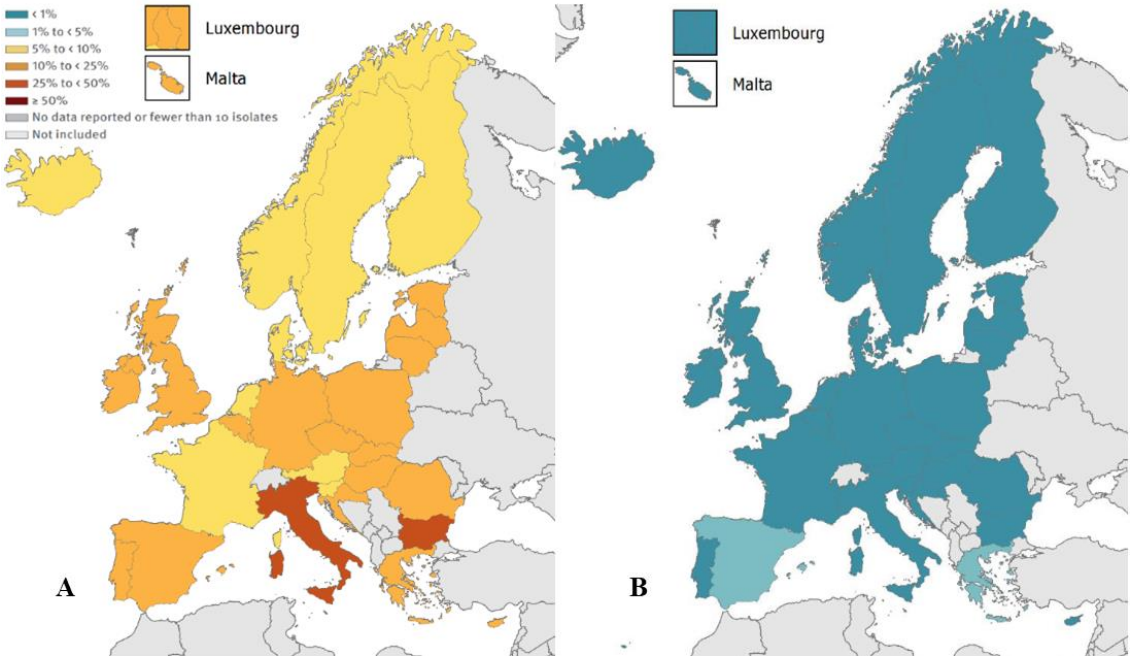

Figure 1.3 – Surveillance in Europe, in 2019 of invasive isolates of *E. coli* by country **A** – Isolates with 3rd generation cephalosporins resistance; **B** – Isolates with carbapenem resistance (55).

In 1996, the first KPC determinant, encoding a carbapenemase, was identified in a *K. pneumoniae* isolate in North Carolina (217). In the following decades KPC producing pathogens, including not only *K. pneumoniae* but also *E. coli*, *Klebsiella oxytoca*, *C. freundii*, *Enterobacter aerogenes*, and other species belonging to Enterobacteriaceae Family, expanded globally, leading to KPC predominance worldwide in both hospital and community. The KPC dissemination is believed to have derived from the spread of carbapenem-resistant high-risk clones, associated with hospital outbreaks, which contributed to the spread of this antimicrobial resistance gene.

One of the most well-known high-risk clones in *K. pneumoniae* is characterized by the multilocus sequence type 258 (ST258). In 2009 this clone was the predominant clone in United States, where it represented 70% of all the *K. pneumoniae* harboring KPC determinants. Noteworthy, this clone carried, additionally, multiple antimicrobial resistance determinants providing resistance to aminoglycosides, β-lactam, fluroquinolones, macrolides, chloramphenicol, trimethoprim and sulfonamide (133). In Portugal, the first KPC (KPC-3) was identified in 2009 in a *K. pneumoniae* ST11 collected from a pediatric unit in Lisbon and this is still the most predominant carbapenemase in this country. Currently, KPC-3 producers are found in a high diversity of healthcare settings in all regions of Portugal (127).

Another high-risk clone associated to the spread of a different resistance determinant – ESBLs - is *E. coli* ST131. Isolates belonging to ST131 have been associated to hospital and community acquired urinary tract infections. This clone was first described in 2008 (152) in eight different countries (France, Portugal, Spain, Switzerland, Lebanon, India, Korea and Canada) from patients with different infections, such as urinary tract infections, intrabdominal infections, bacteriemia, among others and carried only the CTX-M-15 ESBL resistance gene. However, nowadays, most of the ST131 strains are multidrug resistant. Moreover, they are known to carry several different virulence factors and to have an increased fitness, which have been contributing to the dissemination of *E. coli* ST131 strains harboring *bla*CTX-M-15 in several settings, such as the community, hospitals, long-term-care facilities and companion animals. (20, 68, 133).

On the other hand, the OXA-48, encoding a carbapenemase, was first described in 2001 in a patient in Turkey (164). Since then, sporadic cases or outbreaks have been described in hospitals in most European countries, including Portugal, and worldwide (79). Since 2014, OXA-48 producing *K. pneumoniae* demonstrated a high dissemination capacity, especially in the Mediterranean region, and became endemic in Turkey and Malta (75). In Portugal, since the first description of OXA-48 producing Enterobacteriaceae in a catheterized patient, in 2014 (131) some sporadic cases of OXA-48 producing pathogens were identified in Lisbon and in the northern region (126). However, OXA-181, that differs from OXA-48 in four amino acids, is nowadays one of the most prevalent carbapenemases in Portugal (5).

The presence of AmpC producing Enterobacteriaceae in humans has been reported worldwide, frequently associated to cephalosporins resistance (98). According to some surveillance studies, CMY-2 enzymes are currently one of the most widespread variants in the world (71, 157). In Portugal, between 2002 and 2008, DHA-1 was the most prevalent AmpC identified, followed by CMY-2 (71). Since then, no more studies were performed to evaluate the dissemination of AmpC in Portugal. Although the molecular epidemiology of *ampC* genes is understudied, the knowledge on the distribution and activity of these enzymes is becoming more relevant, given the numerous reports on resistance to carbapenems in Enterobacteriaceae that is associated to AmpC hyperproduction (98).

1.5.2 Enterobacteriaceae in the farm setting and epidemiological links with hospital

Enterobacterales are found both in humans and animals and antibiotics are used in both hospital and farms, leading to the emergence of antimicrobial resistant bacteria. However, the extent of dissemination of antibiotic resistant Enterobacterales between humans and production animals is not well explored. The situations in which dissemination is more likely to occur include those in which humans are in close contact with production animals.

In particular, there are several cases in which Enterobacteriaceae from colonization in pigs were linked to infections in humans, especially in farm workers. In 2016, in Cameroon, a high risk clone of *K. pneumoniae* ST307 was identified colonizing both pigs and farm workers (69). An isolate of this clone was previously reported in the Netherlands in 2008 from a human urine sample, but the first clinical strain reported in the literature was collected in a Pakistan hospital, in 2009 (88). Since then this clonal type has spread worldwide; in 2019 it was identified in an extensive hospital outbreak of a drug-resistant *K. pneumoniae* in Germany (90).

Another example of dissemination of drug resistant Enterobacteriaceae between food-producing animals and infections in humans, was observed in *E. coli* ST131 clone, identified in eight different countries. This clone was found causing different infections in humans (urinary tract infections, intrabdominal infections, bacteriemia, among others) and also colonizing the gastrointestinal tract of food producing animals, like poultry (20, 68).

Besides the dissemination of antibiotic resistant bacteria, dissemination of resistance between humans and animals can also be achieved through the spread of antibiotic resistance genes. This can occur for example, when animal commensal bacteria transfer their resistance genes to bacteria

that are able to colonize and cause infection in humans. For this to occur these two types of bacteria will have to, at least transitorily, inhabit the same environment.

Examples of antibiotic resistance determinants disseminated worldwide that are found in both humans and animals, are CTX-M-1, CTX-M-15 and CMY-2, being the last one considered the AmpC enzyme more predominant in animals. Additionally, carbapenemases, such as VIM-1, NDM-1, frequently found in humans, were also reported in animals (129).

1.5.3 Human colonization as a risk factor for infection

Some studies have shown that humans colonized with Enterobacteriaceae have a higher risk of contracting a disease, when compared to non-colonized (135, 198). Human microbiota plays an important role in the defense of the immune system, but the use of antibiotics when patients are severely affected by a disease, result in the decrease of the normal flora, promoting the infection by opportunist pathogen, especially antimicrobial resistant pathogens. However, the disruption of the flora by the intake of antibiotic is not the only risk factor for the infection by Enterobacteriaceae. Other risk factors for respiratory infection in people with high rates of oropharynx colonization include the chronic alcoholism, heroin addiction and diabetes mellitus (128). Moreover, it was found that women who use diaphragms for contraception have higher probability to have a urinary tract infection, since they present higher rates of vaginal colonization with *E. coli* and others Enterobacteriaceae Family members (87).

The high prevalence of carbapenemases and ESBL producing Enterobacteriaceae in hospitals, as also in animals, makes all staff, such as medical, veterinary or farmers, prone to a higher risk of infection by this type of bacteria (48, 162).

In general colonization is a requirement for infection, however the percentage of colonized patients that will have an active infection is still unknown (198). According to a study, involving CRE from several hospitals between 2014 and 2015, 16.5% of patients colonized with CRE progressed to clinical infection and colonization was associated with 10% mortality (198). Although, the colonization of antimicrobial resistant Enterobacteriaceae was mainly defined by gastrointestinal tract carriage, the most frequent clinical manifestation in previously colonized patients was pneumoniae followed by urinary tract infection (198). In a study carried out in Manhattan, United States of America (USA) hospital in 2013, where the adverse consequences of colonization with CRE in intensive care units (ICU) were analyzed, colonized patients had a higher mortality within 90 days of sampling than the non-colonized patients (33% of mortality for colonized patients; 15% of mortality for non-colonized patients) (135). To circumvent the risk

of colonization by CRE, decolonization with antimicrobial combinations or fecal microbial transplantation has been tested to restore a healthy microbiome (135).

1.6 Molecular characterization of Enterobacteriaceae based on DNA sequencing

1.6.1 Identification of Enterobacterales by molecular techcniques

In addition to biochemical methods, molecular-based techniques have brought a new insight into bacterial identification. The DNA-DNA hybridization (DDH) techniques are the gold standard for classification of bacterial species. This technique is based on thermal stability of DNA and include some methods such as S1 endonucleases method and DNA microarrays among others. All of them are time-consuming and laborious (172). For that reason, some sequencing-based approaches have been developed as an alternative. Among them amplification-based methods and sequencing-based.

Polymerase chain reaction (PCR) is one of the most important techniques for bacterial identification that use standardized gene regions as target for identification. After the amplification of the gene target, the PCR products are sequenced and the results are compared to known databases, such as National Center for Biotechnology Information (NCBI) database. For Enterobacterales identification it was showed that 16S rRNA gene and *rpoB* gene, encoding RNA polymerase β subunit are ideal target for detection by PCR amplification (62, 70). Although the 16S rRNA gene sequencing technique was considered a standard method that is use in some clinical settings (70), some studies showed that this molecule is too conserved to provide a reliable identification at the species level, since 16S rRNA gene from different species are ~99% identical (38, 102).

Currently, the calculation of the pair-wise average nucleotide identity (ANI) has been proposed to replace the DDH as the gold standard for classification of bacterial. ANI consist in the overall similarity between two genome sequences, the ANI value is the mean of the identity values of each fragment of the genome sequence when compared with a second genome sequence, usually a reference (218). Despite the fact that ANI is an easy and fast method with reliable results (39), this method involve high costs associated with the whole genome sequencing (WGS), as well as advanced computational skills.

1.6.2 Molecular typing methods

Infections caused by antibiotic resistant Enterobacterales have increased in recent years in hospitals. One of the most effective ways of decreasing the spread of resistant bacteria is to search and destroy their reservoirs and routes of transmission. For that purpose, several typing techniques have been developed that enable to distinguish bacteria that are similar and are epidemiologically related from those that are different and unrelated. There are several different molecular typing techniques developed to characterize Enterobacterales.

Traditional molecular typing methods were based on the use of restriction enzymes, such as pulsed-field gel electrophoresis (PFGE), or polymerase chain reaction, such as ribotyping. But the most used typing methods nowadays are those based on the analysis of DNA sequence, either focusing on a single gene, multiple genes or the entire genome.

Multilocus sequence typing (MLST) is one of the most popular molecular typing techniques, frequently used to identify the bacterial genetic background. This method was first proposed in 1998 (130) and is based on the sequencing of an internal region of seven housekeeping genes scattered along the chromosome. For each gene sequence a specific allele number is attributed and the combination of the seven alleles defines an allelic profile that corresponds to a sequence type (ST). Isolates can then be compared according to their allelic profiles and classified as related or not according to the number of differences in alleles. This method has the great advantage of being portable and comparable between different laboratories. However, MLST schemes have to be optimized and validated for each species and not all bacterial species have an approved MLST scheme (183).

1.7 Whole genome sequencing (WGS) and its importance in clinical settings

1.7.1 WGS molecular characterization in the clinical settings

Whole-genome sequencing (WGS) is a methodology that enables the analysis of the entire genome of an organism. This is achieved by the use of sequencers that can have different technologies (Illumina, Ion Torrent, PacBio, Nanopore). In particular, sequencers can be divided into two major groups, according to the length of the sequencing reads that are produced: short reads (50-150 bp) (Illumina, Ion Torrent) or long reads (10-100 Kb) (PacBio, Nanopore) (114).

Among all, Illumina sequencing is the most accepted and used technology, accounting for more than 90% of entire sequencing data being generated. For using this technology quality DNA is extracted and fragmented and adapters are added. These adapters will serve as reference during amplification, sequencing, and analysis. The modified DNA is loaded into the flow cells and adapters will pair with the flow cell nucleotide sequences. The DNA will be then amplified by bridge amplification PCR generating the so-called "clusters". When the cluster generation is complete the next step is the sequencing. This is done by DNA polymerase and fluorescently labeled modified nucleotides. The Illumina technology detects the fluorescently labeled nucleotide that is incorporated in the DNA fragments. The emission wavelength and intensity are used to identify the corresponding nucleotide that is being sequenced. The reaction occurs over millions of fragments until all reads are obtained. After the sequencing process is finished, the raw reads obtained are analyzed for quality and adapters are removed. The sequencing analysis involves the alignment of raw reads and their assembly into larger fragments (contigs) (e.g. *de novo* assembly). Raw reads can also be aligned directly against reference genomes (mapping). Further analyses can be performed using different bioinformatics tools to determine bacterial genetic background, content in antibiotic resistance and virulence and relatedness between strains. This process involves several computational steps producing vast quantities of data (10).

Although the first sequencer was developed in 1987, only in 2010 WGS was introduced in clinical settings. Still nowadays, WGS is only part of the routine clinical laboratories in some countries (such as Sweden, France, Australia, United Kingdom and USA). In Portugal, as far as we are concern, WGS is still not adopted as a routine typing method in clinical laboratories. Due to its high discriminatory power, this method has a high potential for use in epidemiological surveillance, infection control and outbreaks detection in clinical settings (16).

Even though WGS is an exceptional diagnostic method, there are some limitations that hinder its fast implementation as a gold standard methodology. One limitation regards to the lack of bioinformatic knowledge of laboratory staff. Although many user-friendly bioinformatic tools have been developed, still there are many tasks that require command-line knowledge. Moreover, although the cost of sequencing the whole genome has been decreasing, is still expensive. Furthermore, there is the need for a high capacity of data storage and computer processing, usually not available in typical clinical laboratories. Another drawback is the absence of a standardized workflow for WGS analysis, making more difficult the interpretation and exchange of data (9, 114).

1.7.2 The use of WGS to acess relatedness between strains and cross-transmission events

One of the most useful applications of WGS is tracking transmission events. This is usually done by comparing the core single nucleotide polymorphisms (SNPs) between the draft genomes of different strains. Isolates are then considered epidemiologically related or not, taking into consideration a defined threshold, which should be determined and validated for each species (17, 119).

This approach was successfully used to manage an outbreak of *Acinetobacter baumannii* in a British hospital in 2010 (119). Furthermore, in 2011, in Germany, WGS was essential to understand the transmission routes within an outbreak of *E. coli* O104:H4 causing bloody diarrhea and hemolytic uremic syndrome that resulted in 50 deaths (82). In both situations the use of WGS allowed a rapid response and helped to control the outbreaks and decrease mortality.

The MLST is also an important technique for global epidemiology, like the analysis of geographic clonal dissemination, however, since it is based in the analysis of sequences of a limited number of conserved genes, it is not appropriated for tracing transmission routes. The core genome MLST (cgMLST) is an approach similar to MLST technique that uses a much higher number of core-genome genes. The cgMLST has a higher resolution than MLST, what makes it appropriate, as alternative to SNPs analysis, for tracing transmission routes and outbreak management (170).

Besides enabling the detection of similar strains WGS can be also used for surveillance and detection of new variants or new reservoirs (16).

1.7.3 The use of WGS to search and identify resistance and virulence determinants

Another main application of WGS is to predict the pathogen antimicrobial susceptibility phenotype and virulence profile by analyzing the genotype. The identification of resistance and virulence markers can be performed using free online platforms like the Centre for Genomic Epidemiology (http://www.genomicepidemiology.org/). This website contains multiple tools for identification of antimicrobial resistance genes [Resfinder (220)], prediction of virulence genes [VirulenceFinder (37)], and mobile genetic elements [PlasmidFinder (32)]. Genes are detected by submitting the sequencing reads or the contigs to the platform that are then aligned to sequences in the database according to a user-defined identity and gene coverage. This platform allows to

analyze one sequence at a time, but other programs, such as ABRicate (187), allows to perform massive screening of genomes contigs.

The prediction of the resistome from WGS allows skipping some of the culturing steps that are part of the traditional phenotypic susceptibility testing, which enables to reduce to time to detect antimicrobial resistant microorganisms, avoiding by this way further transmission events and the use of inappropriate treatments. This is particularly beneficial in slow growing pathogens like *Mycobacterium tuberculosis* (114).

The major challenge on using WGS as phenotype prediction in clinical settings is the need for updated databases. New antimicrobial resistance genes are described frequently and need to be incorporated into the databases in a timely manner. Also usually, the databases contain antibiotic resistance genes for the most common pathogens only, failing to detect the presence of resistance genes in more unusual bacteria. Moreover, there are some mechanisms of resistance that are particularly difficult to detect because they depend on multiple antibiotic resistance genes, mutations, gene copy number or gene expression (16).

1.8 Study objectives

In this study, we aim to establish the epidemiological links between resistant Enterobacterales present in different stages of pig production chain, from live pigs to meat consumption. Moreover, we want to understand if antibiotic resistant Enterobacterales previously associated to infection in hospitals are related with those isolated from the pig production chain. Additionally, we will assess if Enterobacterales works as a trafficker of antimicrobial resistance and virulence genes between production animals and humans, using WGS approaches.

2. MATERIAL AND METHODS

2.1 Farm2Fork bacterial collection workflow overview

The sampling and putative Enterobacterales isolation were previously performed in the Laboratory of Bacterial Evolution and Molecular Epidemiology (MOSTMICRO, ITQB NOVA-Institute of Chemical and Biological Technology António Xavier, Oeiras, Portugal) and the Centre for Interdisciplinary Research in Animal Health (Faculdade de Medicina Veterinária, Universidade de Lisboa, Lisbon, Portugal).

2.2 Bacterial collection from a pig's production chain

The bacterial collection included in this master thesis was obtained from two independent samplings performed in a pig slaughterhouse in the Lisbon region, Portugal. Samples were collected along the pig's production chain, with the objective of covering all steps of production chain, from the live animal to handling of raw meat by human consumers at home.

The collection used for this thesis included 26 samples collected in 2019. The samples were recovered from ears and rectum of live healthy pigs in the slaughterhouse entrance (n=3), from where pigs followed the slaughterhouse procedure (electrical stunning, followed by bleeding and evisceration), and final animal chop. In this step, samples were collected from different meat pieces (n=3), specifically from shoulder, belly and trimmings of deboning lot. Additionally, samples were collected from cutting room surfaces in contact with the meat, before and after sanitizing (n=5), and from the workers hands involved in the bleeding and chopping process (bleeding zone operator and splitting zone operator), before and after disinfection (n=3). Furthermore, shoulder meat pieces collected at a butcher shop, were cut in steaks and given to human consumers. Samples were also collected from consumers dirty hands (n=4), from the clean hands, after disinfection (n=5) and from the hands after meat manipulation (n=3) (see Figure 2.1). The samples were collected and processed with different protocols according to their source. The pig rectum samples were performed using a swab, the remaining samples were collected using gauze sponges soaked in peptone water (20 g/L) (Scharlau, Barcelona, Spain) by swabbing a respective portion of the site to be analyzed. Each gauze was homogenized in peptone water and transported. In the laboratory, a volume of 100 µL from each sample was grown in Violet Red

Bile Dextrose agar (Scharlau, Barcelona, Spain) supplemented with meropenem (4 mg/L) and Violet Red Bile Dextrose agar supplemented with ceftazidime (4 mg/L). Ten colonies of putative carbapenem-resistance and cephalosporins resistance Enterobacterales from each sample was conserved in Tryptic Soy Broth (TSB, Bacto ™ Tryptic Soy Broth, Becton Dickinson, Sparks, MD, USA) (see Annex 1), supplemented with 15% glycerol. A total of 117 isolates were gathered and analyzed in this study.

Moreover, a series of controls, previously identified and characterized by WGS, were used throughout this work [OND228A (*K. pneumoniae*), OND299A (*C. freundii*), OND218 (*K. pneumoniae*), OND255A (*K. pneumoniae*), OND255B (*E. coli*), OND105e1 (*E. cloacae*), OND25e1 (*Phytobacter/Metakosakonia*), OND396 (*E. coli*)] (Faria *et al*., *unpublished*).

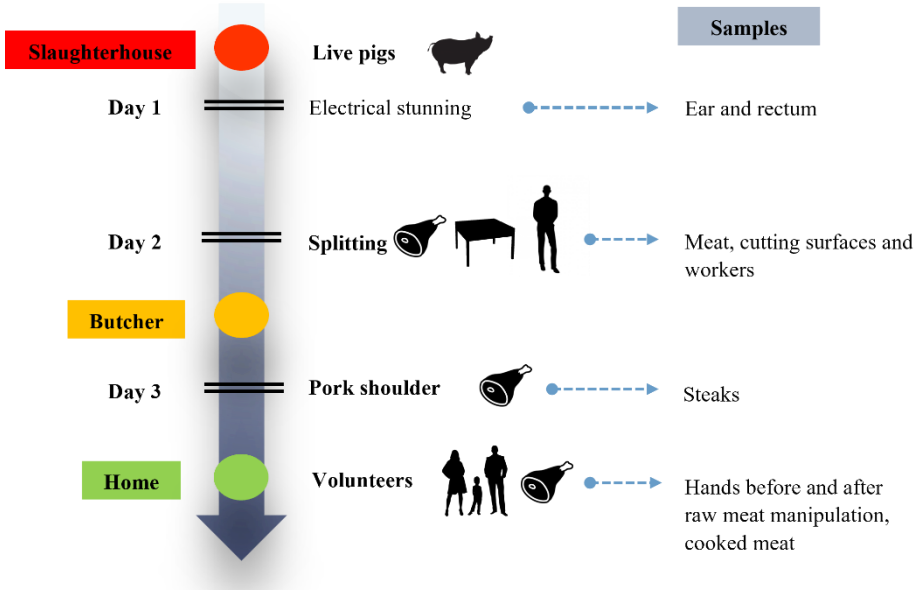

Figure 2.1 – Schematic representation of sampling process collection throughout the pork production chain.

2.3 Phenotypic characterization

2.3.1 Selective and chromogenic media: CHROMagar™ ESBL and CHROMagar™ KPC

All isolates (n=117) were grown at 35ºC during 18 hours (overnight) in two selective chromogenic media: CHROMagar™ ESBL (CHROMagar, Paris, France) and CHROMagar™ KPC (CHROMagar, Paris, France) (see Annex 1), for the selection of Gram-negative bacteria producing extended-spectrum β-lactamases (ESBL) and Gram-negative bacteria with reduced susceptibility to carbapenems, respectively. To ensure the quality control of the test, control stains

were used (OND228A, OND299A, OND218, OND255A, OND255B, OND105e1, OND25e1, OND396*)*. Furthermore, based on the colonies' colour, it was possible to do a presumptive bacterial identification according to the manufacturer's interpretation guidelines. Isolates presenting a pink to reddish or metallic blue colour, corresponding to *E. coli* and KEC group (*Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp.), respectively, were selected for further characterization. Isolated colonies were transferred to a fresh rich media until a pure culture was obtained.

2.3.2 Lactose fermentation

All isolates presumptively identified as *E. coli* or KEC in the chromogenic media, were grown in the Drigalski agar media (Drigalski Lactose Agar, Liofilchem, Roseto d. Abruzzi, Italy) (see Annex 1), at 35ºC during 18 hours (overnight). Drigalski agar media is a selective differential medium for Gram-negative rods (Enterobacterales and certain non-fermenting agents) that is able to distinguish lactose-fermenting bacteria (yellow colonies/medium) from non-fermenting bacteria (blue-grey to blue-green colonies/medium). All pure isolates able to grow on Drigalsky were classified as lactose producers/non-producers according to the colonies colour and streaked in Muller-Hinton II agar (BBL™ Mueller Hinton II Agar, Becton Dickinson, Sparks, MD, USA) (see Annex 1) and grown at 35ºC during 18 hours. To ensure the quality control of the test, control stains were used (OND228A, OND299A, OND218, OND255A, OND255B, OND105e1, OND25e1, OND396*)*. Bacteria were then conserved in Tryptic Soy Broth (TSB, Bacto ™ Tryptic Soy Broth, Becton Dickinson, Sparks, MD, USA), supplemented with 15% glycerol, at -80ºC for further analysis. All isolates that did not have the capacity to grow in Drigalski medium were not included in the study.

2.3.3 Urease production

To test for urease production, all isolates were streaked in the Christensen's Urea Agar (Christensen's Urea Agar, Sigma Aldrich, Steinheim, Germany) (see Annex 1), which was supplemented with urea (Merck, Darmstadt, Germany), according to manufacturer indications. Plates were incubated at 35ºC, for a maximum of 48h. A positive result corresponded to the occurrence of a medium colour change from yellow to bright pink (fuchsia), as a result of pH increase caused by the enzyme urease. On the other hand, the urease test was considered negative when there was no change in medium colour, indicating that the bacteria did not metabolize urea.

Control strains were used to verify the functionality of the media (OND228A, OND299A, OND218, OND255A, OND255B, OND105e1, OND25e1, OND396*).*

2.3.4 Indole test

In order to evaluate the capacity to convert tryptophan into indole, all isolates were grown in liquid medium, Muller-Hinton broth (Difco Mueller Hinton Broth, Becton Dickinson, Sparks, MD, USA) (see Annex 1), at 35ºC for 24h; indole presence detection was performed by adding three drops of the Kovacs Reagent (Sigma-Aldrich). The formation of a red ring on the medium surface corresponds to a positive reaction, while the absence of colour change was classified as a negative reaction. A quality step was performed using different control strains (OND228A, OND299A, OND218, OND255A, OND255B, OND105e1, OND25e1, OND396*).*

2.3.5 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing was performed using the disk diffusion and broth microdilution methods.

2.3.5.1 Disk diffusion method

Antimicrobial susceptibility testing was performed by Kirby-Bauer disk diffusion according to the methodology described by EUCAST (European Committee on Antibiotic Susceptibility Testing) (58). A panel of 14 different antibiotics, covering all major relevant antibiotic classes, were selected as follows: ticarcillin (TIC, 75 µg), temocillin (TEM, 30 µg), amoxicillin + clavulanic acid (AMC, 20/10 µg), piperacillin + tazobactam (TZP, 30/6 µg), trimethoprim + sulfamethoxazole (SXT, 1.25/23.75 µg), cefoxitin (FOX, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 14 µg), ceftazidime + avibactam (CZA, 30 µg), imipenem (IMP, 10 µg), meropenem (MEM, 10 µg), ertapenem (ERT, 10 µg), gentamicin (CN, 10 µg), ciprofloxacin (CIP, 5 µg).

All bacterial isolates were plated in Muller-Hinton II agar (BBL™ Mueller Hinton II Agar, Becton Dickinson, Sparks, MD, USA) and incubated overnight at 35ºC. From these fresh cultures, a 0.5 McFarland ($1x10^8$ CFU/ml) bacterial suspension (Suspension turbidity detector Densitometer DEN-1B, Sai Biosan, Riga, Latvia), was prepared in a sterile saline solution of

0.85% NaCl. Seven antibiotic disks (Oxoid, Basingstoke, United Kingdom) were distributed in each plate, six disks in a hexagonal distribution with a disk in the centre. Plates were incubated at 35ºC for 18h-20h. Diameters of inhibition halos were recorded and interpreted according to EUCAST 2021 guidelines (58) (see Table 2.1 and Table 2.2), wherein three levels of susceptibility are considered: susceptible (S), susceptible to increased exposure (I) and resistant (R) to each antibiotic.

Table 2.1 – Clinical breakpoints for Enterobacterales according to EUCAST 2021 guidelines.

Antibiotic	**Disk antibiotic content (µg)**	**Class**	**Interpretation Criteria (mm)**		
			S ≥	**I**	**R <**
Ticarcillin	75	Penicillin	23	20-22	20
Temocillin	30		50	17-49	17
Amoxicillin + Clavulanic acid	20/10	Penicillin/ β-lactamase inhibitor	19	-	19
Piperacillin + Tazobactam	30/6		20	-	20
Cefoxitin	30	Cephalosporin 2nd	19	-	19
Cefotaxime	30	Cephalosporin 3rd	20	17-19	17
Ceftazidime	30		22	19-21	19
Ceftazidime + Avibactam	10/4	Cephalosporin 3rd/ β-lactamase inhibitor	13	-	13
Imipenem	10	Carbapenems	22	19-21	19
Ertapenem	10		25	-	25
Meropenem	10		22	16-21	16
Gentamicin	10	Aminoglycosides	17	-	17
Ciprofloxacin	5	Fluoroquinolones 2nd	25	22-24	22
Trimethoprim + Sulfamethoxazole	1.25/23.75	Sulfonamides/ Dihydrofolate reductase	14	11-13	11

Table 2.2 - Clinical breakpoints for *Aeromonas* spp. according to EUCAST 2021 guidelines.

Antibiotic	Disk antibiotic content (µg)	Class	Interpretation Criteria (mm)		
			S ≥	I	R <
Ceftazidime	30	Cephalosporin 3rd	24	22-23	21
Trimethoprim + Sulfamethoxazole	1.25/23.75	Sulfonamides/ Dihydrofolate reductase	19	17-18	16
Ciprofloxacin	5	Fluoroquinolones 2nd	27	25-26	24

2.3.5.2 Broth microdilution method

For the evaluation of colistin susceptibility, the minimum inhibitory concentration (MIC) was determined by broth microdilution following EUCAST recommendations (56). For this purpose, a 0.5 McFarland bacterial suspension (NaCl 0.85%), from an overnight culture in Muller-Hinton broth (Difco Mueller Hinton Broth, Becton Dickinson, Sparks, MD, USA), was prepared using a spectrophotometer (LAMBATM 25 UV/Vis Spectrometer, PerkinElmer, Wilmington, DE, USA) in order to obtain a final OD (optical density) of 0.08. The suspension was then further diluted to a final concentration of $1x10^6$ CFU/ml (1:100) and added to colistin solutions with concentrations ranging from 1 to 16 µg/ml in a 96-well microtiter plate (BRANDplates® pureGrade™ S 96-Wellwith U-Bottom, BrandTech Scientific, Wertheim, Germany). All plates were incubated at 35ºC in static condition (without shaking) for 18-20h. For MIC determination, the OD was measured at 595 nm in a microtiter plate reader (Infinite F200 Pro, TECAN, Lifescience). According to EUCAST guidelines from 2021 a bacterium was considered susceptible when the MIC ≤ 2 and was considered resistant when the MIC > 2. The positive control corresponding to bacteria suspension without colistin and negative control to growth medium without inoculum. A second positive control was added correspondent to a colistin resistant isolate previously identified (OND396). For each strain four technical replicas were performed. Isolates were considered to be heteroresistant when the antibiotic concentration exhibiting the highest inhibitory effects is eight-fold, or more, higher than the highest non-inhibitory concentration (52).

2.3.5.3 Detection of resistance mechanisms

In order to predict antibiotic resistance mechanisms of clinical and/or epidemiological importance we used EUCAST criteria that is based on the combination of results obtained for susceptibility to different antibiotics by disk diffusion method (57) and we also looked for antibiotic induction, such as carbapenems and ceftazidime, as previously described (74). According to these criteria, the isolates were classified as: possible carbapenemase producers, possible ESBL producers possible AmpC carriers, reduced temocillin susceptibility, and with relevant antibiotic induction phenomena (see Table 2.3). Furthermore, isolates were considered multidrug-resistant if they were resistant to three or more antibiotic classes.

Table 2.3 – Screening methods for specific resistances considered epidemiological important.

Epidemiological Group	Characterization
Possible carbapenemase producers	Diameter of the resistance zone to ertapenem less than 25 mm; Diameter of the resistance zone to meropenem less than 28 mm; Diameter of the resistance zone to meropenem between 25-27 mm if resistant to temocillin.
Possible ESBL producers	Diameter of the resistance zone to cefotaxime less than 21mm; Diameter of the resistance zone to ceftazidime less than 22 mm.
Possible AmpC carriers	Diameter of the resistance zone to cefoxitin less than 19 mm if resistance to ceftazidime or cefotaxime.
Antibiotic induction	Exhibit induction between the antibiotic class carbapenems and ceftazidime; Exhibit induction between the antibiotic class cephalosporins and amoxicillin + clavulanic acid.
Temocillin susceptibility	Diameter of the resistance zone to temocillin less than 17 mm.

2.3.6 Biofilms formation

In order to evaluate the biofilm production ability, isolates were resuspended in TSB (Bacto ™ Tryptic Soy Broth, Becton Dickinson, Sparks, MD, USA) and incubated overnight at 37ºC with shaking at 100 rpm. Initial inoculum was prepared from the overnight culture adjusted to 0.5 McFarland (Suspension turbidity detector Densitometer DEN-1B, Sai Biosan, Riga, Latvia), which was further diluted in TSB supplemented with 1% of glucose (1:100; 5 x 10^6 cells per well).

Afterwards, 200 µl of the cell suspension was added in each well of a 96-well microtiter plate (Corning® 96-well Clear Flat Bottom Polystyrene TC). All plates included a negative control (culture medium without inoculum), and six positive controls (OND228A, OND299A, OND218, OND255A, OND255B, OND105e1, OND25e1, OND396*)*. Plates were incubated for a period of 18h, at 37ºC, in static conditions. For each strain four technical replicas and three biological replicas were performed.

After the incubation period, nonadherent bacteria were removed by washing each well three times with deionized water, followed by an incubation period of 60 min at 60ºC to fix adherent cells. In order to perform cell staining 200 µl of a 0.7% (w/v) crystal violet solution dissolved in deionized water was added to each well and incubated at 60ºC for 12 min to promote heat-fixation. After the staining steps, plates were rinse in deionized water at a 45º angle to promote the exceeding staining removal without disturbing biofilm; this process was repeated three or four times, until the wash solution was cleaned. To quantify the crystal violet adherent to bacterial cells a 200 µl of a 30% (v/v) acetic acid solution was added to each well. Optical density of the biofilm was measured at a wavelength of 595 nm (OD_{595}) using a multiwell plate reader (Infinite F200 Pro, TECAN, Lifescience).

The classification of the isolates according to their ability to form biofilms was based in the procedure described by Ramos-Vivas *et al*. (2019) (175). The isolates were classified as follows: non-adherent, $OD \leq 0.05$; weak producers, $0.05 < OD \geq 0.1$; moderate $0.1 < OD \geq 0.3$; strong biofilm producers, $OD > 0.3$.

2.4 Molecular characterization

2.4.1 DNA extraction

Genomic total DNA from pure isolates was extracted using Dneasy® Blood and Tissue kit (QIAGEN, Hilden, Germany), according to manufacturer's instructions, with the following optimisations steps: the strains were inoculated in Luria-Bertani (LB) agar (Difco™ Dehydrated Culture Media: LB Agar, Miller Becton Dickinson, Sparks, MD, USA) (see Annex 1) and incubated at 35ºC, overnight. The pre-treatment usually used for Gram-positive bacteria was applied. Briefly, one loopful of culture was resuspended in 1X PBS (see Annex 2) and centrifuged at 8000 rpm for 1 min. Bacterial pellet was suspended in lysis buffer composed of: 162 µL of LP1 buffer (see Annex 2), 10 µL of lysozyme at 20 ng/µL (Sigma-Aldrich) 5 µL of lysostaphin at 10 mg/µl (Sigma-Aldrich) and 3 µL of RNase at 10 ng/µL (Sigma-Aldrich) (see Annex 3). The

mixture was incubated at 37ºC for 1h. Afterwards, 40 µL of proteinase K and 200 µL of buffer AL were added, followed by an additional incubation at 56ºC for 1h. After that time, 200 µl of absolute ethanol was added.

The mixture was transferred to the mini columns and washing steps were performed as described in the kit protocol. For the DNA recovery step, 50 µL of 10mM Tris-EDTA pH 8.5 was added (see Annex 2). A second elution step was performed to recover residual DNA from mini columns. The DNA was subsequently stored at -20ºC.

DNA purity was assessed using the Nanodrop apparatus (NanoDrop® ND-1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, USA) as well as A260/A280 and A260/230 ratios. Furthermore, concentration of double stranded DNA was determined by fluorometry (Qubit® 2.0 Fluorometer, Thermo Fisher Scientific, Wilmington, DE, USA).

DNA integrity was verified on a 1% agarose gel (Seakem, LE, Lonza, Rockland, ME, USA) in TAE 1X (see Annex 2), supplemented with 0.002% v/v of GreenSafe (GreenSafe Premium, NZYTech – Genes & Enzymes, Lisbon, Portugal) and run at 6.25 V/cm for 25 min (Biometra Standard Power Pack P25, Biometra GmbH, Gottingen, Germany). DNA bands were visualized under ultraviolet (UV) (Gel-Doc TM EZ Imager, Bio-Rad, Hercules, California, USA) and images recorded. The molecular weight ladder GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific™, Vilnius, Lithuania) was used.

2.4.2 Amplification of 16S rRNA gene by polymerase chain reaction

Species identification was performed by 16S rRNA gene sequencing, as previously described (209). An internal fragment of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) (MiniAmp™ Plus Thermal Cycler, Thermo Fisher Scientific, Wilmington, DE, USA) with primers, 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (CTACGGCTACCTTGTACGA) (Invitrogen by Thermo Fisher Scientific, Wilmington, DE, USA), The reaction mixture was prepared as follows: in 50 µL of total volume, containing 1X GoTaq Flexi PCR buffer (Promega, Madison, WI, USA), 1.5 mM of $MgCl^2$ (Promega, Madison, WI, USA), 16 µM dNTPs each (BIORON, Ludwigshafen, Germany), 0.2 µM of 27F primer, 0.24 µM of primer 1492R, 0.025 U/µL of Go Taq polymerase enzyme (Promega, Madison, WI, USA), 0.5 µg/50 µL of genomic DNA and sterile MiliQ H_2O. The negative control corresponded to the reaction mix with no DNA.

The PCR reaction included an initial denaturation at 95ºC for 5 min, followed by 30 amplification cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds, and extension at

72ºC for 1 min, final extension at 72ºC for 10 min, and soaking at 16ºC. Samples were stored at 4ºC until electrophoresis.

Amplification products were visualized on conventional agarose gel electrophoresis as described in 2.4.1.

2.4.3 PCR products purification

The PCR products were purified using the High Pure PCR products Purification kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Briefly, a total of 50 µL of PCR product was mixture with 250 µL of binding buffer, transferred to a column and centrifuged at 14000 rpm for 1 min. After that, two washing steps were performed. For the DNA recovery step, 50 µL of elution buffer was added to the column.

PCR products were visualized in a conventional gel electrophoresis as described in 2.4.1 and their concentration estimated by comparison with the bands 500 pb and 1500 pb of the molecular weight ladder GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific™, Vilnius, Lithuania).

2.4.4 Sanger sequencing and bioinformatic analysis

DNA Sanger sequencing was performed by an outsourced company, Eurofins Genomics Sanger sequencing (Ebersberg, Germany). PCR purified products were prepared according to company instructions: 12.5 ng/µL of purified PCR product plus 2.5 ρmol/µL of primer were added; The primers used for DNA sequencing were the same as those used in PCR reaction. Both forward (F) and reverse (R) strands were sequenced.

Nucleotide sequences were analysed using the SeqMan program v7.0.0 from DNASTAR Lasergene software package (Lasergene Inc, Madison, USA). Forward and Reverse Sanger sequences were scanned for quality and trimmed for low quality nucleotides. Sequences were then assembled and checked for mismatches. Consensus sequence was recorded in FASTA format for later analysis.

2.4.5 Whole genome sequencing and genome assembly

A representative subset of 19 isolates were selected for WGS. Total genomic DNA extracted as described in point 2.4.1, was prepared according to sequencing services facility requirements (pure DNA; 10 µL at 10 ng/µL). At sequencing services, NEXTERA XT DNA libraries were prepared (2x250bp paired end reads) and sequenced on an Illumina NextSeq 500 apparatus.

Raw reads provided by sequencing facilities were first compiled and converted into the two typical paired ended files which are readable by downstream analysis software, using an in-house python script (M. Machado, unpublished). Reads were then assembled into contigs using the INNUca v3.1 software (https://github.com/B-UMMI/INNUca) (145) a bioinformatics pipeline for bacterial genome assembly. INNUca pipeline performs: quality control check of reads using FastQC v0.11.5; *de novo* draft genome assembly using SPAdes v3.11.0; contigs quality assessment with Pilon v1.18; and contamination detection/species confirmation with MLST 2 v2.4.

Draft genomes were then used to perform a full molecular characterization, including, species identification, MLST prediction, identification of antimicrobial resistance genes, virulence genes, and mobile genetic elements, namely plasmids.

2.4.6 Species identification

For species identification, 16S rDNA amplification nucleotide sequences obtained for each isolate were compared against National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST). Species were identified considering 100% identity and 100% coverage between query sequence and the database. When identity and coverage were below 100%, the top hits were recorded and compared with species identification obtained using other methodologies, namely those based in genomic data described below.

INNUca assembly pipeline includes a final step wherein MLST is used. This method based on the sequencing of internal fragments of seven housekeeping genes can be used to infer species identification and detect eventual contaminations, since MLST schemes are defined specifically for each species and species dedicated databases are available online. However, the MLST schemes are available only for certain bacterial species (see PubMLST public repository (https://pubmlst.org/) (103).

Draft genomes obtained from INNUca pipeline were also submitted to Pathogenwatch platform (160) (https://pathogen.watch/), which uses Speciator, an in-house tool for species assignment, which refers to NCBI RefSeq database (http://www.ncbi.nlm.nih.gov/RefSeq/) (155).

Species identification was also carried out using the OrthoANI algorithm, recurring to an online calculator (https://www.ezbiocloud.net/tools/ani) (218), which allows bacterial identification by calculating the average nucleotide identity (ANI) values of two prokaryotic genome sequences. Our draft genome sequences from the pork collection were compared with genomes from NCBI Datasets (https://www.ncbi.nlm.nih.gov/datasets/genomes/). Reference genomes chosen were based on the identification obtained by the 16S rRNA sequencing approach. Access numbers of the reference genomes used: GCF_008693605.1 (*Citrobacter portucalensis*); GCF_003795375.1 (*Citrobacter europeaus*); GCF_016503095.1 (*Citrobacter youngae*); GCF_000210775.1 (*Enterobacter hormaenchei*); GCF_000799205.1 (*Enterobacter asburiae*); GCF_000770155.1 (*E. cloacae*); GCF_000364385.3 (*K. pneumoniae*); GCF_000612605.1 (*Pantoea septica*); GCF_002077695.1 (*Pantoea latae*); GCF_003860325.1 (*Pantoea aglomerans*); GCF_004768745.1 (*Serratia nematodiphila*); GCF_008364325.2 (*Serratia liquefaciens*); GCF_017309605.1 (*Serratia ureilytica*); GCF_003516165.1 (*S. marcescens*); GCF_011617105.1 (*H. alvei*); GCF_011754525.1 (*Hafnia paralvei*); GCF_014639435.1 (*Hafnia psychrotolerans*); GCF_017310215.1 (*Aeromonas hydrophila*); GCF_000783715.2 (*Aeromonas caviae*); GCF_000157115.1 (*E. coli*).

The phylogenetic relatedness determination was processed using the software CSIPhylogeny v1.4 available at the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) based on single nucleotide polymorphism (SNP) in the core genome (nucleotides present in all isolates in the analysis). We performed a general analysis with all the isolates sequenced by WGS and performed a more specific analysis with only the isolates from the same genus. In order to visualize the results obtained the software Microreact was used (https://microreact.org/).

2.4.7 Antimicrobial resistance genes screening

Antimicrobial resistance determinants search was performed through massive screening of draft genomes with ABRicate v1.0 command line program (187) using Resfinder (220) database. Furthermore, to extract β-lactamases nucleotide sequences in FASTA format we used ResFinder v4.1, web-server, available at Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/ResFinder/). Selected settings for both methods were an identification (ID) threshold of 80% and a gene coverage (COV) threshold of 60%.

β-lactamases sequences identified and saved in FASTA format were aligned with each other and with an in-house database using the pairwise alignment tool in CLC Genomics Workbench v9 (Qiagen, Netherlands). The in-house database used in these alignments contained all the alleles of the β-lactamases identified in our collection, which are contained in the Bacterial Antimicrobial Resistance Reference Gene Database, Accession Number PRJNA313047 (https://www.ncbi.nlm.nih.gov/bioproject/313047). Results were visualized in the form of a phylogenetic tree (UPGMA method, Jukes-Cantor for substitution model). In order to determine if nucleotide differences observed in β-lactamases could impact gene function, their nucleotide sequence was translated into proteins and the resulting proteins were also aligned.

2.4.8 Virulence genes screening

Virulence genes search was performed though massive screening of draft genomes with ABRicate v1.0 (187), using VFDB (37) database. Moreover, VirulenceFinder v2.0 web-server, available at Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/VirulenceFinder/) was used. Selected settings for both methods were ID threshold of 80% and a COV threshold of 60%.

2.4.9 Plasmids screening

Plasmids types were screened through massive screening of draft genomes contigs with ABRicate v1.0 (187), using PlasmidFinder (33) database. Furthermore, PlasmidFinder v2.1 web-server, available at Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/PlasmidFinder/), was also ran. Selected settings for both methods were an ID threshold of 80% and a COV threshold of 60%. The chromosome/plasmid location of class C β-lactamases for *E. coli* was assessed using the software MLplasmid (https://sarredondo.shinyapps.io/mlplasmids/)(13).

3 RESULTS

3.1 Antibiotic susceptibility profile of the isolates from a pig production chain

In order to evaluate if the pig production chain could constitute a reservoir of antibiotic resistant Enterobacterales we collected samples from live pigs at slaughterhouse entrance, pig meat, slaughterhouse surfaces and workers. Moreover, we delivered raw meat to healthy human volunteers and sampled their hands before and after hand washing and meat handling. Presumptive Enterobacterales isolates were obtained by growing samples in selective media (n=94 isolates). Isolates were collected from 26 different samples and some of them were collected from the same sample.

To assess the antibiotic susceptibility profile of 94 putative Enterobacterales we tested the susceptibility to a panel of 14 antibiotics by disk diffusion and tested the susceptibility to colistin by the microdilution assay in a selection of isolates. This phenotypic characterization revealed that 73.4% (n=69) of the isolates were resistant to amoxicillin + clavulanic acid (AMC), 57.4% (n=54) to ticarcillin (TIC), 53.2% (n=50) to cefoxitin (FOX), 34% (n=32) to cefotaxime (CTX), 31.9% (n=30) to ceftazidime (CAZ), 26.6% (n=25) to ertapenem (ERT), 18% (n=17) to piperacillin + tazobactam (TZP), 18% (n=17) to trimethoprim + sulfamethoxazole (SXT), 12.8% (n=12) to temocillin (TEM) and 9.8% (n=9) were resistant to ciprofloxacin (CIP) (see Figure 3.1). Furthermore, some isolates were susceptible to increased exposure to particular antibiotics, namely to TEM, TIC, CTX, CAZ, IMP, and CIP (see Figure 3.1). No resistance to ceftazidime + avibactam (CZA), gentamicin (CN), imipenem (IMP) and meropenem (MEM) was observed.

Overall, 55% of the isolates (n=52/94) showed resistance to three or more antibiotic classes, being classified as multidrug resistant isolates (see Annex 4).

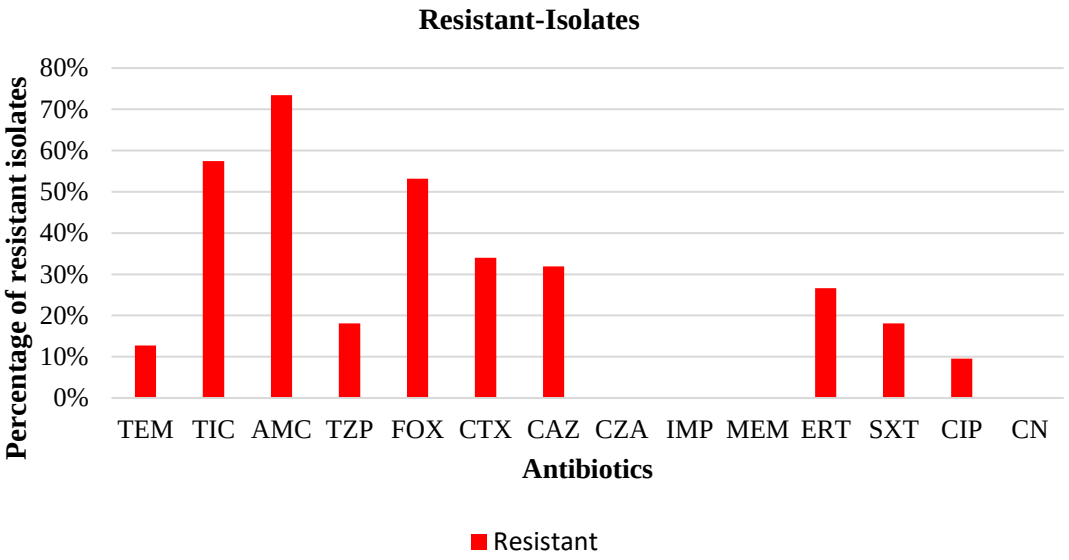

Figure 3.1 – Antimicrobial susceptibility testing of the 94 putative Enterobacterales isolates collected in a pig production chain. Abbreviations: AMC – amoxicillin with clavulanic acid, TZP – piperacillin with tazobactam, TEM – temocillin, TIC – ticarcillin, FOX – cefoxitin, CTX – cefotaxime, CAZ – ceftazidime, IMP – imipenem, MEM – meropenem, ERT – ertapenem, SXT – trimethoprim with sulfamethoxazole, CIP – ciprofloxacin, CN – gentamicin, CZA – ceftazidime with avibactam.

We observed that resistance to the different antibiotics appears to be differentially distributed among live pigs, the different sampling sites after slaughter (meat, workers and surfaces) and human consumers. Considering that a sample was resistant to an antibiotic if at least one isolate collected from that sample was resistant to that antibiotic, we observed that all samples from live pigs (n=3 samples) were resistant to 3rd generation cephalosporins, penicillin, penicillin with β-lactamase inhibitor and sulfonamides with dihydrofolate reductase. Moreover, two out of three pig samples were resistant to 2nd generation cephalosporins, and one sample was resistant to fluoroquinolones and carbapenems (see Table 3.1).

In the group of samples collected after slaughter (meat, workers, surfaces) (n=11), five samples were resistant to 2nd generation cephalosporins, penicillin and penicillin with β-lactamase inhibitor, two samples were resistant to 3rd generation cephalosporins and carbapenem and one was resistant to sulfonamides with dihydrofolate reductase.

Regarding samples from human consumers (n=12), eight isolates showed to be resistant to 2nd generation cephalosporins, seven were resistant to penicillin, six were resistant to penicillin with β-lactamase inhibitor, and five were resistant to 3rd generation cephalosporins and carbapenems.

Table 3.1 – Resistance profile along the pig production chain. The samples were classified as live pigs when collected before the slaughter; after slaughter when collected from the meat pieces, surfaces and from the workers, and human consumers when collected from consumers hands.

	Antibiotics (nº samples / total samples)						
Type of sample	**Fluoroquinolones**	**3rdG Cephalosporins**	**Penicillin**	**Sulfonamides/ dihydrofolate reductase**	**2ndG Cephalosporins**	**Carbapenems**	**Penicillin/ B-lactamase inhibitor**
Live Pigs	1/3	3/3	3/3	3/3	2/3	1/3	3/3
After slaughter	5/11	2/11	5/11	1/11	5/11	2/11	5/11
Human consumers	0/12	5/12	7/12	0/12	8/12	5/12	6/12

3.1.1 Resistance mechanisms epidemiologically important

To select among the 94 putative Enterobacterales, which isolates were epidemiologically relevant to proceed for further studies, the antimicrobial susceptibility testing results were analyzed following the classification proposed by EUCAST (57) (see Table 3.2), wherein isolates are grouped according to their antimicrobial susceptibility profiles. Following this approach, a total of 34% of the isolates (n=32/94) were included in the group of possible ESBL producers, 27% (n=25/94) were integrated in the group of possible carbapenemase carriers, 20% (n=19/94) fitted in the characteristics of possible AmpC carriers, 16% (n=15/94) in the group of antibiotic induction and, finally, 13% (n=12) in the group of resistance to temocillin.

Overall, 47% (n=44/94) of the isolates were considered epidemiologically important, since the susceptibility profile could be assigned to one or more of these groups. These 44 isolates were further analyzed for species identification (section 3.2) and colistin resistance (section 3.1.2)

Table 3.2 – Number of isolates considered epidemiological important and respective characteristics.

Epidemiological Group	N	Characteristics
Possible carbapenemase producers	25	Ertapenem resistance – less than 25 mm; Meropenem resistance – less than 28 mm; Meropenem between 25-27 mm if temocillin resistance.
Possible ESBL producers	32	Cefotaxime resistance – less than 21 mm; Ceftazidime resistance – less than 22 mm.
Possible AmpC carrier	19	Cefoxitin less than 19 mm if ceftazidime or cefotaxime resistance.
Antibiotic induction	15	Induction between carbapenems and CAZ; Induction between cephalosporins and AMC;
Temocillin susceptibility	12	Temocillin resistance – less than 17 mm.

3.1.2 Colistin resistance

To complete the antimicrobial susceptibility testing, the minimum inhibitory concentration (MIC) for colistin was additionally determined for the 44 isolates considered as epidemiologically important (see Annex 4 – A.4B). Our results showed that 45% (n=20/44) were resistant to colistin and had MIC > 8 mg/L, among which 17 isolates had an MIC > 16 mg/L. The remaining isolates were susceptible and had an MIC < 2 (n=24/44) (see Table 3.3). Furthermore, a colistin heteroresistance phenotype was identified in six isolates.

Table 3.3 – Distribution of the minimum inhibitory concentration (MIC) for colistin of the 44 putative Enterobacterales isolates that were considered epidemiologically important according to their antimicrobial susceptibility profile.

MIC variation	Number of isolates	Percentage (%)
[0 ; 1 [	20	45
[1 ; 2 [	4	9
[2 ; 4 [	0	0
[4 ; 8 [	0	0
[8 ; 16 [	3	7
[> 16 [	17 *	39

* 6/17 isolates presented a heteroresistant profile

Taking into consideration the species identification obtained (see section 3.2), we found that the 20 colistin resistant isolates, belonged to four different genera, including *Enterobacter spp* (n=7), *Serratia* spp. (n=7), *Hafnia* spp. (n=4) and *Aeromonas* spp. (n=2) (see Figure 3.2). These results are in agreement with the fact that *Serratia* spp. is intrinsically resistant to colistin. Moreover, the six isolates presenting a heteroresistance phenotype were either *E. asburiae* (n=5) or *H. alvei* (n=1).

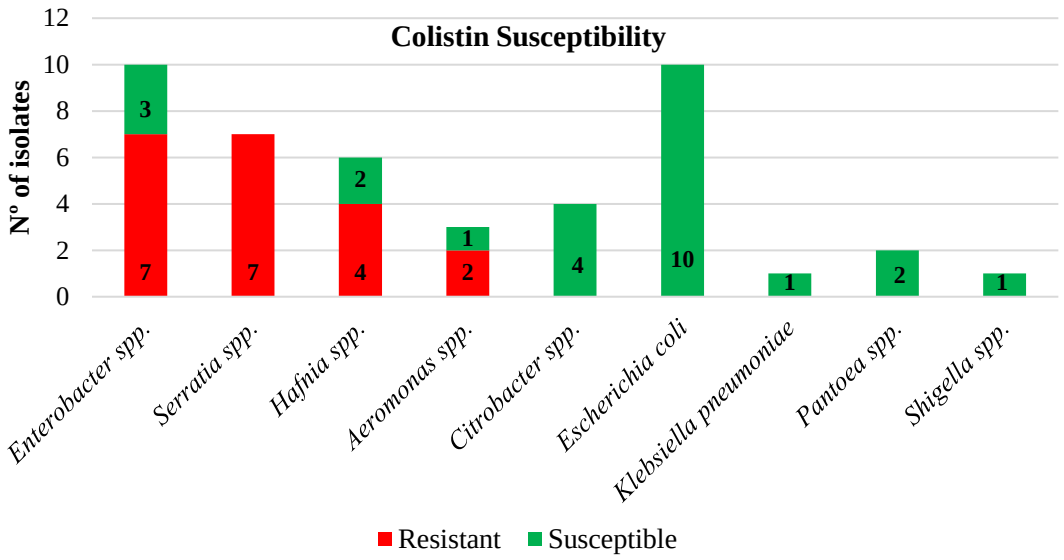

Figure 3.2 – Genus/species identification of the 20 colistin resistant isolates.

3.1.3 Antimicrobial resistance genes

Among the 44 isolates that were considered epidemiologically relevant, we selected a representative group of 19 isolates for whole genome sequencing. The 19 isolates were selected to include the highest genetic diversity in terms of species, to encompass the entire pork production chain and to include at least one isolate from each sample. In order to characterize their content in antibiotic resistant genes, the sequencing reads obtained were assembled into contigs and a massive screening of draft genomes was performed with ABRicate v1.0 command line program (187) using Resfinder (220) database. We found that all the isolates carried at least one antimicrobial resistance gene, when an ID > 80% was considered. Moreover, as many as 79% of the isolates (n=15/19) carried β-lactamases (see Table 3.4 – identified in blue).

β-lactamases from all four molecular classes were identified in our collection. The β-lactamases more represented in this study were those of class C, better known as AmpC, that were found in 74% (n=14) of the isolates. A total of 10 different *ampC* genes were detected including *blaACC*-

3, *blaACC-7*, *blaACT-2*, *blaACT-5*, *blaCFE-1*, *blaCMY-2*, *blaCMY-53*, *blaMOX-4*, *blaMOX-6* and *blaSST-1*. Class A β-lactamases were only found in two isolates being encoded by genes *blaSHV-187* and *blaCARB-2*. Class D β-lactamases were identified in two isolates (*blaOXA-427* and *blaOXA-724*), and a single isolate carried a gene associated to class B metallo-β-lactamase (a carbapenemase encoded by *cphA7*).

For other antibiotic classes, different resistance determinants were identified. In 68.4% (n=13/19) of the isolates we identified genes associated to fluroquinolones resistance, including the *oqxB* (n=9), *qnrE1* and *oqxA* (n=3, each) and *qnrB27* (n=2). Moreover, 15.8% of the isolates carried genes conferring resistance to aminoglycosides (n=3/19), namely *aadA2*, *ant(3'')-Ia* and *aac(6')-Ic*, and genes conferring resistance to tetracycline resistance (*tet(41)* and *tet(A*). Furthermore, we found three isolates carrying the *mcr-10* gene, conferring resistance to colistin (see Table 3.4 – identified in green).

Although less represented, we also found genes associated to resistance to sulfonamides, macrolides, trimethoprim, chloramphenicol, fosfomycin, and disinfectants.

Interestingly, the vast majority of the described genes (85.1%; 46/54) did not present 100% nucleotide identity with reference gene, constituting thus genes homologous to resistant genes or possibly new resistance genes.

Each isolate contained between one and 11 resistance genes (see Table 3.4). The isolate with the highest number of resistance genes was an *E. coli* isolate, collected from a live pig, harboring a total of 11 genes conferring resistance to β-lactams (penicillins, cephalosporins, carbapenems), aminoglycosides, sulfonamides, trimethoprim, chloramphenicol, macrolides and tetracycline.

All the isolates with ertapenem resistance (n=12) have at least one *ampC* gene. However, in 16 isolates we found inconsistencies, observed between the resistance profile obtained by the antimicrobial susceptibility testing and the genes detected (see Figure 3.4). In some isolates, such as the case of isolate 2, the phenotypic tests showed that the isolate was resistant to colistin, but no resistance gene or other colistin-resistance mechanism were found. This type of inconsistencies was identified in isolates resistant to temocillin (n=4), cefoxitin (n=4), amoxicillin with clavulanic acid (n=3), colistin (n=2) and ticarcillin (n=1). In five colistin resistant isolates, correspondent to *Serratia* spp., no gene that confers colistin resistance was found, however, *Serratia* spp. is intrinsically resistance to colistin (185). The other type of inconsistencies, identified in 14 isolates, corresponded to the detection of antimicrobial resistance genes without a corresponding resistance phenotype (see Table 3.4). This was identified in isolates carrying fluoroquinolones resistance genes (n=13) and aminoglycoside resistance genes (n=3).

Table 3.4 – Antimicrobial resistance genes detected in 19 putative Enterobacterales collected from the pig production chain.

Isolate	Origin	Species	Resistant Antibiotic	Gene present (ID)
1	Dirty Table 2	*A. caviae*	TIC; AMC; ERT	***blaMOX-6*** (97.92); ***blaOXA-427*** (86,00).
2	Dirty Table 2	*A. hydrophila*	TIC; AMC; TZP; ERT	***blaMOX-4*** (84.55); ***blaOXA-724*** (97.36); ***cphA7*** (94.38).
			Colistin	No gene present
3	Dirty hands F9	*C. europaeus*	TIC; AMC; TZP; FOX; CTX; CAZ; ERT	***blaCFE-1*** (99.22);
			No resistance detected	*qnrB27* (99.69).
4	Clean hands F9	*C. europaeus*	TIC; AMC; TZP; FOX; CTX; CAZ; ERT	***blaCFE-1*** (99.22);
			No resistance detected	*qnrB27* (99.69).
5	Dirty Table 2	*C. youngae*	TIC; AMC; FOX; CTX; CAZ; ERT	***blaCMY-53*** (86.56).
6	Pig 6	*E. coli*	TEM; TIC; AMC; TZP; FOX; CTX; CAZ; ERT	***blaCARB-2*** (100.00); ***blaCMY-2*** (100.00);
			SXT	*sul3* (100.00); *dfrA16* (100.00);
			CIP	*mdf(A)* (98.78);
			No resistance detected	*aadA2* (99.88); *ant(3'')-Ia* (99.63); *cmlA1* (99.92); *tet(A)* (100.00); *sitABCD* (99.57); *formA* (81.08).
7	Dirty hands F6	*E. asburiae*	TEM; TIC; AMC; TZP; FOX; CTX; CAZ; ERT	***blaACT-2*** (99.65);
			Colistin	***mcr-10*** (100.00);
			No resistance detected	*oqxB* (89.31); *qnrE1* (93.48).
8	Dirty hands F6	*E. asburiae*	AMC; FOX; ERT	***blaACT-2*** (99.65);
			Colistin	***mcr-10*** (100.00);
			No resistance detected	*qnrE1* (93.48).

9	Dirty hands F6	*E. asburiae*	TIC; AMC; FOX; CTX; CAZ; ERT	***blaACT-2*** (99.65);
			Colistin	***mcr-10*** (100.00);
			No resistance detected	*qnrE1* (93.48).
10	S. Worker 1	*E. hormaechei*	TIC; AMC; FOX; CTX; CAZ; ERT	***blaACT-5*** (98.95);
			No resistance detected	*fosA* (96.71), *oqxA* (86.32); *oqxB* (89.10).
11	Hands after meat handling F4	*H. alvei*	TEM; TIC; AMC; TZP; FOX; CTX; CAZ;	***blaACC-3*** (99.73).
			Colistin	No gene present
12	Dirty Table 2	*H. paralvei*	AMC; TZP; CAZ; ERT	***blaACC-7*** (99.15).
13	Pig 10	*K. pneumoniae*	TEM; TIC; TZP; FOX	***blaSHV-187*** (99.42);
			No resistance detected	*oqxA* (99.32); *oqxB* (98.91); *fosA5* (96.91).
14	Dirty hands F7	*P. séptica*	TEM; FOX;	No gene present
			No resistance detected	*oqxB* (84.15).
15	Dirty table 1	*S. liquefaciens*	TEM; AMC; FOX;	No gene present
			Colistin	Intrinsically resistant
			No resistance detected	*oqxB* (80.16).
16	S. Worker 3	*S. liquefaciens*	TEM; AMC; FOX;	No gene present
			Colistin	Intrinsically resistant
			No resistance detected	*oqxB* (80.03).
17	Meat Pieces 2	*S. liquefaciens*	TEM; TIC; AMC; FOX;	No gene present
			Colistin	Intrinsically resistant
			No resistance detected	*oqxB* (80.16).
18	Clean hands F9	*S. ureilytica*	TEM; TIC; AMC; FOX;	***blaSST-1*** (95.16);
			Colistin	Intrinsically resistant
			No resistance detected	*aac(6')-Ic* (92.52); *oqxB* (82.67); *tet(41)* (92.68).
19	Hands after meat handling F9	*S. ureilytica*	TEM; TIC; AMC; ERT	***blaSST-1*** (95.16);
			Colistin	Intrinsically resistant
			No resistance detected	*aac(6')-Ic* (92.52); *oqxB* (82.67); *tet(41)* (92.68).

3.1.4 Analysis of β-lactamases diversity

Considering that the vast majority of the AmpC β-lactamases found had an ID of less than 100%, when compared to the reference sequences, we hypothesized that the AmpC β-lactamases identified could constitute putative new antibiotic resistance genes. To explore this hypothesis, all *ampC* gene alleles available in NCBI database (https://www.ncbi.nlm.nih.gov/bioproject/313047) were retrieved and aligned against the AmpC sequences obtained in our study using the CLC Genomics Workbench. In Figure 3.3 there is an example of a phylogenetic tree constructed from the alignment of sequences from our study with sequences of the *blaMOX* alleles. The alignments with the remaining *ampC* genes were included as annexes (see Annex 6). The number of nucleotides and amino acid differences between AmpC from our study and the closest *ampC* gene previously described were included in Table 3.5.

Excluding the gene alleles promptly identified (ID=100%), the remaining genes presented nucleotide differences that ranged between one and 44 nucleotide differences, when compared to the closest described allele, which corresponded from one to nine differences in the amino acids sequence of the translated protein. The only exception was *blaMOX-4* that was distantly located in the tree when compared to the remaining gene alleles and showed a significant number of differences (218 nucleotides; 84.55% of ID) (see Figure 3.3 and Table 3.5). In the case of *blaSHV-187* gene, seven alleles were identified that corresponded to a single amino acid difference in the translated protein (see Table 3.5).

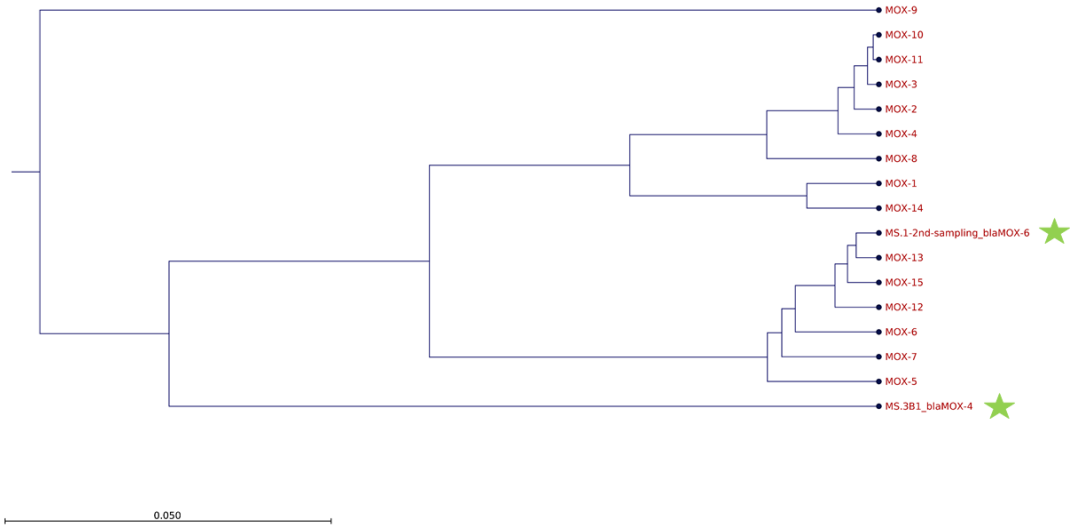

Figure 3.3 – UPGMA phylogenetic tree constructed from Jukes-Cantor showing the relatedness of *blaMOX* alleles (15 sequences included) with the *blaMOX* genes identified in our study (MS.1-2nd-sampling_*blaCFE-1* and MS.3B1_*blaMOX-4*, depicted with a star).

Table 3.5 – Number of differences in nucleotides and amino acids between *ampC* genes and proteins identified in this study with the closest gene alleles described.

Gene detected by ResFinder	Nt ID (ResFinder)	Closest gene by phylogenetic analysis	Nº different nt w/ closest gene	Nº different aa w/closest gene
blaCARB-2	100.00	*blaCARB-2*	0	0
blaSHV-187	99.42	*blaSHV-40*	2	1
		blaSHV-56	2	1
		blaSHV-79	2	1
		blaSHV-85	2	1
		blaSHV-89	2	1
		blaSHV-209	2	1
		blaSHV-224	2	1
blaACC-3	99.73	*blaACC-3*	3	2
blaACC-7	99.15	*blaACC-7*	10	4
blaACT-2	99.65	*blaACT-34*	1	1
blaACT-5	98.95	*blaACT-47*	4	2
blaCFE-1	99.22	*blaCFE-1*	9	2
blaCMY-2	100.00	*blaCMY-2*	0	0
blaCMY-53	86.56	*blaCMY-157*	14	4
blaMOX-4	84.55	*blaMOX-8*	218	81
blaMOX-6	97.92	*blaMOX-13*	8	4
blaSST-1	95.16	*blaSST-1*	11	9
blaOXA-724	97.36	*blaOXA-950*	15	2
blaOXA-427	86.00	*blaOXA-780*	25	9
cphA7	94.38	*cphA1*	44	7

3.1.5 Plasmids

One of the main means of spread of antimicrobial resistance determinants is through the transmission of plasmids carrying antibiotic resistance genes. To understand which was the content of plasmids in isolates collected from the pork production chain, the 19 isolates for which the whole genome was available were screened for the presence of the plasmids through massive screening of draft genomes contigs with ABRicate v1.0 (187), using PlasmidFinder (33) database, a software that allows the identification of plasmid replicons. This is a specific region that encodes key plasmid functions involved in activation and control of plasmid replication (32). When

applying this approach, a total of 22 different replicons were identified in 80% (n=15/19) of the isolates (Table 3.6). The identity of the replicons varied between 81.28% and 100%. The two most common family of plasmid replicons were the IncF family (66.7%; 10/19) and the Col plasmids family (40%; 6/19), known to contain genes encoding bacteriocins. The most common replicon was the Col(pHAD28), present in 42.1% (n=8/19) of the isolates (Table 3.6 – identified in blue), followed by Col440II present in 36.8% (n=7/19) (Table 3.6 – identified in green).

To understand if the *ampC* genes found in isolates collected in our study could be carried in plasmids, we assessed if the assembled contigs containing the *ampC* genes coincided with the contigs containing the plasmids replicons. However, in every case, the *ampC* genes were located in contigs that were different from the ones containing replicons. Nevertheless, when we applied the MLplasmids (https://sarredondo.shinyapps.io/mlplasmids/), a software that can predict the chromosome/plasmid location of assembled contigs for *E. coli* we verified that the gene *blaCMY-2* identified in the isolate 6 was carried by a plasmid.

We also determined the distribution of the different plasmids in the 19 isolates, to evaluate the possibility of plasmid spread among different strains and species (see Figure 3.4). This analysis showed that the plasmids Col(pHAD28) and Col440II were both disseminated among four different genera, including *Pantoea* spp., *Enterobacter* spp., *Citrobacter* spp. and *Serratia* spp. corresponding to five and four different species, respectively. Moreover, the plasmid Col(MGD2) was disseminated among *H. paralvei*, *P. septica* and *S. liquefaciens*, the plasmid IncFIB(K) was disseminated in *E. asburiae* and *K. pneumoniae* and the plasmid IncR was disseminated in *E. asburiae* and *E. coli*.

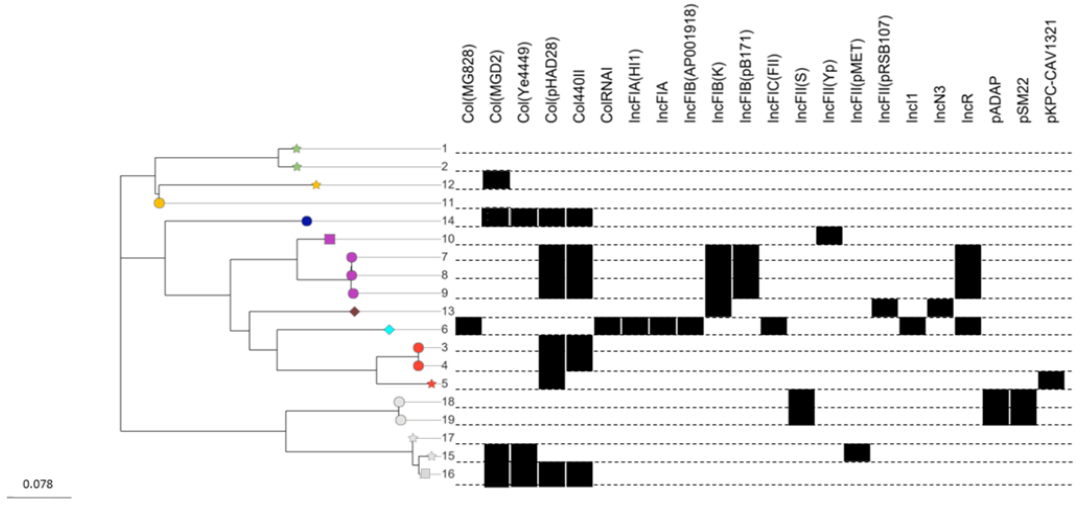

Figure 3.4 – UPGMA phylogenetic tree constructed from the core single nucleotide polymorphisms of 19 isolates collected from the pork production chain and distribution of plasmid replicons. Legend: stars – surfaces; circle – human consumers; squeare – workers; diamond – pigs; green – *Aeromonas* spp.; yellow – *Hafnia* spp.; dark blue – *Pantoea* spp.; purple – *Enterobacter* spp.; brown – *Klebsiella* spp.; light blue – *Escherichia* spp.; red – *Citrobacter* spp.; grey – *Serratia* spp. Black – presence; white – absence.

Table 3.6 – Plasmid replicons detected in 19 isolates collected from the pig production chain.

Isolates	Origin	Species	Nº plasmids	Plasmid replicons (ID%)
3	Dirty hands F9	*C. europaeus*	2	**Col(pHAD28)** (94.66); **Col440II** (83.39).
4	Clean hands F9	*C. europaeus*	2	**Col(pHAD28)** (94.66); **Col440II** (83.39).
5	Dirty Table 2	*C. youngae*	2	**Col(pHAD28)** (88.55); pKPC-CAV1321 (100.00).
6	Pig 6	*E. coli*	8	Col(MG828) (95.00); ColRNAI (88.55); IncFIA(HI1) (98.97); IncFIA (99.74); IncFIB(AP001918) (98.39); IncFIC(FII) (84.06); IncI1 (100.00); IncR (99.60).
7	Dirty hands F6	*E. asburiae*	5	**Col(pHAD28)** (96.12); **Col440II** (83.75); IncFIB(K) (98.93); IncFIB(pB171) (90.62); IncR (99.60).
8	Dirty hands F6	*E. asburiae*	5	**Col(pHAD28)** (96.12); **Col440II** (83.75); IncFIB(K) (98.93); IncFIB(pB171) (90.62); IncR (99.60).
9	Dirty hands F6	*E. asburiae*	5	**Col(pHAD28)** (96.12); **Col440II** (83.75); IncFIB(K) (98.93); IncFIB(pB171) (90.62); IncR (99.60).
10	S. Worker 1	*E. hormaechei*	1	IncFII(Yp) (99.56).
12	Dirty Table 2	*H. paralvei*	1	Col(MGD2) (90.10).
13	Pig 10	*K. pneumoniae*	3	IncFIB(K) (99.64); IncFII(pRSB107) (82.07); IncN3 (88.26).
14	Dirty hands F7	*P. séptica*	4	Col(MGD2) (86.14); Col(Ye4449) (81.86); **Col(pHAD28)** (87,60); **Col440II** (83.75).
15	Dirty table 1	*S. liquefaciens*	3	Col(MGD2) (89.11); Col(Ye4449) (89.69); IncFII(pMET) (100.00).
16	S. Worker 3	*S. liquefaciens*	4	Col(MGD2) (87.13); Col(Ye4449) (90.21); **Col(pHAD28)** (94.66); **Col440II** (84.45).
18	Clean hands F9	*S. ureilytica*	3	IncFII(S) (81.28); pADAP (84.44); pSM22 (91.33).
19	Hands after meat handling F9	*S. ureilytica*	3	IncFII(S) (81.28); pADAP (84.44); pSM22 (91.33).

3.2 Identification and distribution of Enterobacterales in a pig's production chain in Portugal

3.2.1 Genus identification based on phenotypic tests and 16S rDNA

To identify the 44 epidemiologically important Enterobacterales isolates at genus and species level, we used both phenotypic and genotypic approaches. Phenotypic characterization was done by testing the ability of the isolates to perform lactose and urease fermentation and to metabolize indole. Moreover, results obtained were complemented with 16S rRNA gene sequencing and whole-genome based sequencing approaches to reach a final species identification.

The analysis of 16S rRNA gene sequences obtained showed that the isolates belonged to nine different genera of five Families (Enterobacteriaceae, Aeromonadaceae, Yersiniaceae, Hafniaceae and Erwiniaceae). Aeromonadaceae belong to Aeromonadales Order, the remaining Families belong to Enterobacterales Order (see Figure 3.5). The most prevalent genus among Enterobacteriaceae were *Enterobacter* spp. and *Escherichia* spp. (n=10, each), followed by *Citrobacter* spp. (n=4), *Klebsiella* spp. and *Shigella* spp. (n=1, each). In Aeromonadaceae Family we only found isolates belonging to *Aeromonas* spp. (n=3) genus, in Erwiniaceae we identified isolates belonging to the genus *Pantoea* spp. (n=2). In Yersiniaceae Family we identified isolates from *Serratia* spp. (n=7), and in Hafniaceae Family we identified isolates belonging to *Hafnia* spp. (n=6) (see Figure 3.5).

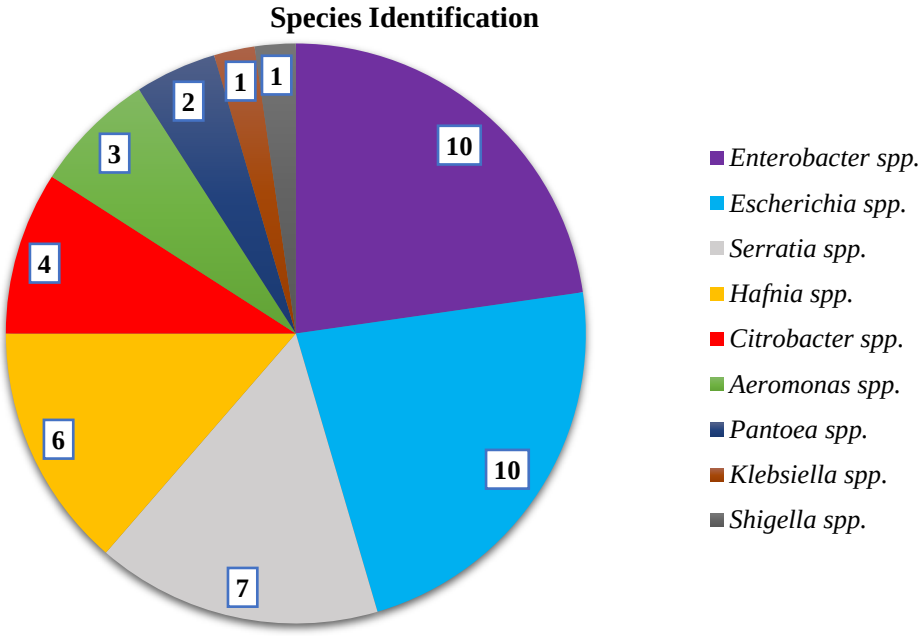

Figure 3.5 – Identification of the 44 presumptive Enterobacterales by 16S rRNA gene sequencing at genus level.

The phenotypic tests performed were in accordance with the identification at the genus level by 16S rRNA gene sequencing in 70% of the isolates (see Table 3.7). The cases in which a concordance was not observed were depicted by an asterisk in the Table 3.7, such as the case of *Escherichia* spp. with a negative reaction to indole.

Analysis by the 16S rRNA sequencing did not allow identifying the majority of isolates [72.7% (32/44)] at the species level due to the fact that alignment of 16S rRNA gene sequences with NCBI database by BLAST algorithm provided similar nucleotide identity percentages for different species.

Table 3.7 – Genus identification by 16S rRNA gene sequencing and biochemical reactions of 44 presumptive Enterobacterales.

Genus identification (16S rRNA gene)	**Biochemical tests**			**No isolates**
	Lactose	Urease	Indole	
***Aeromonas* spp.**	-	-	-	1 *
	-	-	+	2
***Citrobacter* spp.**	+	+	-	3 *
	+	-	-	1 *
***Enterobacter* spp.**	+	+	-	8
	-	+	-	2
***Escherichia* spp.**	+	+	+	3 *
	+	-	+	5
	+	-	-	2 *
***Hafnia* spp.**	-	-	-	6
***Klebsiella* spp.**	+	-	-	1 *
***Pantoea* spp.**	+	+	+	1 *
	+	-	-	1
***Serratia* spp.**	-	-	-	7
***Shigella* spp.**	+	-	+	1 *

Legend: (+) positive reaction; (-) negative reaction; * non-concordance between phenotypic tests and 16S rRNA gene sequencing.

3.2.2 Species identification based on WGS

Since the combination of 16 rRNA sequencing and phenotypic methods only allowed identifying isolates at the genus level, we used the whole-genome sequencing data obtained for 19 representative isolates, to identify these isolates at species level. For this purpose, whole genome data was assembled and analyzed by INNUca software and draft genomes obtained were submitted to PathogenWatch in order to identify species. Moreover, the average nucleotide identity (ANI) was calculated. Results obtained allowed to confirm that the 19 isolates belonged to the five Families and eight Genera previously identified.

A total of 13 different species were identified (see Table 3.8) among the 19 isolates, belonging to Enterobacteriaceae (n=9), Aeromonadaceae (n=2) Yersiniaceae (n=5), Hafniaceae (n=2) and Erwiniaceae (n=1) Families. The most common species were *E. asburiae* and *S. liquefaciens* (15.8%, 3/19 isolates, each). ANI results were in concordance with those obtained using PathogenWatch for the great majority of strains (16/19). The only three exceptions regarded to isolates 12, 18 and 19. In the first case, isolate 12 was identified as *H. alvei* by PathogenWatch and by *H. paralvei* by ANI. In the other two cases (isolates 18 and 19), isolates were identified as *S. marcescens* by PathogenWatch and as *S. ureilytica* by ANI. The final species identification used in this study corresponds to the one retrieved from ANI results.

When analyzing the distribution of the different genus and species along the food processing chain we observed that some isolates from specific genus were exclusively identified in a single sampling site whereas others were found in different sites. In particular, *Aeromonas* spp. were only identified in surfaces, *Escherichia* spp. in live pigs, *Pantoea* spp. in human consumers and *Klebsiella* spp. in live pigs. On the other hand, *Hafnia* spp. were identified in both surfaces and human consumers, *Enterobacter* spp. were identified in the workers hands and human consumers, *Citrobacter* spp. in surfaces and human consumers and *Serratia* spp. in surfaces, workers hand, meat pieces and human consumers.

Table 3.8 – Species identification of the 19 isolates collected in the pig processing chain according to PathogenWatch and ANI values.

Isolates	Origin	PathogenWatch (Speciator tool)	ANI	ANI Values (%)
1	Dirty Table 2	*A. caviae*	*A. caviae*	97.78
2	Dirty Table 2	*A. hydrophila*	*A. hydrophila*	96.89
3	Dirty hands F9	*C. europaeus*	*C. europaeus*	99.08
4	Clean hands F9	*C. europaeus*	*C. europaeus*	99.11
5	Dirty Table 2	*C. youngae*	*C. youngae*	98.71
6	Pig 6	*E. coli*	*E. coli*	96.96
7	Dirty hands F6	*E. asburiae*	*E. asburiae*	96.88
8	Dirty hands F6	*E. asburiae*	*E. asburiae*	96.95
9	Dirty hands F6	*E. asburiae*	*E. asburiae*	96.94
10	S. Worker 1	*E. hormaechei*	*E. hormaechei*	95.97
11	Hands after meat handling F4	*H. alvei*	*H. alvei*	98.97
12	Dirty Table 2	***H. alvei***	***H. paralvei***	95.86
13	Pig 10	*K. pneumoniae*	*K. pneumoniae*	99.00
14	Dirty hands F7	*P. septica*	*P. septica*	95.98
15	Dirty table 1	*S. liquefaciens*	*S. liquefaciens*	98.97
16	S. Worker 3	*S. liquefaciens*	*S. liquefaciens*	98.93
17	Meat Pieces 2	*S. liquefaciens*	*S. liquefaciens*	98.96
18	Clean hands F9	***S. marcescens***	***S. ureilytica***	98.71
19	Hands after meat handling F9	***S. marcescens***	***S. ureilytica***	98.68

Access numbers of the reference genomes used for ANI vales: GCF_008693605.1 (*C. portucalensis*); GCF_003795375.1 (*C. europeaus*); GCF_016503095.1 (*C. youngae*); GCF_000210775.1 (*E. hormaenchei*); GCF_000799205.1 (*E. asburiae*); GCF_000770155.1 (*E. cloacae*); GCF_000364385.3 (*K. pneumoniae*); GCF_000612605.1 (*P. septica*); GCF_002077695.1 (*P. latae*); GCF_003860325.1 (*P. aglomerans*); GCF_004768745.1 (*S. nematodiphila*); GCF_008364325.2 (*S. liquefaciens*); GCF_017309605.1 (*S. ureilytica*); GCF_003516165.1 (*S. marcescens*); GCF_011617105.1 (*H. alvei*); GCF_011754525.1 (*H. paralvei*); GCF_014639435.1 (*H. psychrotolerans*); GCF_017310215.1 (*A. hydrophila*); GCF_000783715.2 (*A. caviae*); GCF_000157115.1 (E. coli).

3.2.3 Assessment of within-host genetic diversity and occurrence of transmission events

To understand the within-host genetic diversity and the possible occurrence of transmission of antibiotic resistant bacteria between different samples, we did a SNPs analysis, wherein we compared, individually, the genomes of isolates of each genus. We found that *E. asburiae* isolates all collected from the same human consumer differed in a low number of SNPs. In particular, isolates 7 and 8, differed between them in five SNPs, isolates 8 and 9 differed in six SNPs and isolates 7 and 9 in seven SNPs (see Annex 5 – Table A.5). Similarly, *C. europaeus* isolates 3, collected from dirty hands and isolate 4 collected from clean hands in the same human consumer differed in only two SNPs (see Annex 5 – Table A.6), and *S. ureilytica* isolates 18, collected from clean hands and 19 collected from hands after meat handling from the same human consumer, differed in a single SNP (see Annex 5 – Table A.7) The results suggest that transmission of *C. europaeus* and *S. ureilytica* have occurred between the different sampling sites (see Figure 3.6). The remaining isolates of the same species had a high number of SNPs difference (>SNPs).

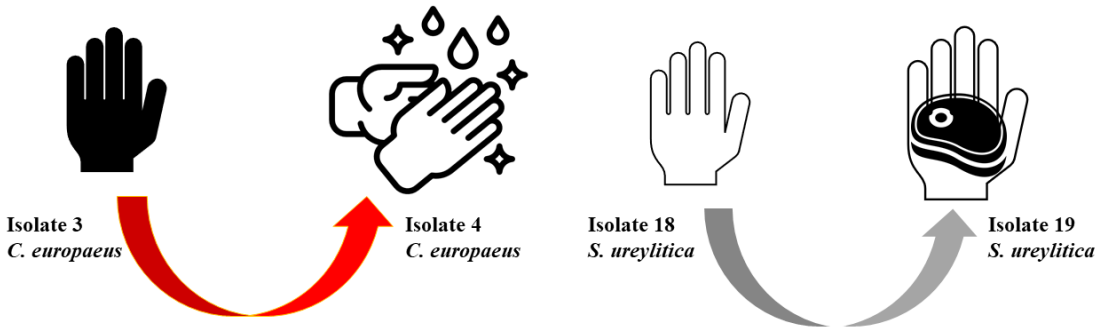

Figure 3.6 – Transmission of *C. europaeus*, between dirty hands and clean hands from the same human consumer. Transmission of *S. ureylitica* between dirty hands and meat through meat handling.

3.3 Molecular characterization of genetic background

To characterize the clonal types of the 19 isolates that were sequenced, the assembled sequencing contigs were used to determine MLST, using the INNUca pipeline. This approach was used for five out of the 13 species identified in our collection, for which a MLST scheme was developed (see Table 3.9), namely for *K. pneumoniae*, *E. coli*, *E. cloacae*, and *Aeromonas* spp.

We found that the unique *K. pneumoniae* isolate belonged to ST60 (clonal complex 16) and that the *E. coli* isolate belonged to ST410 (clonal complex 88), being both isolates collected from a pig's ear. Moreover, the three *E. asburiae*, collected from the dirty hands of human consumers, were identified as ST1249 (clonal complex 384). Although no MLST scheme exists for this

species, we used for *E. asburiae* the scheme previously developed and validated for *E. cloacae* species as was done before by some authors (92). Finally, the *A. caviae* and *A. hydrophila* isolates, collected from dirty tables, belonged to two new STs. In *A. caviae* three non-described alleles were identified, while in *A. hydrophila* six, out of seven alleles, were novel.

Table 3.9 – Multilocus sequence typing of seven isolates collected in the pig production chain and belonging to *Aeromonas* spp., *E. coli*, *E. asburiae* and *K. pneumoniae* isolates.

Isolates	Species	ST	Allelic Profile						
			gltA	*groL*	*gyrB*	*metG*	*ppsA*	*recA*
1	*A. caviae*	New	(~92)	610	627	(~235)	(~104)	(~216)
2	*A. hydrophila*		(~349)	(~287)	(~273)	(~366)	(~443)	(~465)
			Adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*
6	*E. coli*	ST410	6	4	12	1	20	18	7
			dnaA	*fusA*	*gyrA*	*leuS*	*pyrG*	*rplB*	*rpoB*
7	*E. asburiae**	ST1249	347	15	39	134	47	65	10
8									
9									
			gapA	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*
13	*K. pneumoniae*	ST60	2	1	2	1	4	4	8

* The MLST scheme used was *E. cloacae* although the species identified was *E. asburiae*.

3.4 Assessment of pathogenicity in Enterobacterales collected in a pig production chain

To assess the pathogenic potential of Enterobacterales collected in the pork production chain we characterized all the isolates, considered epidemiologically important according to the antimicrobial resistance profile, for the ability to form biofilm (n=44). Moreover, we detected the content in virulence genes of all the 19 isolates for which the whole genome was sequenced.

Biofilms were quantified through spectrophotometry and classified according to Ramos-Vivas *et al*. (2019) (175) (see Annex 4 – A.4C). Overall, 20% (n=9/44) of the isolates were considered as nonproducers, 18% (n=8) were weak producers, 39% (n=17) were moderate producers and 23% (n=10) were considered as strong biofilm producers (see Figure 3.7). The weak producer isolates included *Hafnia* spp. (n=3) and *Escherichia* spp. (n=5); moderate producers included *Aeromonas* spp. (n=1), *Citrobacter* spp. (n=4), *Enterobacter* spp. (n=1), *Escherichia* spp. (n=5), *Hafnia* spp.

(n=3), *Klebsiella* spp. (n=1), *Pantoea* spp. (n=1) and *Shigella* spp. (n=1); and strong producers included *Aeromonas* spp. (n=1), *Enterobacter* spp. (n=2), *Pantoea* spp. (n=1) and *Serratia* spp. (n=6).

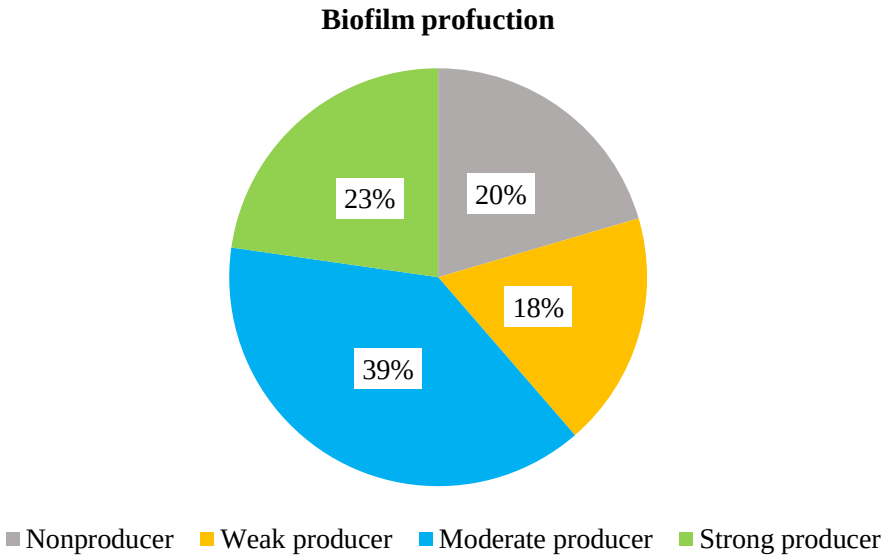

Figure 3.7 – Classification of 44 Enterobacterales collected from the pork production chain according to their ability to produce biofilms. Legend: nonproducer - OD ≤ 0.05; weak producer - 0.05 < OD ≥ 0.1; moderate producer - 0.1 < OD ≥ 0.3; strong producer - OD > 0.3.

The screening of the 19 Enterobacterales for the presence of virulence genes was done through massive screening of draft genomes with ABRicate v1.0 (187), using VFDB (37) database. A total of 263 different virulence genes were identified distributed among eight different classes: 47.5% (125 genes) were included in motility, such as flagella, pilli and fimbriae genes; 18.2% (48 genes) were associated to secretion systems; 14.0% (37 genes) were related to iron acquisition; 10.3% (27 genes) to capsule genes; 3.4% (9 genes) were related to enterotoxins and other toxins like hemolysins; 3.4% (9 genes) were associated to chemotaxis; 2.7% (7 genes) were linked to efflux pumps; and 0.4% (1 genes) were relate to porins (see Figure 3.6).

The isolate with the highest number of virulence genes was the *A. hydrophila* (isolate 2) with 146 genes followed by the *K. pneumoniae* (isolate 13) with 88 genes, and *A. caviae* (isolate 1) with 74 genes. The genes that were present in a higher number of isolates were: *rcsB*, encoding a transcriptional regulatory protein, that was identified in 17 (89.4%) isolates; *gnd* encoding for 6-phosphogluconate dehydrogenase, present in 13 (68.4%) isolates; *fliN*, encoding a flagellar motor switch protein identified in 11 (57.9%) isolates; and *acrB*, encoding for a multidrug efflux pump subunit, present in 11 (57.9%) isolates and *ompA*, encoding for an outer membrane protein A precursor, present in 10 (52.6%) isolates. Noteworthy, toxins were found in three isolates belonging to *A. hydrophila*, *A. caviae* and *E. coli* species.

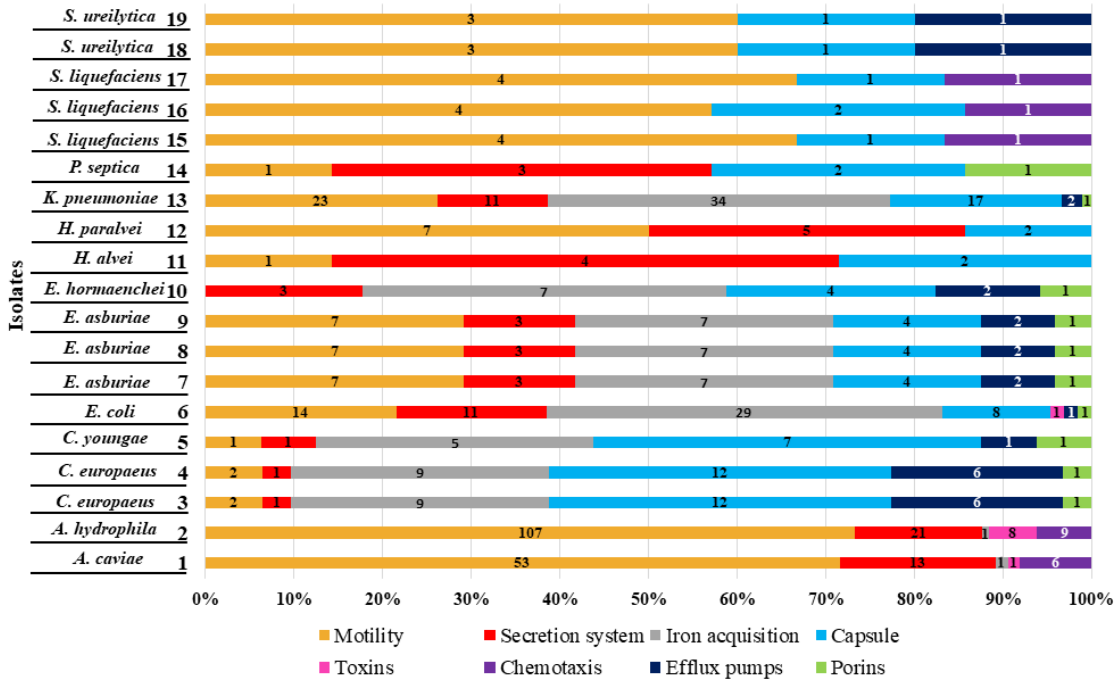

Figure 3.8 – Class diversity of virulence genes found in 19 isolates collected in the pig production chain.

4 DISCUSSION

4.1 Enterobacterales collected from the pig production chain are highly diverse

The description of bacteria resistant to antimicrobials in food-production animals is not a novelty. However, there is a reduced number of studies analyzing the extent of spreading of these bacteria between production animals/food and humans or that evaluate their capacity to cause disease.

In this study, we analyzed a total of 94 presumptive Enterobacterales isolates collected from the pig meat production chain. Among these 94 isolates we identified a group of 44 that were considered epidemiologically important, according to their antimicrobial resistance profile, having at least one of the following characteristics: possible carbapenemase producers, ESBL producers, AmpC carriers, with antibiotic induction, and resistance to temocillin. The induction between carbapenems and ceftazidime is an important feature because it enables a better detection of AmpC β-lactamases (31); induction between cephalosporins and amoxicillin with clavulanic acid is important, because it allows to better detect ESBLs; finally, resistance to temocillin is a marker for the presence of OXA-48 like carbapenemase producers.

The initial species identification of these 44 isolates was performed using biochemical tests, and 16S rRNA gene sequencing. The use of biochemical tests, alone, for identification purposes can become very difficult to interpret due to the occurrence of the convergence and divergence phenomena. Convergence is observed when isolates from different species have similar phenotypic characteristics, as with *Hafnia* spp. and *Serratia* spp., both showing negative results to lactose, urea and indole tests (see Table 1.1). Divergence is observed when isolates from the same species show different phenotypic characteristics (177), as is the case of *Citrobacter* spp. that can have variable results in the lactose test. The use of biochemical tests for identification of bacterial species is based on the assumption that the great majority of isolates from that species have a certain biochemical feature. However, for the establishment of these taxonomic rules not all the diversity within the bacterial populations was sampled and it is expected that outliers might exist. This fact may help to partially explain the reason why we only retrieve a 70% of concordance between the data from the biochemical tests and the 16S rRNA gene sequencing results, regarding the identification of the genus.

The application of the 16S rRNA gene sequencing allowed to identify with confidence the genus, but frequently failed to identify isolates at the species level. This was due to the fact that some isolates had more than 99% of identity in 16S rRNA gene sequences with two different species within the same genus (102).

From the 44 isolates that were epidemiologically important we selected a representative collection of 19 isolates for WGS analysis. For these isolates, species was identified using the software Speciator, from the Pathogenwatch platform, and the average nucleotide identity (ANI) with reference strains. The application of these methods allowed to identify the species in all the 19 isolates tested and, for the great majority, identification was concordant between the two. Moreover, these methodologies allowed to confirm the identification of the genus previously performed when applying 16S rRNA gene sequencing. The only two discrepancies found between ANI results and Pathogenwatch, corresponded to the identification of *S. ureilytica* and *H. paralvei* that in the Pathogenwatch were identified as *S. marcescens* and *H. alvei*, respectively. These differences may result from the fact that these species are not included in the Pathogenwatch database. In a previous study wherein ANI was used for species identification of bacterial type strains, only 3.6% of 141.000 prokaryotic genome from the GenBank were misidentified (39), which supports ANI as a highly reliable method for species identification. Nevertheless, it still presents the disadvantage of requiring the prior knowledge of species, to choose the reference.

Overall, the Enterobacterales from our collection belonged to seven different genus (Enterobacter spp., *Escherichia* spp., *Serratia* spp., *Hafnia* spp., *Citrobacter* spp., *Pantoea* spp. and *Klebsiella* spp.) and 11 species. Despite the fact that *Aeromonas* spp. doesn't belong to Enterobacterales Order, they morphologically resemble members of the Family Enterobacteriaceae. *Aeromonas* spp. belong to the Family Aeromonadaceae, Order Aeromonadales, are Gram-negative, rod-shaped, non-sporulating and facultative anaerobic bacteria. They share similar biochemical characteristics with Enterobacterales but are differentiated by the ability to produce oxidase (176). In our collection we identified two *Aeromonas* species (*A. hydrophila* and *A. caviae*).

All of these species/genus were previously identified in pigs (84). However, in our collection the number of different genus/species was higher than that observed in other studies, in which *E. coli* frequently dominates (20, 30, 69, 72). For example, in Switzerland a total of 91 isolates, collected from food-producing animals in slaughterhouses, were ESBL producing and 98% were identified as *E. coli* (72).

4.2 Antibiotic resistant Enterobacterales were found among different steps of the pig production chain

Among the isolates collected from live pigs, three genera were identified, including *Escherichia* spp. (n=10), *Klebsiella* spp. (n=1) and *Shigella* spp. (n=1), which is in line with the genus previously identified in the pig rectum colonization (184). Almost all isolates identified in live pigs [92% (11/12)] were multidrug resistant, but only one isolate showed resistance to carbapenems. Moreover, no resistance to colistin was observed. Regarding the pig meat a single *S. liquefaciens* was identified, suggesting that meat is not a rich reservoir of Enterobacterales.

Enterobacterales are frequently isolated from surfaces in contact with meat processing, since they have a natural presence in mammalian intestine, as well as in the natural environment. This was highlighted in this study, where 18% (8/44) of the isolates were found in dirty surfaces. In particular, we found six different genera (*Serratia* spp., *Aeromonas* spp., *Hafnia* spp., *Citrobacter* spp., *Pantoea* spp.) in the slaughterhouse surfaces, but no *Escherichia* spp. or *Enterobacter* spp. were detected in this sampling site, even though they were the genera more commonly found in this study. The bacteria found in surfaces normally have specific properties that allow their survival in severe conditions, including high resistance to disinfectants, ability to produce biofilm and also the capacity to survive at low temperature Previous literature reported genus, like *Serratia* spp. and *Hafnia* spp., found in food processing surfaces that were able to grow at a minimum temperatures of 0.2ºC, whereas others, like *Escherichia* spp., were less commonly found and were only capable of growing at 8ºC (144). At this sampling site, 87.5% (7/8) of the isolates recovered were multidrug resistant, and five isolates were resistant to carbapenems. Regarding the ability to produce biofilms, we found that the isolates from the slaughterhouse surfaces could span all the different levels of biofilm production, from strong producers to non-producers, which is in agreement with what has already been reported (205).

People with frequent contact with pigs, like farmers, are one of groups at a higher risk for carriage of food-borne bacteria, many of them resistant to antibiotics (48). However, in this study only three Enterobacterales isolates [*Serratia* spp. (n=1), *Enterobacter* spp. (n=2)] were collected from dirty hands of the workers and no isolate was found in hands after cleaning, suggesting that hand washing/disinfection was efficient in eliminating Enterobacterales from the slaughterhouse workers. The few isolates identified were all multidrug resistant, and two of them were carbapenem resistant and strong biofilms producers.

Previous studies have demonstrated that improper food handling, preparation and consumption practices at home, have been implicated in foodborne diseases (139). In total 45.5% (20/44) of the isolates analyzed in this study were identified in the hands of human consumers 65% of which

(13/20) were multidrug resistant. These isolates were found in consumer's dirty hands, hands after washing and hands after meat manipulation, suggesting that washing was not efficient to successfully eliminate the bacteria under study.

4.3 No evidence for transmission of Enterobacterales within the pig production chain was found

Although Enterobacterales considered pathogenic for humans were found in life pigs (*E. coli* ST410 and *K. pneumoniae* ST60) and Enterobacterales resistant to antibiotics were found in the different stages of the pig processing chain, namely, in surfaces, workers and meat, overall, we found no evidences for the occurrence of transmission between the different processing steps in the slaughterhouse. This was demonstrated by the fact that no isolates from the same species were found in different samples or, if discovered, differed in a high number of SNPs, suggesting they are unrelated. However, we cannot disregard the fact that a low number of isolates were analyzed, and we might be missing some of the transmission events.

The absence of transmission of Enterobacterales in the slaughterhouse might be related to specific processing steps in the slaughterhouse that eliminate bacteria or decrease their reproduction, like hot water bath treatment, skin removal or use of pressure cleaning system (air intake with high pressure followed by detergent jet) (213). Also, the disinfection procedures of both surfaces and workers appear also to have been effective against Enterobacterales, since no transmission was observed between surface, meat and workers.

We also did not detect any transmission between the meat and consumers during meat manipulation. Moreover, in the sampled meat after being prepared by human consumers (cooked meat), no resistant Enterobacterales were found, which confirms that proper cooking can effectively eliminate bacteria before food consumption, avoiding its ingestion.

However, we found some evidence that inefficient hand washing procedures in households might be a possible source of meat contamination. In particular, in one case, dirty hands of a consumer carried one isolate (isolate 3) that was very similar (2 SNPs) to hands of the same consumer after washing (isolate 4) (see Annex 5 – A.6). Moreover, in another case the isolate from the dirty hands of one consumer (isolate 18) was very similar (2 SNPs) to an isolate from the hands of the same consumer after meat manipulation (isolate 19) (see Annex 5 – A.7). Despite the fact that these isolates were considered probably related since by SNP analysis they differed in a low number of SNPs, they had different susceptibility profiles (see Results 3.1.3 – Table 3.4). While

isolate 18 was resistant to cefoxitin and susceptible to ertapenem, isolate 19 was susceptible to cefoxitin and resistant to ertapenem.

4.4 There was genetic diversity within the same sample

When we compared isolates collected from the same sample, we found that there was some genetic variability in isolates. In particular, we found that isolates from the same sample could belong to different genus, different species and be different strains.

The *E. asburiae* isolates 7, 8 and 9, all found in the same dirty hands of one slaughterhouse worker, were considered probably related since by SNP analysis they differed in a low number of SNPs (5-7 SNPs) (see Annex 5 – A.5). However, isolate 7 and 9 were resistant to penicillin and 3rd generation of cephalosporins, while isolate 8 was not (see Table 3.4). Moreover, isolate 7 had two additional antimicrobial resistance genes, *oqxA* and *oqxB*, then the other *E. asburiae* isolates.

In addition, in the dirty table 2 seven species/genus were found corresponding to *A. caviae* (isolate 1) *A. hydrophila* (isolate 2), *C. youngae* (isolate 5), *H. paralvei* (isolate 12), *Pantoea* spp. (n=1), *Hafnia* spp. (n=1) and *Aeromonas* spp. (n=1). Moreover, isolates belonging to *Escherichia* spp. (n=1), *Shigella* spp. (n=1) and *K. pneumoniae* (isolate 13) were also found in the same pig ear sample.

4.5 Resistance determinants from the food chain were similar to those found in hospitals

To evaluate if the resistance determinants from the food chain were related with the ones existing in the hospital settings, we compared the resistance genes obtained with those reported in the literature to be associated to bacterial isolates causing infections in hospital. Regarding the antimicrobial resistance genes, in the pig production chain, we identified 79% (n=15/19) class A, B, D β-lactamase and AmpC producing Enterobacterales. According to a European surveillance, the prevalence of presumptive *E. coli* ESBL and/or AmpC producers in meat from pigs varies from 0% (in Finland and Netherlands) to a total of 24.4% (recorded in Portugal) (112), a much smaller number than what we found.

Although we found a large variety of β-lactamase genes (a total of 15 different genes), only two presented 100% nucleotide identity with genes deposited in the Resfinder database, *blaCARB-2*

and *blaCMY-2*, a class A β-lactamase and an AmpC, respectively. These two genes were found in the same isolate and were collected from the pig ear. These same genes were carried by *E. coli* and *Salmonella enterica* causing infections in humans (110, 143). Regarding *blaCARB-2* gene, besides being found in bacteria causing infection in humans, it has also been found colonizing pig farm workers, although it has not been detected in pigs (94). The CMY-2 is the most wide spread enzyme in Europe and the second most common AmpC found in Portugal in clinical settings (71). Moreover, the fact that this enzyme is frequently found in animals, and located in mobile elements, especially plasmids, suggests that animals may represent important reservoirs of these resistance genes and a direct risk for public health (129).

The remaining β-lactamase genes, with nucleotide identity ranging between 80% and 99%, were classified as homologues of described resistance genes. To understand how similar AmpC found in isolates from pig production chain were from those found in isolates causing infection in hospitals (reference sequences), we extracted the sequences of the β-lactamases and did an alignment. This analysis showed that the homologues were highly similar to reference genes differing between one and 44 amino acids, suggesting that they may be acting as resistance determinants. However, no further studies were performed to verify if the amino acid differences observed in the enzymes were important for their activity and for the resistance function.

In total, seven [*bla-SHV-187*, *blaMOX-6* (215), *blaOXA-427* (46), *blaCFE-1* (150), *blaACT-2* (21), *blaACT-5* (132), *cphA7* (91)] out of the 13 β-lactamases for which we found homologues, were previously identified in Enterobacterales associated with infections, at the clinical settings. In particular, the *blaSHV-187* gene was described in hospitals from the north of Portugal (161). Additionally, it was found in China, in hospitals and pigs, wherein it is considered the predominant resistance gene (124). Although a total of 15 β-lactamases were identified, only *blaCFE-1* (identified in isolate 3 and 4) and *blaSST-1* (identified in isolate 18 and 19) were found in more than one isolate from different samples. However, these isolates were highly related suggesting that in these cases, isolates, rather than the resistance determinant, are being disseminated.

In all the isolates with a carbapenem resistant phenotype we were able to identify a class C β-lactamase. However, in these isolates only ertapenem resistance was observed when the antimicrobial susceptibility testing was performed. This result is similar to the one obtained by Jacoby *et al*., when they analyzed ertapenem resistant *K. pneumoniae* (99) in which the specific ertapenem resistance phenotype was found to be correlated with the overexpression of *ampC* genes and to the lack of outer membrane porins. Although we were not able to evaluate if the isolates from this collection present any modification in the outer membrane porins, affecting cell

permeability, or if *ampC* genes were overexpressed, it is probable that a similar mechanism is associated to the ertapenem resistance observed in our study.

Regarding the remaining antimicrobial resistance genes identified, only four of them (*sul3, dfrA16, tet(A)* and *mcr-10* presented 100% of nucleotide identity with known genes in the database and have been already found in clinical settings worldwide (3, 12, 156, 206). The remaining genes were homologues that ranged in nucleotide identity between 80.03 and 99.92.

In this study, as far as we are concerned, we described for the first time the presence of the *mcr-10* gene in Portugal. This gene was found in three *E. asburiae* collected from the dirty hands of human consumers. This gene was first described in 2020 and was detected in an *Enterobacter roggenkampii* clinical isolate in a hospital in China. After its initial identification it was described in various countries and in different species of Enterobacteriaceae (206), some of which collected from animals (115). Among our collection, a total of 45% (20/44) of colistin resistant isolates were identified, a much higher frequency than that described among Enterobacteriaceae from pig farms in Portugal (10.6%, 42/398) (40).

In the 20 colistin resistant isolates seven were from *Serratia* spp., that is known to be intrinsically resistant to colistin, due to the constitutive expression of *arnBCADTEF operon*, which is regulated by two-component systems PhoPQ. The operon mediates the synthesis and transfer of 4-amino-4-deoxy-L-arabinose to the lipid A, which result in a more positive charge of LPS leading to colistin resistance (154). Like *Serratia* spp. also the genus *Hafnia spp.* is considered naturally resistant to colistin (101), but in this study we identify two colistin susceptible *Hafnia* spp. with an MIC of 2 μg/ml. From the 20 colistin resistant isolates found in our study, only 10 were selected for whole genome sequence analysis and, besides the *mcr-10* gene (identified in three isolates) and the species naturally resistant to colistin (n=5) no other resistant mechanism was identified among the remaining two isolates (see Results 3.1.3 – Table 3.4). Additionally, a heteroresistant phenotype was found in six isolates belonging to *E. asburiae* and *H. alvei* species. Colistin heteroresistant phenotype has been previously described in *E. asburiae* associated with AcrAB-TolC, a multidrug efflux pump; in our study we detected the presence of *acrA* and *acrB* genes in the *E. asburiae*, however no *tolC* gene could be identified (195).

In general, there was a good correlation between phenotype and genotype. The only discrepancies corresponded to aminoglycoside, fluroquinolones and colistin. Despite the number of quinolones resistance genes (carried by 12 isolates), only one isolate showed resistance to ciprofloxacin; in the case of aminoglycosides no resistance was observed in the Antimicrobial Susceptibility Testing performed although aminoglycoside resistance genes were identified in three isolates belonging to *E. coli* (n=1) and *S. ureilytica* (n=2) species. This discrepancy might be explained by the absence of the activator (RarA or RamA) and/or the repressor (OqxR), in the case of the

efflux pump OqxAB (121). Alternatively, the homologous of resistance genes identified might not be performing the resistance function in isolates carrying them, but this needs further testing for clarification. Regarding colistin resistance, as mentioned above no resistant mechanism was identified in two isolates. In these cases, the resistance could be related to mutation, which were not screened in this study.

4.6 Plasmid transfer between different Enterobacterales occurs in the pig production chain

Plasmids were identified in 80% (n=15) of the isolates, harboring a total of 22 different plasmids as defined by the plasmidFinder software. The most common plasmid was Col(pHAD28) (42%, n=8/19), followed by Col440II (36.8%, n=7/19). The Col(pHAD28) was previously identified in resistant *S. enterica* serovar Panama isolated from farm animals (28). In our study Col(pHAD28) was found in five different species (*P. septica, E. asburiae, C. europaeus, C. youngae* and *S. liquefaciens*) collected from dirty surfaces, dirty hands of workers, and in three different human consumers. The plasmid Col440II was previously identified in *K. pneumoniae* isolates from Austrian rivers and in clinical isolates (118). In our study this plasmid was found in four species (*C. europaeus, E. asburiae, P. septica* and *S.liquefaciens*) collected from dirty hands of workers, and in three different human consumers, suggesting that some of the plasmids were frequently transmitted across different strains, species and genus, contributing to the dissemination of their genetic content.

Although some of the antibiotic resistance genes identified in our study, are known to be carried by plasmids, with the analysis performed in this study it was not possible to know the genetic content of the plasmids identified. Also, we could not confirm the chromosomal or plasmid location of the antibiotic resistance genes with the exception of *blaCMY-2* identified in *E. coli* carried out by a plasmid. To confirm the localization of the antibiotic resistance genes in the remaining isolates would require a more detailed analysis of the contigs containing the resistance genes, namely its annotation. Alternatively, the plasmids could be sequenced, and their sequence analyzed in detail.

4.7 Enterobacterales from the pig production chain carry many virulence genes

Enterobacterales from our study carried a wide variety of virulence factors (263 genes), of which the most represented were those involved in motility (47.5%), secretion (18.2%), and iron acquisition (14.0%). These virulence factors might be important if these Enterobacterales are transmitted to humans and have the chance of developing infection.

Motility is a feature that allows bacteria to move across the environment allowing for the detection and pursue of nutrients. Moreover, motility-associated genes can also be involved in bacterial adherence to epithelium, and interaction with the host (104). The secretion systems have a role in delivering virulence factors, as they function as a channel from the bacterial cytoplasm into the host cell, facilitating the infection (45). Additionally, iron acquisition siderophores (enterobactin, yersiniabactin, aerobactin) allow bacteria to acquire iron from the host (186). Interestingly, the isolates with the highest number of iron acquisition genes were the isolates of *E. coli* and *K. pneumoniae*, belonging to known human pathogenic clones.

The most common virulence factors found were *rcsB, gnd, fliN, acrB* and *ompA*. The *rcsB* gene, is part of a signaling system that controls transcription of numerous genes, including genes involved in the capsule synthesis, biofilm formation, acid stress response, cell division and outer membrane proteins synthesis (35). Among the 17 isolates carrying the *rcsB* gene 76.4% were biofilm producers. The *gnd* gene encodes a 6-phosphogluconate dehydrogenase and is one of the enzymes involved in the oxidative stage in the pentose phosphate cycle, which is part of the carbohydrate degradation and associated with cellular growth (151). The *fliN* is one of the three proteins (*fliN, fliM, fliG*) involved in the basal body of flagella (25). Although the *fliN* was identified in 11 isolates, the *fliM* and *fliG* was only identified in two isolates, in the remaining isolates the motor complex was not formed. Moreover, the *acrB* gene, as previously explained, is a subunit of the major antibiotic efflux pump AcrAB-TolC. This efflux pump consists in three proteins, AcrA, a periplasmatic membrane protein, AcrB, the inner membrane protein and TolC, the outer membrane channel, which are regulated by AcrR, a repressor, and MarA a transcriptional activator (168). Although we identified *acrA* in four isolates and *acrB* in 11 isolates, the genes encoding the remaining proteins of the efflux pump were not identified in any isolate. The *ompA* gene, encoded for an outer membrane protein A precursor. OmpA is the most abundant porin and can be associated with drug resistance, allowing slower diffusion of negatively charged β-lactam antibiotics (201). In this study the *ompA* gene was identified in 66.7% (8/12) of the carbapenem resistant isolates and may be involved together with AmpC in the carbapenem resistance mechanism.

Biofilm production is another important virulence factor that might have an impact in the persistence of bacteria in the pig production chain. The adhesion of bacteria to the surfaces of slaughterhouse equipment and meat and the formation of biofilm provides structural stability, protection from stressful conditions, such as cleaning and disinfection, and allow bacteria to persist in these environments. In this study we found that 80% of the isolates were biofilm producers. Although Enterobacterales that are biofilm producers were found in dirty surfaces, after the disinfection step, no isolate was found, suggesting that the disinfection step was efficient in eliminating the potential biofilms formed.

4.8 The pig production chain is a source of nosocomial pathogens

In our collection a total of 13 different species were identified, however only five of them have a MLST scheme described (103). The *K. pneumoniae* isolates were identified as ST60 (associated to clonal complex 16), *E. coli* isolate identified as ST410 (associated to clonal complex 88), the three *E. asburiae* shared the same ST1249 (associated to clonal complex 384) and the two *Aeromonas* spp. were from a new ST. Among these STs found, and recurring to the information in the MLST database and available bibliography, *K. pneumoniae* ST60 and *E. coli* ST410 have previously been described in human infection (142, 180), and *E. asburiae* ST1249 has been associated to a human colonization (106).

K. pneumoniae ST60 is a single locus variant (SLV) of ST16, presenting only a difference in gene *tonB*, where they differ by six nucleotides (ST60 - *tonB*-4; ST16 - *tonB*-8) (27). *K. pneumoniae* ST60 was previously described as causing disease in China, associated with liver abscess (142), and in New York associated with a complicated bacteremia (159). Furthermore, this clone was previously associated with animals infection, having been found in foals with severe arthritis in the Netherlands (27). The ST16 was associated with high bloodstream infection mortality rates in humans (11). These observations suggest that isolates belonging to this clonal background have the capacity to cause severe disease in both human and animal settings.

E. coli ST410 has been described as a high-risk clone reported worldwide, including China, Italy Denmark, among others (63, 148, 180). *E. coli* ST410 is usually associated with extraintestinal pathogenic *E. coli* with resistance to fluoroquinolones, 3rd generation cephalosporins, and carbapenems, causing urinary tract infections and septicemia (180). Moreover, and with an higher impact for our study, this clone was identified among hemodialysis patients in Lisbon (41) and animal infections were also described in a dog in Portugal (26), which may evidence some

distribution of such clone in the community. *E. coli* ST410 is a SLV of ST88, more precisely with differences in *purA* locus (ST410 *purA-18*, while ST88 *purA-12)*. *E. coli* ST88 has been identified in infection in both human and animals (197, 221), which highlights the capacity of these clonal background to cause severe disease and being distributed through several environments.

The *E. asburiae* is a member of the *E. cloacae* complex (138), and the MLST scheme used for *E. cloacae* also has been used for the *E. asburiae* (92). The *E. asburiae* ST1249, described in our collection, is a SLV of ST384 described in this scheme, with only a single difference in the *dnaA* locus (ST1249 – *dnaA-3471;* ST384 – *dnaA-22). E. asburiae* ST1249 was firstly identified colonizing a 43-year-old women diagnosed with breast cancer (106) and harboring a *blaKPC-2*. These observations suggest that isolates from this genetic background have the capacity to colonize humans and become resistant to important antibiotic classes.

The *Aeromonas* spp. isolates identified in our collection did not belong nor were related to any previously described pathogenic clonal type. Nevertheless, they belonged to species previously found associated to infections in humans (*A. caviae* and *A. hydrophila*), being important causers of gastroenteritis, bacteremia, and wound infections (19, 64) and contained a plethora of virulence and antibiotic resistance, which make them as potential human or animal pathogens.

Altogether these results strongly suggest that the pig production chain represents a reservoir for well-known pathogenic bacteria associated with multidrug resistance and infection in both human and animals and also existing in a wide range of reservoirs. Although no direct transmission could be identified between the different stages of the pig production chain and between these chain and humans, the presence of such potential pathogenic microorganisms represents a worrisome finding that should be brought into attention.

4.9 Limitations of the study

All studies face some limitations during its execution, and this was no exception. It is important to notice that the initial antibiotic used to select the putative KPC was meropenem, however we were not able to identify such phenotypic resistance among our collection. According to EUCAST guidelines (57) meropenem is the best choice for screening carbapenemase-production, since it presents a better balance between sensitivity and specificity, however ertapenem have a higher sensitivity. In order to increase the detection of putative carbapenemases, ertapenem could have been used in the initial screening, which would probably increase the number of positive samples found.

Furthermore, we only determined the whole sequence for a limited number of isolates, which decreased the possibilities of finding evidence of transmission between the different stages of the pig production chain. The inclusion of a higher number of isolates in genomic comparison analysis would help to support the findings from this study that transmission of Enterobacterales along the pig processing chain is not frequent.

Finally, the majority of the software, analysis tools and databases are developed for the most frequent pathogens identified in human diseases, such as *E. coli* and *K. pneumoniae*. This hinders a comprehensive genomic analysis, namely in antibiotic and virulence gene content, in less common species, like some of those identified in our study.

4.10 Future work

In our work we have identified a high prevalence of resistance to ertapenem, but not to other carbapenems, a phenotype that suggests that a mechanism associated to AmpC overexpression and decreased permeability of porins may be involved. In line with this, we found also a high prevalence of AmpC and OmpA in isolates collected from the pig production chain. In the future, we intend to further investigate the mechanism of resistance to ertapenem identified by analyzing AmpC expression level in these isolates through a reverse transcription quantitative real time PCR (RT-qPCR). Additionally, we will do a deeper characterization of OmpA, including sequence analysis, comparing the *ompA* nucleotide sequences obtained in our study with reference sequences associated to resistance. Moreover, in order to assess the contribution of OmpA to the resistant phenotype we intend to construct OmpA null mutants of ertapenem resistant isolates.

Additionally, we intend to expand the collection analyzed by WGS, which will allow a better understanding of the extension of the antibiotic resistant gene reservoir and transmission events occurring in the pork production chain and between this setting and humans.

5 CONCLUSION

Our results suggest that the pig production chain constitutes a reservoir of antibiotic resistance genes and antibiotic resistant bacteria pathogenic to humans. Nevertheless, no evidence of bacterial transmission was observed between the different processing steps, which can probably be associated with a successful disinfection in the slaughterhouse. Moreover, the controlled sanitation measures in slaughterhouse workers were important to prevent colonization/ contamination with Enterobacterales, since the hand washing eliminated all Enterobacterales previously found in worker's dirty hands. In consumers home, cooking the meat was enough to eliminate Enterobacterales, however incorrectly hand washing was a possible source of meat contamination, which highlights the importance of primary hygiene rules.

Our study also showed that Enterobacterales collected from the pig production chain were highly diverse, being the vast majority multidrug-resistant and carrying a plethora of virulence genes. Plasmids were identified in different species evidencing its spread along the pig production chain. Furthermore, class C β-lactamases were probably the antimicrobial resistance genes conferring resistance to cephalosporins and carbapenems.

Although the pig production chain constituted a reservoir of pathogenic Enterobacterales if the sanitation measures in the slaughterhouses are followed and an extra care is taken during the meat preparation, eating meat can be considered safe.

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ANNEXES

Annex 1. Culture media

Table A.1 – Culture media and composition per Liter

Culture media	Composition
CHROMagar ESBL	15 g of agar; 17 g of peptone and yeast extract; 1 g of Chromogenic mix; 0.57 g of selective mix ESBL. pH 7.0 ± 0.2
CHROMagar KPC	15 g of agar; 17 g of peptone and yeast extract; 1 g of Chromogenic mix; 0.4 g of selective mix KPC. pH 7.0 ± 0.2
Drigalski agar	15 g of peptone; 3 g of meat extract; 3 g of yeast extract; 1 g of sodium deoxycholate; 1 g of sodium thiosulfate; 15 g of lactose; 0.005 g of crystal violet; 0.08 g of bromothymol blue; 11 g of agar. pH 7.3 ± 0.2
Christensen's Urea agar	1 g of gelatin peptone; 1 g of D-glucose; 2 g of potassium dihydrogen phosphate; 5 g of sodium chloride; 0.012 g of phenol red; 12 g of agar. pH 6.8 ± 0.2
Muller-Hinton broth	3 g of beef extract; 17.5 g of acid hydrolysate of casein; 1.5 g of starch. pH 7.3 ± 0.2
Muller-Hinton II agar	2 g of beef extract; 17.5 g of acid hydrolysate of casein; 1.5 g of starch; 17 g of agar. pH 7.3 ± 0.2
Tryptic Soy Broth	17 g of pancreatic digest of casein; 3 g of peptic digest of soybean; 2.5 g of dextrose; 5 g of sodium chloride; 2.5 g of dipotassium phosphate. pH 7.3 ± 0.2
LB agar	10 g of tryptone; 5 g of yeast extract; 10 g of NaCl; 15 g of agar. pH 7.0 ± 0.2

Annex 2. Buffers and Solutions

Table A.2 – Buffers and solutions for DNA extraction and electrophoresis

Buffers/Solutions	Composition
PBS 1X	137mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4. pH 7.4
LP1 Buffer	20 mM Tris-HCL, pH 8.0; 2 mM Sodium EDTA; 1.2% Triton x-100
Tris-EDTA	10 mM Tris-HCL; 1mM EDTA pH 8.0
TAE 50X	0.5 M Tris; 0.05 M EDTA pH 8.0; 1 M acetic acid.
Gel-Loading Buffer	0.25% bromophenol blue; 0.25% xylene cyanol; 30% glycerol in MiliQ sterile water.
GeneRuler 1kb Plus DNA Ladder	1 µL of DNA ladder; 1 µL of DNA loading dye; 3 µL of MiliQ sterile water.

Annex 3. Enzymes

Table A.3 – Enzymes for DNA extraction

Enzyme	Composition
Lysozyme	20 mg/mL in MiliQ sterile water. Conserved at -20ºC.
Lysostaphin	10 mg/mL in MiliQ sterile water. Conserved at -20ºC
RNase A	10 mg/mL in MiliQ sterile water. Conserved at -20ºC

Annex 4. Antimicrobial resistance profile, species identification and pathogenicity in Enterobacterales from pork production chain

Table A.4 – **A.** Characterization of 117 isolates from the pig production chain: Antimicrobial susceptibility testing, species identification, biofilm production and genomic content. **B.** OD results for MIC determination (colistin) for each isolate. **C.** OD results for quantification of biofilms.

https://www.itqb.unl.pt/~mariana.araujo/Annex_A.4_Thesis.xlsx

Annex 5. Phylogenetic relatedness of the isolates from pig production chain

Table A.5 – Core SNP analysis of *Enterobacter* spp. from the pig production chain.

		Isolates			
		7	8	9	10
Isolates	7	0	5	7	31212
	8	5	0	6	31217
	9	7	6	0	31212
	10	31212	31217	31219	0

Table A.6 – Core SNP analysis of *Citrobacter* spp. from the pig production chain.

		Isolates		
		3	4	5
Isolates	3	0	2	41791
	4	2	0	41791
	5	41791	41791	0

Table A.7 – Core SNP analysis of *Serratia* spp. from the pig production chain.

		Isolates				
		15	16	17	18	19
Isolates	15	0	16994	18222	37778	37778
	16	16994	0	17670	21993	21993
	17	18222	17670	0	38361	38361
	18	37778	21993	38361	0	2
	19	37778	21993	38361	2	0

Annex 6. Analysis of β-lactamases diversity

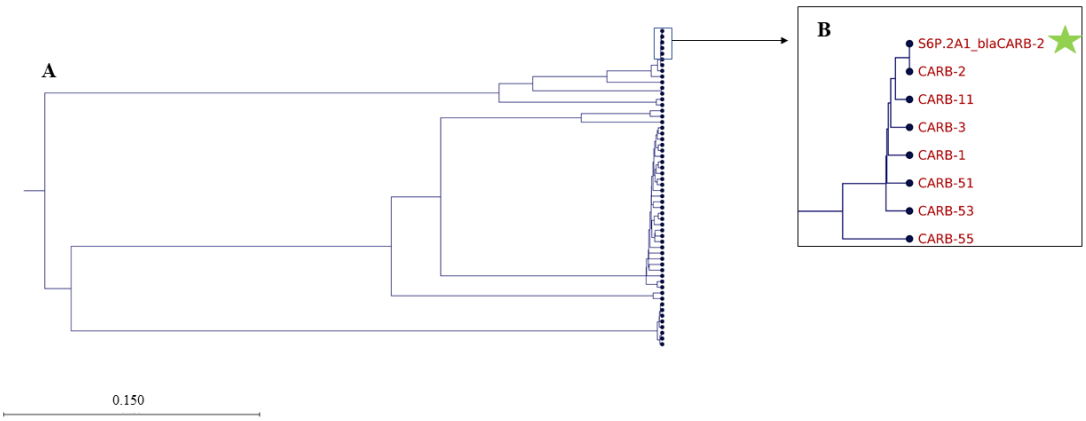

Figure A.1 – **A.** UPGMA phylogenetic tree constructed from Jukes-Cantor showing the relatedness of all alleles of *blaCARB* gene (56 sequences included). **B.** Detailed view of the cluster containing the gene identified in our study (S6P.2A1_*blaCARB–2* identified by a star) and the described allele *blaCARB-2*. Not at scale.

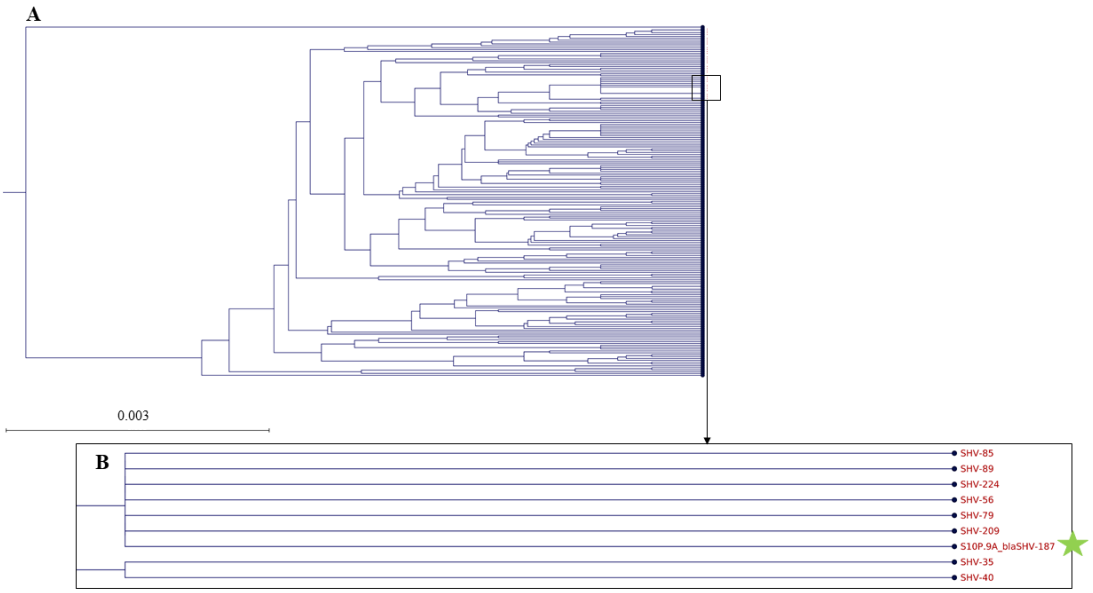

Figure A.2 – **A.** UPGMA phylogenetic tree constructed from Jukes-Cantor showing the relatedness of all alleles of *blaSHV* gene (182 sequences included). **B.** Detailed view of the cluster containing the gene identified in our study (S10P.10_*blaSHV–187* identified by a star) and the described alleles *blaSHV-40, blaSHV-56, blaSHV-79, blaSHV-85, blaSHV-89, blaSHV-209, blaSHV-224*. Not at scale.

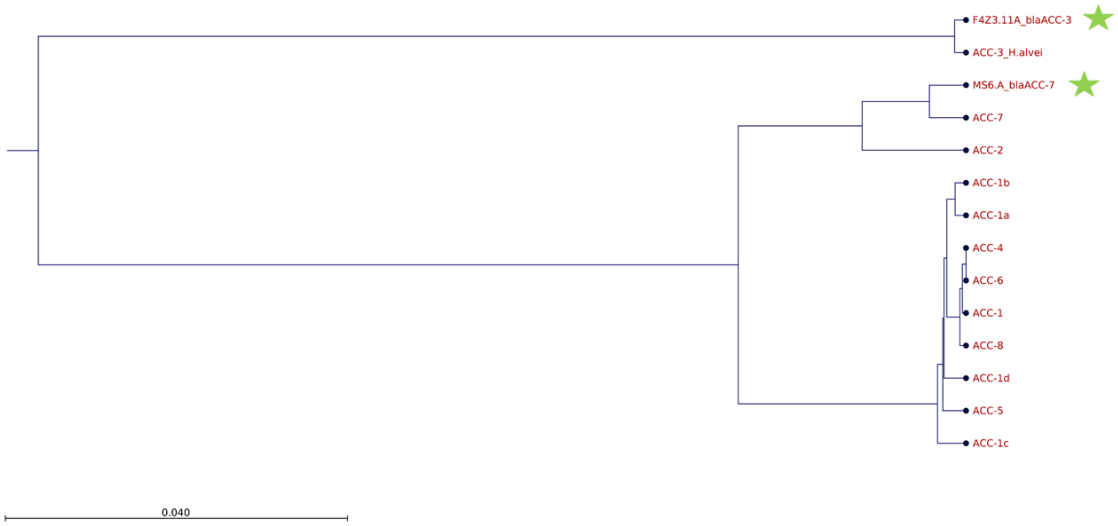

Figure A.3 – UPGMA phylogenetic tree constructed from Jukes-Cantor showing the relatedness of all alleles of *blaACC* gene (12 sequences included) and similarity between the genes identified (F4Z3.11A_*blaACC-3* and MS.6A_*blaACC-7* – identified by a star) and the described alleles *blaACC-3* and *blaACC-7*, respectively.

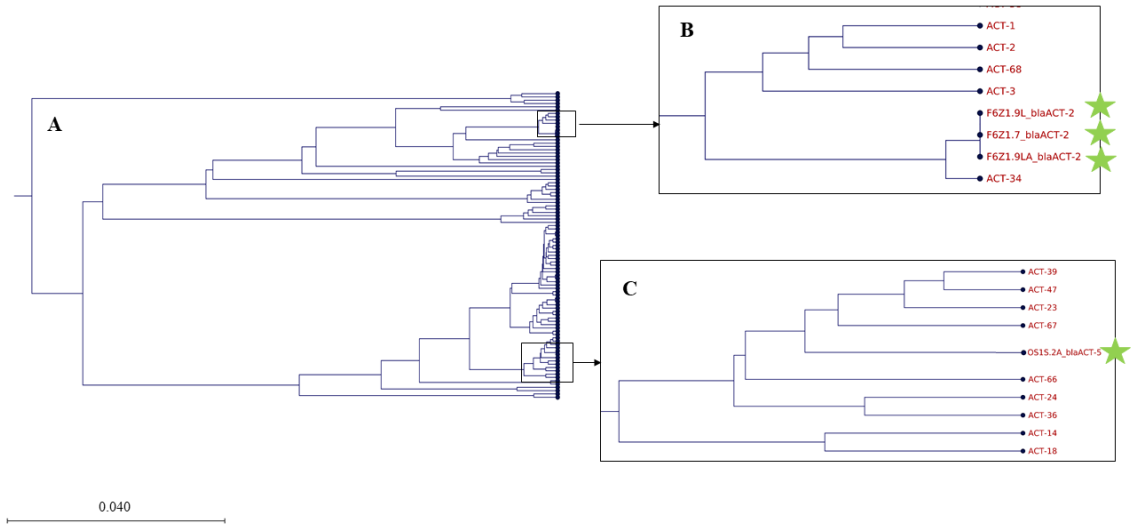

Figure A.4 – **A.** UPGMA phylogenetic tree constructed from Jukes-Cantor showing the relatedness of all alleles of *blaACT* gene (88 sequences included). **B.** Detailed view of the cluster containing the gene identified in our study (F6Z1.9L_*blaACT-2*, F6Z1.7_*blaACT-2*, F6Z1.9LA_*blaACT-2* – identified by a star) and the described allele *blaACT-34*. Not at scale. **C.** Proximity between the gene identified (OS1S.2A_*blaACT-5* – identified by a star*)* and the described allele *blaACC-47*. Not at scale.

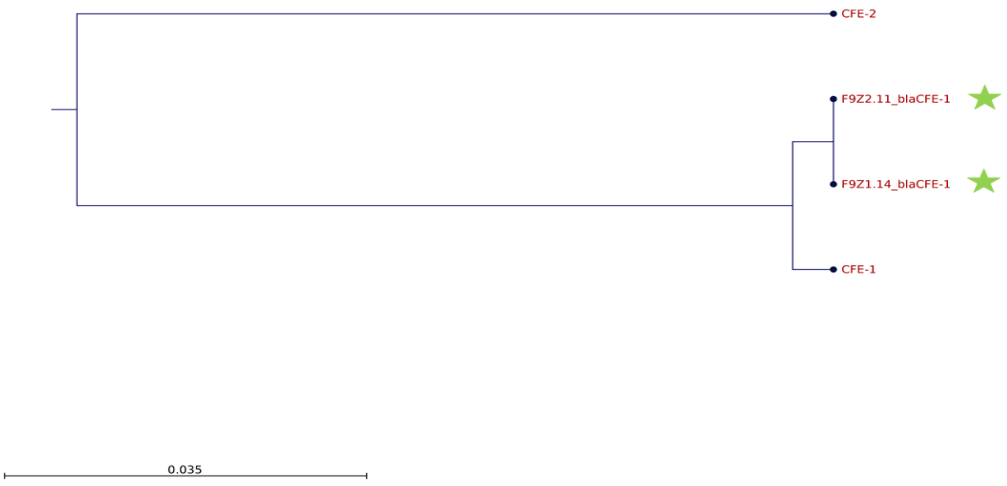

Figure A.5 – UPGMA phylogenetic tree constructed from Jukes-Cantor showing the relatedness of all alleles of *blaCFE* gene (two sequences included) and similarity between the genes identified (F9Z2.11_*blaCFE-1* and F9Z1.14_*blaCFE-1* – identified by a star) and the described allele *blaCFE-1*.

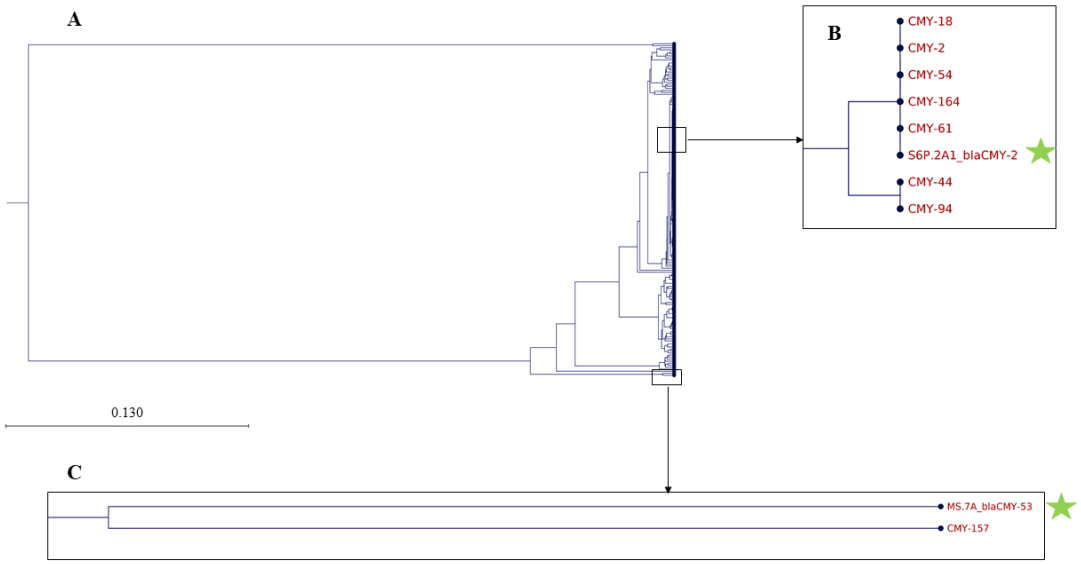

Figure A.6 – **A.** UPGMA phylogenetic tree constructed from Jukes-Cantor showing the relatedness of all alleles of *blaCMY* gene (163 sequences included). **B.** Detailed view of the cluster containing the gene identified in our study (S6P.2A1_*blaCMY-2* – identified by a star) and the described allele *blaCMY-2*. Not at scale. **C.** Proximity between the gene identified (MS.7A_*blaCMY-53*– identified by a star*)* and the described allele *blaCMY-157*. Not at scale.

Figure A.7 – UPGMA phylogenetic tree constructed from Jukes-Cantor showing the relatedness of all alleles of *blaSST* gene (one sequence included) and similarity between the genes identified (F9Z2.18_*blaSST-1* and F9Z2.1B_*blaSST-1* – identified by a star) and the described allele *blaSST-1*.

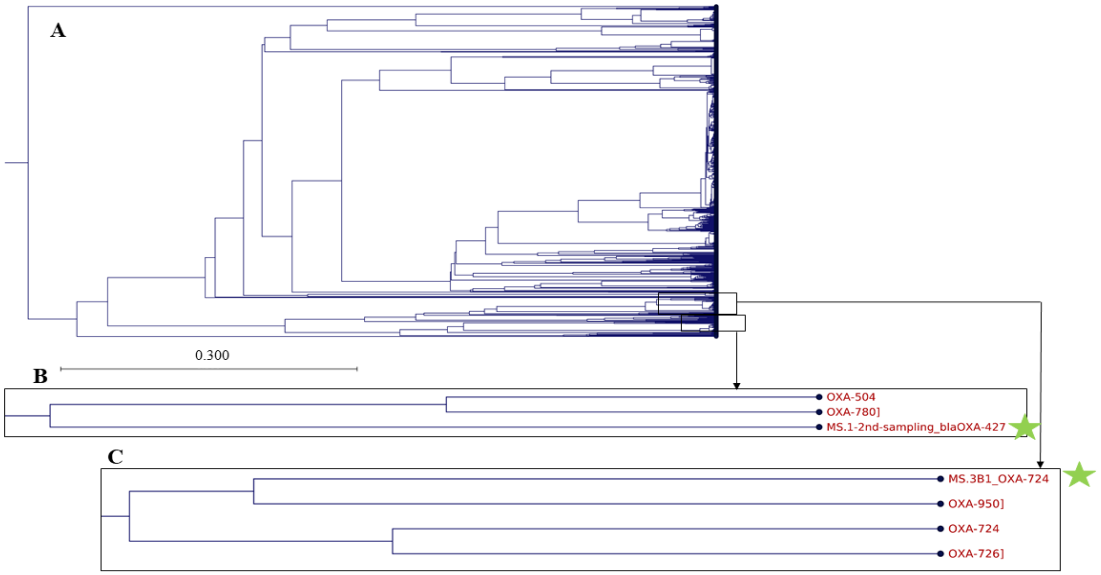

Figure A.8 – **A.** UPGMA phylogenetic tree constructed from Jukes-Cantor showing the relatedness of all alleles of *blaOXA* gene (952 sequences included). **B.** Detailed view of the cluster containing the gene identified in our study (MS.1-2nd-sampling_*blaOXA-427*– identified by a star) and the described allele *blaOXA-780*. Not at scale. **C.** Proximity between the gene identified (MS.3B1_*blaOXA-724*– identified by a star*)* and the described allele *blaOXA-950*. Not at scale.

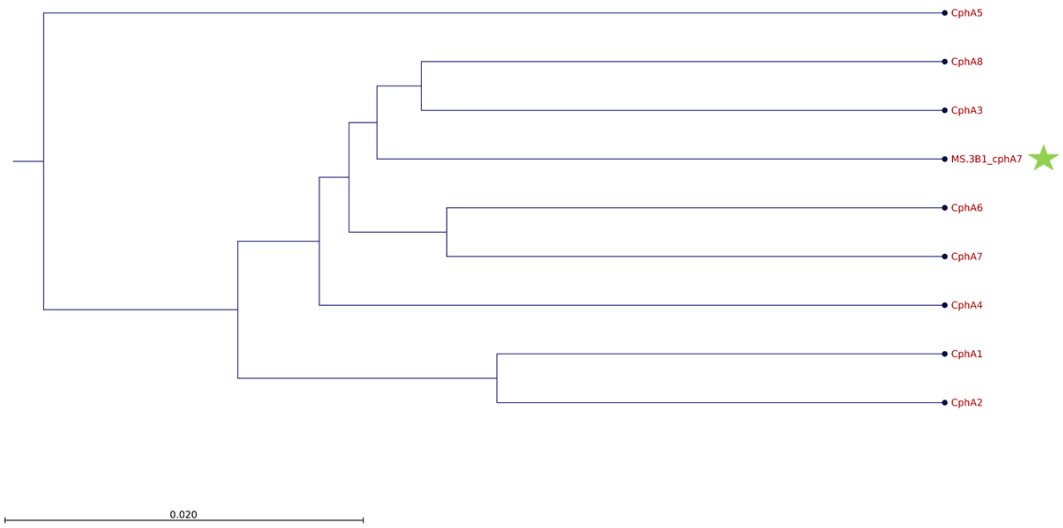

Figure A.9 – UPGMA phylogenetic tree constructed from Jukes-Cantor showing the relatedness of all alleles of *cphA* gene (eight sequences included) and similarity between the gene identified (MS.3B1_*cphA7*– identified by a star) and the described allele *cphA1*.

2021 MARIANA ARAÚJO

ENTEROBACTERALES FROM THE PORK PRODUCTION CHAIN IN PORTUGAL ARE KEY RESERVOIRS OF ANTIBIOTIC RESISTANCE AND VIRULENCE GENES