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Antibiotic Resistant Bacteria in Bovine and Rabbit Meat

Dissertação do 2º Ciclo de Estudos conducente ao Grau de Mestre em Controlo de Qualidade

Trabalho realizado sob a orientação da Professora Doutora Helena Maria Ferreira Neto de Sousa

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1 Abstract

Meat and meat products are one of the most consumed food products around the world. The increase in world population has led to a higher demand of food, thus causing the intensive farming of food animals. These animals are usually confined to tight spaces and are raised in high number to increase profits, augmenting the risk of disease development. In order to prevent and treat these inevitable infections, farmers administrate antibiotics to these animals in bulk (either healthy or sick). This practice leads to the selection of antibiotic resistant bacteria in the animal gut, which will then be excreted to the environment, where they can be disseminated to water and soil.

Antibiotic resistance is a major concern nowadays, due to an increase in the resistance phenotypes of bacteria being higher than the rate of antibiotic development.

In this work, samples of bovine and rabbit meat were analyzed in order to observe the presence of antibiotic resistant bacteria, with special interest in β-lactamase and carbapenemase producing bacteria, as well as non β-lactam resistance. Twenty-one samples of bovine meat with different packaging systems and 3 samples of rabbit meat, were processed, from which 2 isolates were positive for the presence of β-lactamases, 11 samples presented quinolone resistance and 4 isolates showed reduced susceptibility to carbapenems. Through phenotypical analysis of the resistance patterns, most of the isolates were considered multidrug resistant (MDR).

The fact that most of the isolates belonged to *Escherichia coli,* a fecal contamination indicator, points that contaminations with animal or human fecal matter occurred either during slaughtering, transportation or even at retail markets. These bacteria, as well as the other types encountered on this study, can cause infections to consumers with debilitated immune systems, which, with the multirresistant pattern observed, can be difficult to treat and/or promote colonization.

Ultimately, this work highlights the need for a stricter quality control regarding antibiotic resistant bacteria in these products.

Keywords: Meat, multirresistance, quality control

2 Resumo

Carne e produtos cárneos são dos produtos mais consumidos no mundo. O aumento da população mundial levou a uma maior necessidade de alimentos, levando a uma pecuária intensiva. Estes animais estão normalmente confinados a pequenos espaços e são criados em elevado número para aumentar o lucro, levando a um risco acrescido de desenvolverem doenças. De forma a prevenir e tratar estas infeções, os criadores administram antibióticos a estes animais, quer estejam, doentes ou não. Esta prática gera a seleção de bactérias resistentes aos antibióticos no trato gastrointestinal dos animais, que depois são excretadas para o ambiente, onde podem disseminar.

A resistência a antibióticos é uma enorme preocupação atualmente devido ao aumento de bactérias resistentes aos mais diversos antibióticos que é maior que o ritmo de desenvolvimento de antibióticos.

Neste trabalho, amostras de carne bovina e de coelho foram analisadas com o objetivo de observar a presença de bactérias resistentes, com especial interesse em bactérias produtoras de β-lactamases de espectro alargado e carbapenemases, bem como resistentes a antibióticos não β-lactâmicos. Vinte e uma amostras de carne bovina com diferentes processos de embalagem e 3 amostras de carne de coelho foram processadas, das quais dois isolados produtores de β-lactamases, 4 isolados com suscetibilidade reduzida a carbapenemos e 11 amostras com resistência a quinolonas foram encontrados. Através da análise dos padrões de resistência, a maioria destes isolados foram considerados multirresistentes (MDR).

O facto da maioria dos isolados pertencer à espécie *Escherichia coli,* um indicador de contaminação fecal, indica contaminação com matéria fecal animal ou humana no processo de abate ou durante a cadeia de transporte ou mesmo no estabelecimento comercial, com bactérias que poderão causar infeções aos consumidores e/ou colonização.

Em última análise, este trabalho esclarece a necessidade de um controlo de qualidade mais exigente relativamente à presença de bactérias resistentes aos antibióticos nestes produtos.

Palavras-chave: carne, multirresistência, controlo de qualidade

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4 Abbreviations and symbols

AK – Amikacin

AMC – Amoxicillin and Clavulanic Acid

AMP - Ampicillin

ATCC- American Type Culture Collection

ATM – Aztreonam

C – Chloramphenicol

CAZ – Ceftazidime

CIM – Carbapenem Inactivation Method

CIP – Ciprofloxacin

CL - Cloxacillin

CLED – Cystine Lactose Electrolyte Deficient

CLSI - Clinical and Laboratory Standards Institute

CN – Gentamicin

CPT – Ceftaroline

CT – Colistin

CTL – Cefotaxime and Clavulanic Acid

CTX – Cefotaxime

CXM – Cefuroxime

DOP – Protected Origin Denomination

DOR – Doripenem

EDTA – Ethylenediamine tetra acetic acid

EFT – Ceftiofur

ENR - Enrofloxacin

ESBL – Extended-Spectrum β-lactamase

ETP – Ertapenem

EUCAST – European Committee for Antibiotic Susceptibility Testing

F – Nitrofurantoin

FEP – Cefepime

FOT – Fosfomycin

FOX – Cefoxitin

g – grams

h – hours

IMP – Imipenem

I – Intermediate

KPC – *Klebsiella pneumoniae* carbapenemase

MAP – Modified Atmosphere Packaging

MBL – metallo-β-lactamase
MDR – Multidrug Resistant
mg – milligrams
min - minutes
ml – milliliter
mm - millimeters
MRP – Meropenem
MR+BO – Meropenem + Phenyl Boronic Acid
MR+CL – Meropenem + Cloxacilin
MR+DP – Meropenem + Dipicolinic Acid
NA – Nalidixic Acid
NET – Netilmicin
PDR – Pandrug resistant
PRL – Piperacillin
R – Resistant
S – Sensitive
TAE – Tris-Acetic Acid-EDTA
TE – Tetracycline
TEs – Extraction Buffer
TGC – Tigecycline
TO - Temocillin
TOB – Tobramycin
TSB – Tryptic Soy Broth
ug – micrograms
UP – ultra pure
µl – microliter
V - volts
XDR - Extensively drug resistant

5 Introduction

1 Antibiotic use in agriculture

Since the discovery of the first β-lactam antibiotic, penicillin, by Alexander Fleming, several other antibiotics have been developed and its use grew exponentially (1). The misuse and abuse of antibiotics leads to selective pressure, selecting resistant bacteria, either in human or in animals. These are used not only to treat infectious diseases in human population but also in the treatment and prevention of diseases and, in certain countries, these are used as growth promoters in animals (2). In European Union, the use of antibiotic as growth promoters was banned since 2006 (3). However, countries like the United States (US) and China still use them. It is estimated that 80% of the antibiotics used in the US are for animal use, mainly as growth promoters and disease prevention (1). There is an association between the use of sub-therapeutic doses of antibiotics and the selection of resistant bacteria (4).

Although the use of antibiotics as growth promoters is not possible in Europe, these drugs ca be administrated in cases of disease and prevention. Regarding disease treatment, antibiotics are usually administrated to the entire herd, even when there is only one animal showing signs of infection. This occurs since it is logistically more advantageous and efficient to give antibiotics to the entire group of animals rather than looking for signs of infection in each of them. And, just like in growth promoters, this kind of practice leads to the selection of resistant microorganisms in the intestinal flora of the animals (4). Relatively to the use of these drugs as prophylaxis, which consists in the administration of antibiotics to healthy animals to diminish the risk of contracting diseases, the borderline between prevention and uncontrolled administration of these drugs is not well defined (5), leading to concerns on whether the farmers are just giving antibiotics as growth promoters alleging that it is for prophylaxis. Once again, it is evident that selection of resistant strains will occur (4).

The fact that the types of antibiotics used both in animals and in humans are the same or similar, poses great risk for maintaining the value of these compounds to treat human infections (5).

1.1 Antibiotic Resistance – The One Health perspective

In the One Health perspective, human health relates to the health of animals and the environment. Antibiotic resistance is connected to all of these three (6). As perceived in Figure 1, there are innumerous ways that antibiotic use is linked to antibiotic resistance. For example, in the US, antibiotics are administrated in the egg and in broilers through feed and water in order to promote growth, prevent or treat infections. The daily low-dose administration of antibiotics (for growth promotion) leads to the selection of antibiotic

resistant bacteria that are passed through different generations of chicken. Moreover, these animal's excrements contain not only partially degraded antibiotics but also resistant bacteria from the gut, which can contaminate the soil, water and plants (if the excrements are used as manure) that are consumed by people. Animals may also contaminate the workers of these facilities and their meat may become contaminated by gut bacteria through processing in butcheries. If water becomes contaminated, fish can be colonized with these types of bacteria. Ultimately, people will consume both water, plants, fish and meat that, if

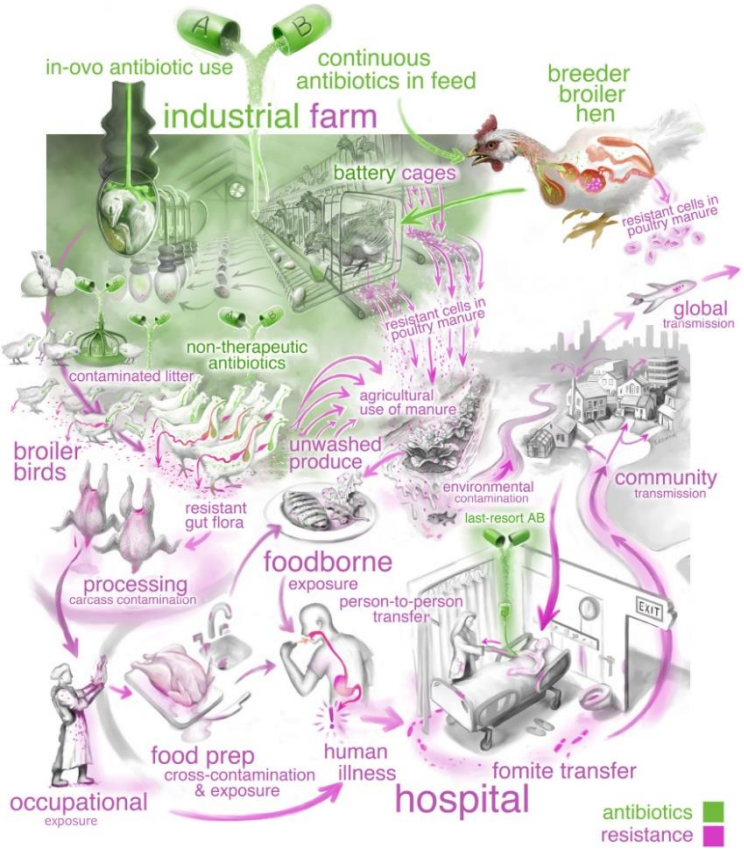

Figure 1 - Diverse pathways of dissemination of antibiotic resistance in a One Health perspective (adapted from Koch et al., 2017)

containing antibiotic resistant microorganisms, can cause antibiotic resistant infections in humans. These can persist and be passed through the community, leading to a broader dissemination (7).

1.2 Factors influencing antibiotic resistance in bovine and rabbit meat

At retail stores, meat is usually found in several forms, either with a packaging or in a butcher's desk ready to be carved and packed for consumers to purchase. This is the last step separating consumers' homes from farms. This meat comes from several production systems where animals are intensively grown into appropriate weight for consumption (8).

Between the production systems and retail stores, several steps exist that can contribute to the contamination of meat with antibiotic resistant bacteria. These steps will be divided into the following: farming, slaughtering, cutting, packaging and transportation (9).

As mentioned in section 1.1, regarding antibiotic use in agriculture, antibiotic resistant bacteria can appear in farming through the intake of veterinary use antibiotics, either for the treatment of diseases or as prophylaxis (4). Nevertheless, there are other ways that these animals may become colonized while growing, such as drinking contaminated water or through the ingestion of contaminated grass, in the particular case of free-range meat animals (10). Both water and grass can be contaminated by several routes as depicted in Figure 1.

The slaughtering and cutting processes can also influence colonization, either through poor cleaning of cutting surfaces and machines, which can create biofilms that can persist and contaminate the meat (11). Moreover, workers at these facilities can either contaminate the meat through improper hygiene or be colonized while cutting the meat.

Packaging and transportation are the last steps before meat arrival to retail stores. Lack of packaging and poor storage (uncontrolled temperature and ventilation) can lead to a proliferation of pathogenic, spoilage and of antibiotic resistant bacteria. These bacteria, now in higher densities, will be able to colonize and infect consumers, retail workers and others.

One way to reduce the risk of infections and subsequently use of antibiotics for treatment is by implementing favorable conditions such as vaccination, limited co-mingling, controlling temperature and ventilation systems and through the implementation of quality control programs. However, these measures require substantial financial resources as well as training of workers and thus are not carried out most of the times (12).

1.3 Mechanisms of action of antibiotics

Antibiotics can act through several mechanisms such as inhibition of cell wall synthesis, inhibition of DNA and RNA synthesis, inhibition of protein synthesis, affecting membrane structure and inhibition of metabolic pathways (13,14). The different mechanisms depend on the class of antibiotics used (Figure 2). For instance, beta-lactams, such as penicillins and carbapenems, act on inhibiting cell wall synthesis by binding to PBP (Penicillin-Binding Proteins). These are proteins that are essential for the synthesis of peptidoglycan and their binding to beta-lactams lead to bacterial lysis (15). Aminoglycosides, tetracyclines, chloramphenicol and others inhibit protein synthesis by acting on ribosomal subunits 30S and 70S, affecting translation. Quinolones inhibit nucleic acid synthesis by targeting DNA gyrase subunit A in Gram-negative or topoisomerase IV in

Gram-positive bacteria, important enzymes in replication and transcription. Finally, sulfonamides and trimethoprim interact with distinct stages of folic acid metabolic pathway, ultimately interfering with nucleic acid synthesis (16).

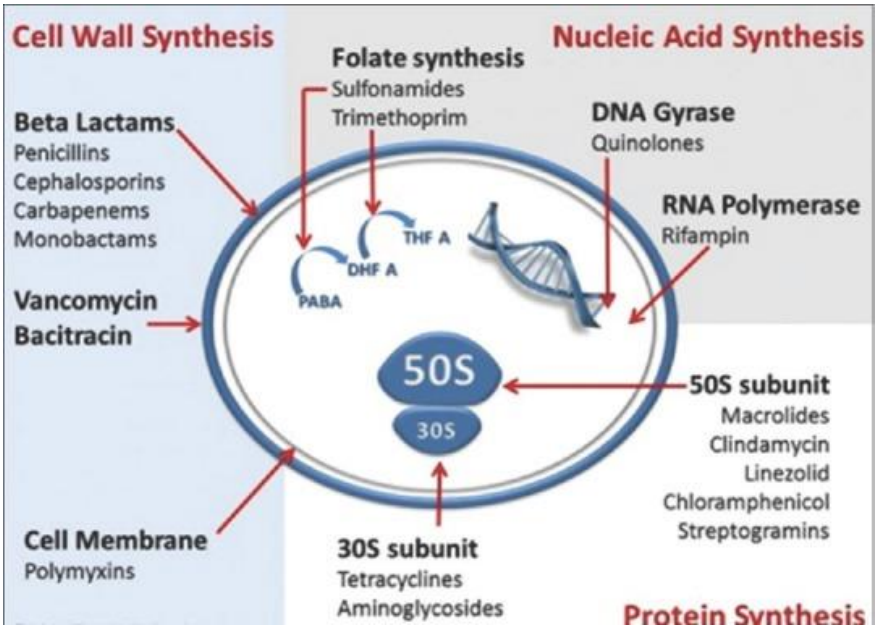

Figure 2- Antibiotics mechanism of action (adapted from Kapoor et al., 2017)

1.4 Mechanisms of resistance to antibiotics

Although antibiotics present diverse manners of targeting bacteria, these have evolved in order to defend themselves against these. These mechanisms can be by changing their surface permeability, through efflux pumps, change in metabolic pathways, enzymatic inactivation or modification of target site (5,14).

Permeability changes might be one of the most common resistance forms, happening either naturally (some bacteria have an intrinsic resistance mechanism such as this) or through acquired resistance genes. The changes in the outer membrane permeability are caused by the presence of efflux proteins or through porin loss (14), that can lead to the entrance of a smaller amount of antibiotic, decreasing its concentration thus turning the drug ineffective (5).

Efflux pumps, as above described can impact on the concentration of antibiotics. This is achieved due to their action in removing drugs and other toxic compounds from the cell interior, diminishing their levels and thus aiding to the cell's survival. These transporters can be effective against several or just one type of these compounds (13).

Some compounds, such as sulfonamides and trimethoprim, interfere with metabolic pathways. Facing this, bacteria can evolve and create a novel pathway to surpass the

antibiotic action. These changes in metabolic pathways can pass through the production of altered metabolites, affecting the affinity with the antibiotics targets (5).

Some microorganisms can change their target site so that the antibiotic cannot recognize and bind to them. An example of this is the modification of PBP's structure, which does not allow the binding of antibiotics like penicillin. Another example can be of a non β-lactam resistance, such as the mutation of DNA gyrase, one of the targets of fluoroquinolones (5).

Enzymatic inactivation usually occurs when bacteria produce one or more enzymes that act on the antibiotic by modifying or degrading their structure, thus inactivating them. Normally the enzymes are specific to a type or class of compounds. This is the case of β-lactamases, enzymes that degrade the β-lactam ring of β-lactam antibiotics (13,14). The production of these enzymes has led to concerns due to their effectiveness in hydrolyzing antibiotics and the ease that they are acquired by several bacteria. Given the importance of this resistance mechanism, a more detailed description about the different types of β-lactamases is contemplated in section 1.5 (β-lactamases types and classification).

1.5 Methods of antibiotic resistance dissemination

Antibiotics can be ineffective through a variety of ways as mentioned above. However, these resistance mechanisms would have minor impact if it were not for the rapid manner that the resistance can spread through other microorganisms. Antibiotic resistance can be intrinsic (degradation by action of proteins naturally present in the bacteria or inexistence of the antibiotic target site) or extrinsic (acquired through different methods). The mechanisms associated with the latter are described below.

1.5.1 Selective pressure

In selective pressure (Figure 3), a stress factor, such as the use of antibiotics to eliminate a type of bacteria, will act on the entire population, eliminating susceptible bacteria. However, some will randomly survive. These bacteria will be able to quickly multiply since there is not competition for nutrients, therefore producing a resistant progeny (5).

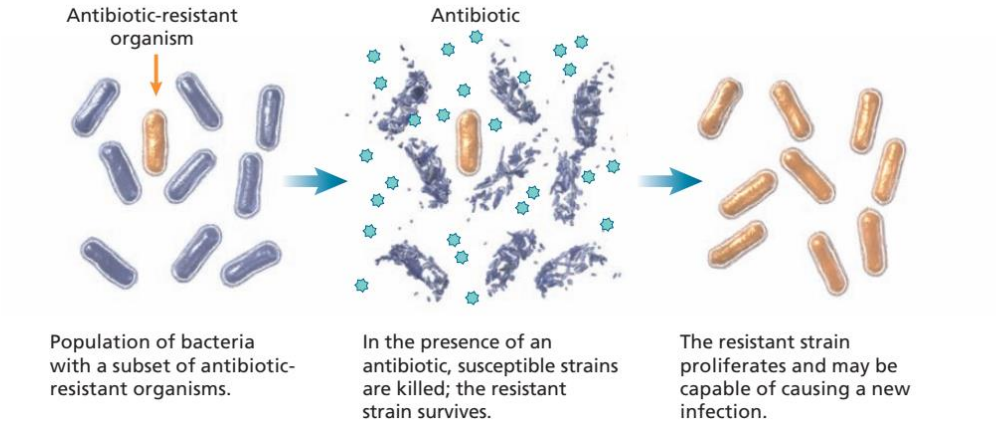

Figure 3 - Effect of antibiotic selective pressure on bacteria (adapted from Mulvey et al., 2009)

1.5.2 Vertical gene transfer

In vertical gene transfer (Figure 4), a spontaneous mutation in the bacterial chromosome occurs leading to the alteration of the antibiotic target, that is transmitted to the next generation. Although these mutations are rare, the high rate of replication of bacteria increases the probability of appearing resistant bacteria (17).

1.5.3 Horizontal gene transfer

Horizontal transfer (Figure 4) can occur either through the same species or even with different niches (Figure 3). This type of transfer can be achieved through conjugation, transduction and transformation and is only possible due to the presence of mobile genetic elements like transposons, integrons, plasmids and gene cassettes (17).

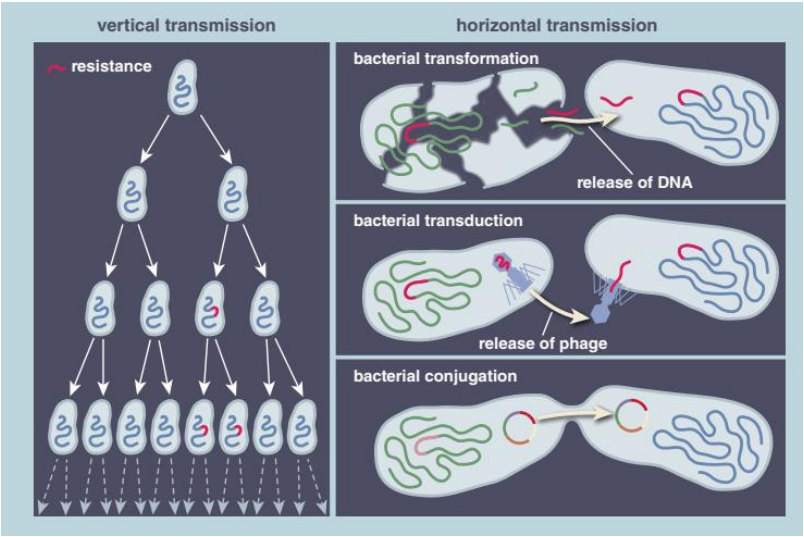

Figure 4 - Vertical and horizontal transfer of antibiotic resistant genes (adapted from Dantas and Sommer, 2014)

Conjugation

Conjugation is the most common type of horizontal gene transfer, in which there is a switch of DNA in extrachromosomal plasmids or transposons, through a connection with

a receptor cell (this connection is called “pilus”) that allows the passage of DNA fragments containing resistance genes, allowing the multiplication of resistance throughout the following generations (17).

Transduction

In transduction, the resistance genes transfer from a bacterial cell to another usually occurs through bacteriophages (virus) that infect bacteria. This mechanism happens when the particles that form through replication inside a receptor cell incorporate a DNA segment containing resistance genes that are freed into nature, after bacterial lysis, and can infect other bacteria, that possibly can incorporate the resistance genes into their genome (17).

Transformation

Transformation is the ability of bacteria to incorporate DNA segments found in the environment from bacteria that suffered lysis into their genome. These fragments can contain resistance genes that will be acquired by either pathogenic or non-pathogenic bacteria, that can then pass this resistance to other cells (13).

1.6 β-lactamases types and classification

As previously mentioned in section 1.3, β-lactamases hydrolyse the β-lactam ring, making β-lactams ineffective. There are a variety of different β-lactamases that depending on their homology between amino acids and nucleotide sequences and functional characteristics, can be classified into separate classes according to Bush-Jacoby-Medeiros. This classification is based on previous schemes by Ambler and it englobes β-lactamases into 4 functional groups and into 6 sub-groups depending on substrate affinity, inhibitors and molecular structure (18,19):

a) **Class C – Group 1** (Cephalosporinases – AmpC class)

Comprising the β-lactamases that can inhibit the action of most β-lactams with exception of carbapenems and that are poorly inhibited by clavulanic acid. These β-lactamases can be chromosomic, or plasmid mediated.

b) **Class A and D – Group 2** (Penicillinase and Oxacillinase)

In this group we find all the penicillinases, cephalosporinases and carbapenemases, that are inhibited by clavulanic acid, including ESBLs.

c) Class B – Group 3 (Metallo-β-lactamases)

Metallo-β-lactamases are carbapenemases that are capable to hydrolyse beta-lactams, with exception of monobactams, and are inhibited by EDTA.

d) Group 4

This group comprises all penicillinases that do not match with the previous classifications.

1.7 Extended Spectrum β-Lactamases (ESBLs) – definition

ESBLs are a branch of enzymes that can hydrolyze most penicillins, cephalosporins and monobactams, but cannot act on carbapenems nor cephamycin and are usually inhibited by β-lactam inhibitors, such as clavulanic acid, sulbactam and tazobactam(19,20). There is an enormous diversity in terms of β-lactamases coded for different *bla* (β-lactamase) genes. Their differences rely on the amino acid structure. Nonetheless, these can be grouped into families according to genetic similarities. Bacteria can possess more than one *bla* gene, broadening their resistance spectrum (20). These β-lactamases belong to group 2 of Bush-Jacoby-Medeiros classification.

1.7.1 TEM type β-lactamases (Temoniera):

Since the first β-lactamase was discovered, more than 200 derivatives have been found with small differences in amino acids and, until recently, it was considered the most common type of these enzymes in *Enterobacteriaceae* (19).

TEM-1 is able to hydrolyze penicillin and first generation cephalosporins, but it was not until the end of the 1980's that the first ESBL phenotype was exhibited by a TEM derivative, TEM-3, conferring resistance to even third generation cephalosporins, due to mutations in blaTEM-1 gene (22).

The first derivative of TEM-1, TEM-2, with a difference in a single amino acid caused a shift in the isoelectric point although it did not change the substrate profile (19).

1.7.2 SHV (sulphydryl variable)

SHV enzymes seem to have derived from *Klebsiella s*pp.. The first beta lactamase of this family, SHV-1 is universally found in *Klebsiella pneumoniae* and appears to have evolved from a chromosomal gene to be incorporated in a plasmid, spreading itself to other *Enterobacteriaceae*.

The SHV enzymes possess similarities to TEM beta lactamases and just like them, their derivatives are a result in minor mutations in amino acids. SHV-1 is responsible for

20% plasmid mediated ampicillin resistance in *Klebsiella pneumoniae*. These enzymes confer high resistance to ceftazidime but not to cefotaxime and cefazolin (5).

1.7.3 OXA (oxacillinases)

OXA β-lactamases were initially considered to be rare and always plasmid mediated. These enzymes confer resistance to penicillin and cephalosporins, although some can even confer resistance to carbapenems, like the OXA-48 enzyme. They are also characterized by their ability to hydrolyze oxacillin and for their poor inhibition by clavulanic acid (23). These β-lactamases have been commonly found in *Enterobacteriaceae,* in *Pseudomonas aureginosa* and in *Acinetobacter* spp.

1.7.4 CTX-M (cefotaximase)

The first CTX-M enzymes were more effective against cefotaxime and ceftriaxone than ceftazidime, hence its name, Cefotaximase

These β-lactamases are nowadays, the most common type in the world, being mediated by plasmids, allowing easy transfer to other bacteria. Opposing to TEM and SHV, which possess some similarities, these enzymes are quite different from them and from each other, forming an heterogeneous and complex family that only due to small amino acid sequence differences can be divided into 6 phylogenetic sub-groups: CTX-M group 1, CTX-M group 2, CTX-M group 8, CTX-M group9, CTX-M group 25 and more recently KLUC group (24,25). CTX-M determinants were found in chromosomal genes of *Kluyvera* spp. The prevalence of each one of these groups varies according to geographic region (26).

Some of the most important enzymes of this group are CTX-M-15 and CTX-M-14, due to their widespread in the globe. CTX-M-15- producing *E.coli* and *Klebsiella pneumoniae* are the predominant encountered species. The first is easily disseminated through the community and the second is related to nosocomial problems (27).

1.8 Carbapenemases

Carbapenemases are enzymes with the ability to hydrolyze most β-lactams, including carbapenems, conferring its host resistance to these antibiotics. These enzymes are included in Bush-Jacoby-Medeiros groups 2 and 3 or Ambler's class A, B and D, being most of them plasmid-mediated. These are mainly found in *Pseudomonas aureginosa, Acinetobacter baumannii* and in *Enterobacteriaceae* (28).

1.8.1 Class A carbapenemases

This class of β-lactamases hydrolyzes carbapenems and is inhibited, although partially, by clavulanic acid. It comprises chromosomally encoded enzymes, such as SME (*Serratia marcescens* enzyme), NmcA (not metalloenzyme carbapenemase A), SFC-1 (*Serratia fonticola* carbapenemase-1), IMI-1 (imipenem-hydrolyzing β-lactamase), and plasmid-mediated β-lactamases like KPC (*Klebsiella pneumoniae* carbapenemase), IMI (imipenemase) and GES enzymes (Guiana extended spectrum) (29). From all these carbapenemases, KPC is considered the most prevalent due to the significant number of outbreaks communicated worldwide (29). This gene was first identified in 1996 in *Klebsiella pneumoniae*. There are a variety of variants of these gene, with little modifications but with the same spectrum of activity (28). KPC producers have been found to be multidrug resistant to β-lactams, limiting the treatment options of KPC producers infections and allowing their dissemination throughout the globe (29).

1.8.2 Class B carbapenemases

Class B carbapenemases are denominated metallo-β-lactamases, which are characterized by their ability to hydrolyze carbapenems and inhibition by EDTA (ethylenediaminetetraacetic acid), a chelator of zinc and other divalent cations (29). This inhibition occurs due to their mechanism of action being through the interaction of zinc and the β-lactams with the active site of the enzyme. This class of β-lactamases includes NDM (New Delhi metallo- β-lactamase), IMP (imipenem resistant Pseudomonas), VIM (verona integron-encoded metallo- β-lactamase), SIM (Seoul imipenemase) and GIM (German imipenemase) (29). This carbapenemase confers resistance to all β-lactams, with exception of aztreonam, and the gene that codifies for this resistance is typically confined to conjugative plasmids (28).

1.8.3 Class D carbapenemases

This type of enzymes is poorly inhibited either by EDTA or other β-lactam inhibitors and comprise serine β-lactamases of the OXA type. These carabapenemases are found in *Acinetobacter* spp., such as *Acinetobacter baumanni* and *Pseudomonas* spp., like *Pseudomonas aureginosa*. This group of enzymes poses major concern due to their rapid mutation ability and therefore their extension in the activity spectrum (29). The main carbapenemase of this group is OXA-48, first identified in a carbapenem resistant *Klebsiella pneumoniae* isolated in Turkey in 2001 (30).

1.9 Non β-lactam antibiotic resistance

1.9.1 Quinolone and fluoroquinolone resistance

Fluoroquinolones derive from quinolones, which are synthetic antibiotics. The first quinolone described was nalidixic acid, a byproduct of chloroquine synthesis, in the 1960's. After that, several other first-generation quinolones were synthesized. Second-generation quinolones, the fluoroquinolones, such as ciprofloxacin, levofloxacin, norfloxacin, were described in 1980's, differed from the quinolones due to the introduction of a fluorine in the C6 position, hence the designated name (31). Norfloxacin was the first broad spectrum fluoroquinolone produced, but due to their poor penetration, ciprofloxacin was elected the quinolone of choice (32).

The first application of these compounds was to treat uncomplicated urinary tract infections but with the discovery of the second generation quinolones, its use expanded to numerous diseases such as sexually transmitted, skin infections, nosocomial pneumonia and even tuberculosis (31). These antibiotics act on the DNA synthesis enzymes like topoisomerase IV and DNA gyrase (type II topoisomerases).

Relatively to the mechanisms of resistance (Figure 4) that bacteria can create towards fluoroquinolones, these can be divided in four: Upregulation of the efflux pumps which decreases the accumulation of the antibiotic; Decreased expression of outer membrane proteins; Chromosomal mutation of gyrA, gyr B and parC; PMQR genes (Plasmid-Mediated Quinolone Resistance).

As previously mentioned, quinolones target two major bacterial enzymes, DNA gyrase and topoisomerase IV. Both enzymes are heterotetramers and act by causing a dsDNA break, passing another strand of DNA through it and sealing it. Quinolones act by blocking the resealing of DNA, which will inhibit enzyme activity which will ultimately inhibit DNA replication (32). Gyrase is the only topoisomerase II that can add negative supercoils to DNA. Topoisomerase IV can remove knots that accumulate in chromosomes (31). DNA gyrase and topoisomerase IV possess 2 gyrA, 2 gyrB and 2 parC and 2 parE subunits, respectively. Mutations in aminoacids in the subunits of these enzymes can lead to quinolone resistance. The most common mutations occur in gyrA in the case of gyrase and in parC in the case of topoisomerase IV, although it can also occur but to a lesser extent in both gyrB and parE. Nevertheless, the action of the quinolone is dependent on the sensitivity of the enzymes to the compound. Normally, gyrase is the most sensitive enzyme in Gram negative bacteria, while topoisomerase IV is more sensitive in Gram positive bacteria (32).

Plasmid-mediated quinolone resistance genes were described in several species of Enterobacteriaceae, in *Escherichia coli, Klebsiella pneumoniae* and *Enterobacter cloacae*.

Regarding the resistance mediated by plasmids, qnrA was the first protein to be discovered and with this, qnrS, qnrB, qnrC and qnrD were found. These genes differ from each other by around 35%. These are normally found in plasmids of multirresistant microorganisms, such as ESBL producers. Qnr genes seem to be rare in non-fermenting organisms. They have been found also in Gram positive bacteria, although in these bacteria they are chromosomal genes and not plasmid mediated. These genes have been reported not only in hospital isolates but also in wild and domestic animals (33). Qnr proteins act by decreasing the binding between DNA and DNA gyrase or topoisomerase IV. With this, a limited number of enzymes on the chromosome is available for quinolones to target. Moreover, Qnr proteins can bind to these enzymes, preventing quinolone action (31).

The Aac(6')-Ib-cr (aminoglycoside acetyltransferase) enzyme modifies (acetylates) quinolones containing an amino nitrogen on piperazinyl ring (like ciprofloxacin and norfloxacin), decreasing drug activity (31). Usually found in a cassette in an integron of a multirresistant plasmid, which can contain other PMQR genes and is typically associated with ESBL producers. In *Enterobacteriaceae* this enzyme is more common in *E.coli* and it is encountered in higher prevalence than Qnr genes in some samples (32).

Another group of PMQR genes are the plasmid-mediated efflux pumps, QepA 1 and 2 and OqxAB. These efflux pumps act by decreasing the entrance of quinolones such as ciprofloxacin and norfloxacin. QepA1 and QepA2 are usually found in human bacterial infections, whereas OqxAB is typically found in animal bacterial infections (31).

In order to reach their targets, quinolones must cross the membrane of bacteria. In Gram-negative bacteria, these have to pass through an extra membrane. Gram-negative bacteria can then downregulate porin expression in order to decrease the influx of quinolones into the cell. Moreover, both Gram-negative and Gram-positive bacteria can upregulate chromosome-encoded efflux pumps. This mechanism is due to mutations in regulatory proteins. Both of these mechanisms lead to low-level quinolone resistance (31,34).

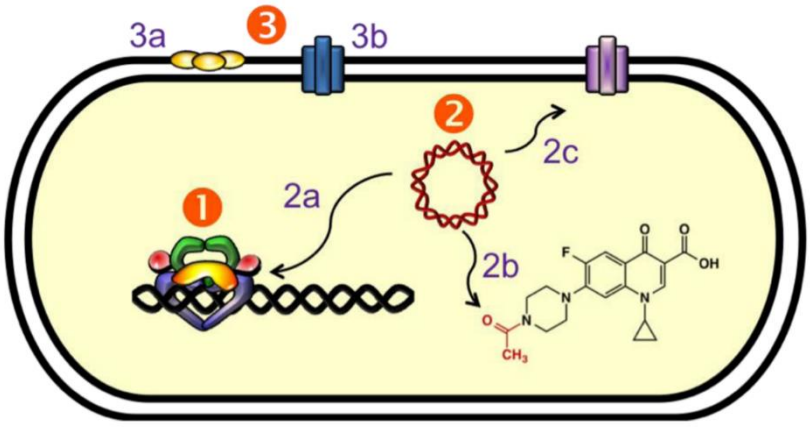

Figure 5- Methods of antibiotic resistance dissemination 1) Target mediated resistance with mutations to chromosomal DNA gyrase and topoisomerase IV. 2) Plasmid-mediated resistance. 2a) Action of qnr genes; 2b) aac(6')-Ib-cr action; 2c) Plasmid-mediated efflux pumps. 3) Chromosomal resistance. 3a) Downregulation of porins. 3b) Overexpression of efflux pumps. (adapted from Aldred et al., 2014)

1.10 *Enterobacteriaceae*

Enterobacteriaceae is a family of Gram-negative bacteria with members like *Escherichia coli, Klebsiella* spp., *Salmonella* spp., *Shigella* spp. and *Yersinia pestis*. These bacteria are non-spore fermenting bacillus, facultative anaerobes, glucose and other sugars fermentors and they reduce nitrate to nitrite. Most members of this family are catalase positive but oxidase negative (35). Depending on the species these can be motile or non-motile, through the presence of peripheric flagella (36).

Some of these bacteria are commensal, inhabiting the gastrointestinal tract of humans and animals, but they can also cause different types of infections, from respiratory tract, urinary, skin, among others (35). This is the case of extra-intestinal bacteria, which can possess virulence factors. Moreover, commensal bacteria can become virulent through the acquisition of such virulence factors, being able to colonize the gastrointestinal tract and cause infections (36).

In order to treat infections caused by this family, β-lactams and non β-lactams, like fluoroquinolones, are prescribed. The bacteria that cause more concern and are more prevalent in hospitals and such are *Escherichia coli* and *Klebsiella pneumoniae*.

Focusing on *Escherichia coli,* this bacterium is naturally present in the intestines of humans and animals. Although most of the strains existent in the intestine are commensal, some pathogenic and opportunistic bacteria can also be encountered, which can lead to infections. Infections by *E.coli* are usually acquired by the consumption of contaminated food and water, as well as by direct contact with contaminated animals (37).

1.11 Antibiotic resistance in bovine and rabbit meat

Antibiotic resistance is indeed a growing concern nowadays, since it limits the possibility of treatment of bacterial infections both in humans as in animals. This resistance connects both animal, human and environmental health, because it can be easily disseminated through all these channels and reach each one of them. Regarding antibiotic resistance in animals, with special focus on bovine and rabbit meat, several reports have been found that demonstrate the presence of resistant bacteria in animal feces and most importantly in animal meat from retail. For instance, Randall et al., in 2017, processed fruit, vegetables and meat samples from retail markets of 5 different regions in order to evaluate the presence of ESBL-producing and carbapenem-resistant *E.coli*. From the 159 samples of beef processed, 1.9% tested positive for ESBL-producing *Escherichia coli*, presenting TEM-1b and TEM-52 genes (38). Another study, conducted by Chen et al., in 2017, studied 208 samples of beef carcasses from 2 different slaughterhouses, regarding ESBL-producing *Escherichia coli*. From the samples analyzed, 45 multidrug isolates were found, from which 42.2% were ESBL producers. These isolates contained CTX-M genes belonging to groups 1 and 9 (39).

In 2017, Nisar et al., studied the antibiotic susceptibility patterns of *Camplylobacter* spp. isolated from retail meat samples from Pakistan. From the 200 beef samples analyzed, 31 (15.5%) were positive for *Campylobacter* spp. . Antibiotic susceptibility tests using 9 antibiotics commonly used in veterinary were performed. Results showed that from the 31 *Campylobacter* spp. isolates found, 26 (83.9%) were resistant to ciprofloxacin, 24 (77.4%) were resistant to enrofloxacin and 23 (74.2%) showed resistance to colistin. The authors believe this could be due to an uncontrolled use of antibiotics in animals, posing a threat to the human health (40). Related with this report, a study by Premarathne et al., searched for tetracycline resistance and prevalence in *Campylobacter jejuni* and *Campylobacter coli* from beef meat acquired in wet and hypermarkets as well as in cattle feces. The prevalence of *Campylobacter* spp. in the 340 samples analyzed was of 17.4%. From these isolates, antibiotic susceptibility tests were performed using antibiotics used in veterinary and in hospitals. Around seventy-nine percent of the *Campylobacter* spp. isolates were resistant to tetracycline, 69.2% were resistant to ampicillin, and only 7.9% presented resistance to chloramphenicol, highlighting the need for a better surveillance and regulation procedures for the control of *Campylobacter* spp. contamination (41).

Nguyen et al., reported the prevalence of ESBL and AmpC-producing *Escherichia coli* from food samples in Vietnam. In this study, 330 samples of chicken, pork, beef and fish/shrimp meat were acquired from slaughterhouses, wholesale markets and supermarkets. A hundred and fifty samples (45%) were positive for ESBL and AmpC-producing *E.coli*.. Three hundred and forty-two isolates were found within the 150 samples,

of which 38 were from beef. Molecular characterization of the beef isolates showed that 31.6% harbored the CTX-M-9 gene, 26.3% contained the CTX-M-1, 3.4% the SHV-12 and 36.8% the CIT gene, the former being an indicator of plasmid-mediated AmpC, demonstrating the potential of these food products as reservoirs of antibiotic resistant bacteria (42).

Relatively to antibiotic resistance in rabbit meat, as far as its aware there are no reports about antibiotic resistant bacteria from retail meat samples. However, there are a few reports regarding this topic through analysis of fecal samples.

Silva et al., in 2010, studied 77 wild European rabbit fecal samples in order to evaluate antibiotic resistant bacteria. In 57.1% of these samples, *Escherichia coli* isolates were detected. Enterococci were also detected in 83.1% of samples (43).

In 2018, Freitas-Silva et al., described the first occurrence of mcr-1 producing *Escherichia coli* from meat rabbits in Portugal. These were detected in the 3 multirresistant isolates retrieved from intestinal content of necropsied rabbits from 2 intensive production systems (44). Besides this study, there was only one other work, from Italy, that found mcr-1 *Escherichia coli* isolates in rabbits (45).

1.12 Objectives

The goal of this exploratory work was to analyze the presence of antibiotic resistant bacteria in bovine meat, with special interest in ESBL-producing, carbapenemase producing bacteria, as well as non β-lactam antibiotic resistance. With this study, a wider knowledge on the spread of these resistance phenotypes and how this can impact human health is meant to be achieved. For this, bacteria were selected in antibiotic supplemented culture media and their resistance phenotype was studied. Moreover, molecular characterization was performed to evaluate the presence of genes that encoded for β-lactamases, carbapenemases and genes that encoded for non β-lactam antibiotic resistance. Finally, a more detailed molecular characterization was carried out for the *Escherichia coli* isolates resistant to quinolones, regarding genes that encoded for colistin resistance, virulence factors and phylogenetic groups.

2 Materials and Methods

2.1 Samples

2.1.1 Processing of meat samples

Bovine meat samples (n = 21) and rabbit samples (n = 3) were purchased from retail and local butcheries. The samples were from different cuts, origins and different packaging systems, including unpackaged products. The description of each sample is located on Table 1. Briefly, in aseptic conditions, 4g of meat were homogenized in 40ml of TSB for 2-3 min and posteriorly 100 µl were inoculated in MacConkey agar media with and without Ampicillin (10µg/ml), Cefotaxime (2µg/ml), Ciprofloxacin (2µg/ml) and Meropenem (2µg/ml). Plates were incubated at 37ºC for 18-24h. This inoculation was performed before and after enrichment of the meat with the broth at 37ºC for 18h-24h. Only resistant strains should survive in antibiotic supplemented mediums. In order to control if the antibiotic supplemented medium were working, an *E.coli* ATCC 25922 (ATCC – American Type Culture Collection) strain that is sensitive to antibiotics was also tested in all MacConkey plates.

MacConkey agar was used for this study since it is a selective medium for Gram negative bacteria due to the presence of biliary salts, limiting the growth of Gram-positive colonies. It is also a differential medium, since it contains lactose in its composition. If bacteria ferment lactose, the agar becomes acid and pink and so do the colonies that appear on the plate. Bacteria that do not ferment lactose appear in an amber color. This medium also contains crystal-violet as a way of selection of bacteria. It is typically used to study *Enterobacteriaceae*.

Sample	Product	Type of packaging	Origin	Date	Time
A1B	veal chuck steak	Unpackaged	Spain	28/10/18	19h00
A2B	Steer Azores Burger	Vacuum packaging	Portugal	5/11/18	18h00
A3B	Minced Bovine meat	Unpackaged	Spain	6/11/18	22h00
A4B	Bovine Burger	Modified Atmosphere Packaging (MAP)	Portugal	13/11/18	20h30
A5B	Short Loin Steer meat	MAP	Portugal	21/11/18	17h30

Sample	Product	Type of packaging	Origin	Date of	Time
A6B	Rib Bovine meat, matured	Vacuum packaging	Ireland	25/11/18	18h00
A7B	Minced bovine meat	Unpackaged	Portugal	28/11/18	8h00
A8B	Bovine sirloin Steak	Unpackaged	Portugal	28/11/18	8h00
A9B	Chuck steak	Unpackaged	Portugal	28/11/18	8h00
A10B	Minced bovine meat	Unpackaged	Portugal	3/12/18	19h00
A11B	Bovine sirloin meat	Unpackaged	Portugal	3/12/18	19h00
A12B	Bovine Round Steak	MAP	Portugal	4/12/18	8h30
A13B	Bovine Burger	MAP	Portugal	4/12/18	8h30
A14B	Brisket Steer Meat	Unpackaged	Portugal	7/1/19	9h00
A15B	Brisket Meat	Unpackaged	Portugal	7/1/19	9h00
A16B	veal Round Meat	MAP	Portugal	7/1/19	9h00
A17B	Bovine Meat Carpaccio	Vacuum	Unknown country	21/1/19	20h30
A18B	Chuck steak	MAP	Poland	21/1/19	20h30
A19B	Filet steak	Vacuum	Argentina	21/1/19	20h30
A20B	Minced meat	Unpackaged	Spain	21/1/19	20h30
A21B	Veal rib	Unpackaged	Ireland	21/1/19	20h39
A1C	Rabbit meat	MAP	Portugal	13/11/18	20h30
A2C	Rabbit Hands	MAP	Portugal	7/1/19	9h00
A3C	Rabbit Fricassee	MAP	Portugal	7/1/19	9h00

Table 1 - Samples analyzed in this study - Description of cuts, type of packaging, origin, date and time of sampling

2.2 Determination of antibiotic resistance phenotype

2.2.1 Antibiotic susceptibility test through agar diffusion method

Antibiotic susceptibility tests were performed using the agar diffusion method under the European Committee for Antimicrobial Susceptibility Testing (EUCAST) guidelines, except for Cefotaxime (30µg), which was used under the Clinical and Laboratory Standards Institute (CLSI). This test was performed in pure isolates after confirmation that these were resistant to the antibiotic in which they were selected.

For these tests, fresh cultures were used (with 18h-24h) and suspended in physiologic serum until a 0,5 MacFarland turbidity was achieved. The suspension was inoculated in 45º streaks in Muller-Hinton medium and incubated at 37ºC for 18-24h. The antibiotic discs used for the test with Gram negative bacteria were in the following order: Ampicillin (10µg), Cefotaxime (30µg), Cefoxitin (30µg), Ceftazidime (30µg), Meropenem (10µg), Aztreonam (30µg), Ciprofloxacin (5µg), Cefepime (30µg), Amoxicillin and Clavulanic Acid (30µg) (the latter was used in the middle of the plate). Clean halos were measured and defined as R (resistant), S (susceptible) or I (Intermediate). An *Escherichia coli* ATCC 25922 was used as a control for these tests.

2.2.2 Broader Antibiotic Susceptibility Tests

In order to gather a broader view on the multirresistant phenotype of bacteria, three susceptibility tests were performed for carbapenems, quinolones and aminoglycosides and other antibiotics. These susceptibility tests were performed as mentioned above, and the list of antibiotics used is described below on Table 2.

Carbapenems	Quinolones and aminoglycosides	Others
Ertapenem (5µg)	Enrofloxacin (5µg)	Nitrofurantoin (300µg)
Ceftiofur (30µg)	Nalidixic Acid (30µg)	Nitrofurantoin (100µg)
Meropenem (5µg)	Fosfomycin (200µg)	Piperacillin (100µg)
Ceftazidime (10µg)	Gentamicin (10µg)	Piperacillin/tazobactam (110µg)
Imipenem (5µg)	Amikacin (30µg)	Cefuroxime (30µg)
Doripenem(5µg)	Colistin (10µg)	Tetracycline (30µg)
Ceftaroline (5µg)	Tobramycin (10µg)	Tigecycline (15µg)
Piperacillin (30µg)	Netilmicin (10µg)	Chloramphenicol (30µg)
Piperacillin/Tazobactam (36µg)		Trimethoprim sulfamethoxazole (25µg)

Table 2 - Antibiotics used in the Broad Susceptibility tests. Each of the columns represents the antibiotics used in an agar plate

Carbapenem Inactivation Method (CIM) to isolates presenting reduced susceptibility to carbapenems

This method was used whenever an isolate presented resistance to a carbapenem in the ASTs. In this procedure, a 10µl loop of bacteria was suspended in 400µl of water and a 10µg carbapenem disk (Imipenem or Meropenem) was placed in the suspension and incubated for 2h at 37 ºC. For this method a dry disk was used as negative control and a well-known carbapenemase producing isolate was used as a positive control. Posteriorly, the disks were placed on a Muller Hinton plate inoculated with an *E.coli* ATCC 25922 strain and incubated for 18-24h at 37ºC. If the bacteria produce carbapenemases, the antibiotic disk will be inactivated, and the inhibition halo will not appear. If it does not inactivate the carbapenem, the halo will appear, and it will be considered a negative result.

Combination Disk Test (CDT)

This method was used to understand which type of carbapenemase was being produced in isolates with reduced carbapenem susceptibility and is based on EUCAST guidelines. To perform this test, several disks of carbapenems and meropenem with different inhibitors were placed in Muller Hinton plates inoculated with the presumptive carbapenemase-producing isolates and the plates were incubated at 37ºC overnight. Interpretation of the results were performed as described in Table 3. As quality control of the efficacy of the disks, an *E.coli* ATCC 25922 strain was used as previously mentioned.
The disks used for this test were the following: Meropenem (10), Meropenem+EDTA, Imipenem (10), Doripenem (10), Temocillin (30), Meropenem+ Cloxacillin(MR+CL), Meropenem+ Dipicolinic Acid (MR+DP), Meropenem+ Phenyl Boronic Acid (MR+PB), blank disk with EDTA.

β-lactamase	Synergy observed as diameter increase (mm) with a 10 meropenem disk				TMO zone diameter <11mm (a)
	DP/EDTA	PB	DP+PB	CL	
MBL	+	-	-	-	Variable
KPC	-	+	-	-	Variable
OXA-48-like	-	-	-	-	Yes
AmpC+porin loss	-	+	-	+	Variable

Table 3 - Interpretation of CDT tests for carbapenemase production (adapted from EUCAST). (a) – differentiates between OXA-48-like and ESBL + porin loss enzymes, only when there is no synergy detected. Abbreviations: MBL – metallo-β-lactamase; KPC – *Klebsiella pneumoniae* carbapenemase; TMO – Temocillin; EDTA - Ethylenediamine tetra acetic acid

Combination Disk Test and Double Disk Synergy Test (DDST)

Upon the suspicion of an ESBL (resistance to cefotaxime and/or ceftazidime), a combination of the CDT and DDST tests described by EUCAST was performed. For this, 2 disks of cephalosporins (CTX and CAZ) were incubated in Muller Hinton plates, with approximately 20mm distance center to center from an AMC disk, inoculated with the presumptive ESBL-producing bacteria with and without the addition of 10µg/ml of clavulanic acid at 37ºC overnight. If a synergism was observed, which is seen by the enlargement of the halo towards the AMC disk and the augmentation of the CTL disk in comparison to the CTX disk of (>5mm), the ESBL phenotype is confirmed and resistance genes should be analyzed.

2.3 Bacteria Identification

2.3.1 ID 32 GN

The ID 32GN is an automatic identification system used to determine Gram negative bacilli. For this test, a bacterial suspension of 0.5 Mac Farland is prepared and 200µl of this suspension are homogenized in API AUX medium, a semi solid minimal medium that contains ammonium sulphate, agar monosodium phosphate, potassium chloride , a vitamin solution with a pH of 7. It consists in placing 135µl of the previous suspension in each of the 32 chambers containing a dehydrated carbohydrate substrate. After 24-48h of incubation the growth in each chamber is detected through an automatic system.

2.3.2 Chromogenic medium

Whenever it was necessary, as a preliminary, presumptive identification, pure bacterial colonies were inoculated in a chromogenic medium, CHROMagar Orientation. This medium contains chromogens, which are molecules that contain a chromophore and a substrate. Color appears by the target organism's enzyme cleavage of the molecule and release of the chromophore.

2.4 Polymerase Chain Reaction (PCR)

PCR is a technique from which a DNA sample is amplified. This procedure comprises three main stages: denaturation, annealing and elongation. In the denaturation process, the PCR reaction is heated to 94-98ºC in order to melt the double stranded DNA (dsDNA) into two single stranded DNA (ssDNA) samples. Following this step, each ssDNA is annealed with the primers that will complement for a short sequence. Two primers are used in each

reaction (one for each DNA strand). After annealing, the DNA polymerase will synthetize a new DNA complementary to the DNA template from the end of the primer, with the aid of dNTPs (deoxynucleotide triphosphates), which are single units of nucleotides that will act as building blocks for the new strand.

2.4.1 DNA extraction

Posteriorly to the antibiotic susceptibility tests performed, possible Extended Spectrum Beta-Lactamase producing bacteria, carbapenem resistant bacteria and quinolone resistant bacteria were selected for molecular confirmation of the phenotype. Prior to the molecular methods a loop of bacteria was removed from Muller Hinton susceptibility plates (due to the fact that in this stage, the bacteria were pure and in higher density) and suspended in 500µl of TSB+glycerol and frozen at -20ºC for no longer than 1 month before DNA extraction. Prior to the extraction, bacteria were thawed in CLED medium (cystine lactose electrolyte deficient) a green agar, non-selective but differential in terms of lactose fermentation. The inoculates were incubated for 18h-24h at 37ºC before usage.

The DNA extraction method used was the conventional boiling-centrifugation method, in which lysis of the cells is achieved through boiling. For this method, a dense loop of bacteria was suspended in 20µl of UP water and boiled at 99ºC for 15 min in a thermocycler to achieve cell lysis and protein denaturation. Following this, 180µl of TE were added, homogenized and a centrifugation step was performed at 13000 rpm for 15 min, after which the supernatant containing the DNA was removed and stored at -20ºC until further use.

2.4.2 Molecular characterization of the resistance phenotype

Upon extraction, the isolates were submitted to PCR in order to confirm if the bacteria had the genes that allowed them to express the resistance phenotype. For the ESBLs, two sets of multiplex PCRs were conducted: one for the TEM, SHV and OXA genes and another for the CTX-M groups 1, 2, 8, 9 and 25. The bacteria that presented carbapenem resistance were submitted to a multiplex PCR for the KPC, OXA-48, IMP, VIM and NDM genes. The primers used in these reactions are listed in Table 4.

Gene	Primer designation	Primer Sequence(5'->3')	Size (bp)	Ref.
*bla*TEM	TEM F	5'- CAT TTC CGT GTC GCC CTT ATT C-3'	800	(46)
	TEM R	5' -CGT TCA TCC ATA GTT GCC TGA C-3'		
*bla*SHV	SHV F	5' - AGC CGC TTG AGC AAA TTA AAC -3'	713	
	SHV R	5' - ATC CCG CAG ATA AAT CAC CAC -3'		
*bla*OXA	OXA F	5' - GGC ACC AGA TTC AAC TTT CAA G -3'	564	
	OXA R	5' - GAC CCC AAG TTT CCT GTA AGT G -3'		
*bla*CTX-M group1	CTX-M1 F	5' – AAA AAT CAC TGC GCC AGT TC - 3'	415	(47)
	CTX-M1 R	5' – AGC TTA TTC ATC GCC ACG TT – 3'		
*bla*CTX-M group2	CTX-M2 F	5' – CGA CGC TAC CCC TGC TAT T – 3'	552	
	CTX-M2 R	5' – CCA GCG TCA GAT TTT TCA GG – 3'		
*bla*CTX-M group8	CTX-M8 F	5' – TCG CGT TAA GCG GAT GAT GC – 3'	666	
	CTX-M8 R	5' – AAC CCA CGA TGT GGG TAG C – 3'		
*bla*CTX-M group9	CTX-M9 F	5' – CAA AGA GAG TGC AAC GGA TG – 3'	205	
	CTX – M9 R	5' – ATT GGA AAG CGT TCA CC – '3		
*bla*CTX-M group25	CTX-M25 F	5' – GCA CGA TGA CAT TCG GG -3'	327	
	CTX-M25 R	5' – AAC CCA CGA TGT GGG TAG C – 3'		
*bla*IMP	IMP R	5' - GGT TTA AYA AAA CAA CCA CC - 3'	232	(48)
	IMP F	5' - GGA ATA GAG TGG CTT AAY TC - 3'		
*bla*VIM	VIM F	5' - GAT GGT GTT TGG TCG CAT A-3'	390	
	VIM R	5' - CGA ATG CGC AGC ACC AG - 3'		
*bla*OXA-48	OXA-48 F	5' - GCG TGG TTA AGG ATG AAC AC -3'	438	
	OXA-48 R	5' - CAT CAA GTT CAA CCC AAC CG - 3'		
*bla*NDM	NDM F	5' - GGT TTG GCG ATC TGG TTT TG - 3'	621	
	NDM R	5' - CGG AAT GGC TCA CGA TC -3'		
*bla*KPC	KPC F	5' - CGT CTA GTT CTG TCT TG -3'	798	
	KPC R	5' - CTT GTC ATC CTT GTT AGG CG -3'		

Table 4 - List of primers used for the molecular characterization of β-lactamase and carbapenemase resistance genes. **Abbreviations**: F – Forward; R – Reverse; A – Adenine ; T – Thymine; C – Cytosine ; G – Guanine; Ref – Reference

2.4.3 Characterization of genes that codify for resistance to non β-lactam antibiotics in *Escherichia coli* isolates expressing the resistance phenotype

Characterization of genes that lead to resistance to non β-lactam antibiotics was conducted by PCR, with specific conditions and primers for the PMQR genes aac6'-IB-cr, qnrA, qnrB, qnrC, qnrD and qnrS. Additional PCRs were performed to the subunits gyrA and parC of DNA gyrase and topoisomerase IV, respectively. The list of primers used for these protocols are listed in Table 5.

Gene	Primer designation	Primer Sequence(5'->3')	Size (bp)	Reference
qnrA	qnrA F	5' – GGA TGC CAG TTT CGA GGA - 3'		(49)
	qnrA R	5' – TGC CAG GCA CAG ATC TTG – 3'		
qnrB	qnrB F	5' –GGM ATH GAA ATT CGC CAC TG–3'	264	(50)
	qnrB R	5' – TTT GCY GYY CGC CAG TCG AA – 3'		
qnrC	qnrC F	5'–GGG TTG TAC ATT TAT TGA ATC– 3'	447	(51)
	qnrC R	5' – TCC ACT TTA CGA GGT TCT – 3'		
qnrD	qnrD F	5'–CGA GAT CAA TTTACGGGGAAT A-3'	582	(52)
	qnrD R	5' – AAC AAG CTG AAG CGC CTG -3'		
qnrS	qnrS R	5'–GAT CTA AAC CGT CGA GTT CGG–3'		(49)
	qnrS F	5' – TCG ACG TGC TAA CTT GCG – 3'		
parC	parC F	5' –TGT ATG CGA TGT CTG AAC TG – 3'	264	(53)
	parC R	5'–CTC AAT AGC ACG CTC GGA ATA–3'		
gyrA	gyrA F	5' – ACG TAC TAG GCA ATG ACT GG - 3'	189	
	gyrA R	5'–AGA AGT CGC CGT CGA TAG AAC–3'		
Aac6'-IB-cr	Aac6'-Ib-cr F	5' – TTG CGA TGC TCT ATG AGT GG– 3'	400	(54)
	Aac6'-Ib-cr R	5' – GCG TGT TCG CTC GAA TGC C – 3'		

Table 5 - List of primers used for the molecular characterization of genes that encode for non β-lactam resistance . **Abbreviations**: F – Forward; R – Reverse; A – Adenine ; T – Thymine; C – Cytosine ; G – Guanine; Ref – Reference

2.4.4 Characterization of genes that codify for colistin resistance in *Escherichia coli* isolates with resistance to non β-lactams

Due to the growing attention to plasmid-mediated colistin resistance, the *Escherichia coli* isolates resistant to non-beta lactams were subjected to a multiplex PCR to analyse the presence of mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5 genes (Table 6), according to the novel procedure described by Rebelo et al. (55). A bacterial isolate containing the mcr-1 gene was used as a positive control for this gene and ultra-pure water as negative control.

Gene	Primer designation	Primer Sequence(5'->3')	Size (bp)	Ref.
Mcr-1	Mcr-1 F	5' – AGT CCG TTT GTT CTT GTG GC – 3'	320	(55)
	Mcr-1 R	5' – AGA TCC TTG GTC TCG GCT TG – 3'		
Mcr-2	Mcr-2 F	5' – CAA GTG TGT TGG TCG CAG TT – 3'	715	
	Mcr-2 R	5' – TCT AGC CCG ACA AGC ATA CC – 3'		
Mcr-2	Mcr-3 F	5'–AAA TAA AAA TTG TTC CGC TTA TG–3'	929	
	Mcr-3 R	5' – AAT GGA GAT CCC CGT TTT ATC – 3'		
Mcr-4	Mcr-4 F	5' – TCA CTT TCA TCA CTG CGT TG – 3'	1116	
	Mcr-4 R	5' – TTG GTC CAT GAC TAC CAA TG – 3'		
Mcr-5	Mcr-5 F	5' – ATG CGG TTG TCT GCA TTT ATC – 3'	1644	
	Mcr-5 R	5' – TCA TTG TGG TTG TCC TTT TCT G – 3'		

Table 6 - List of primers used to characterize colistin resistance**. Abbreviations**: F – Forward; R – Reverse; A – Adenine ; T – Thymine; C – Cytosine ; G – Guanine; Ref – Reference

2.4.5 Identification of the phylogenetic groups in *Escherichia coli* resistant to quinolones

Escherichia coli isolates that showed resistance to non-beta lactams, such as ciprofloxacin, were subjected to a multiplex in order to understand which phylogenetic group they belong, A, B1, B2 or D. The technique, described by Clermont et al., allows for a rapid and simple classification through the presence/absence of chuA, yjaA genes and the tspE4.C2 fragment. The classification in each phylogenetic group was based in the following: presence of chuA and yjaA genes – group B2; presence of chuA and absence of yjaA – group D; absence of chuA and presence of tspE4.C2 – group B1; absence of chuA and absence of tspE4.C2 – group A (56).

The multiplex PCR consisted in an initial denaturation at 94ºC for 5min, followed by 30 cycles of denaturation at 94ºC for 30s, annealing at 55ºC for 30s, extension at 72ºC for 30s and a final extension for 7 min at 72ºC. An isolate belonging to the B2 phylogenetic group was used as a positive control. The primers used in this protocol are listed in Table 7.

Gene	Primer	Primer sequence (5'-3')	Size (bp)	Reference
chuA	chuA 1	5'-GACGAACCAACGGTCAGGAT – 3'	279	(56)
	chuA 2	5' – TGCCGCCAGTACCAAAGACA – 3'		
yjaA	yjaA 1	5' – TGAAGTGTCAGGAGACGCTG – 3'	211	
	yjaA 2	5' – ATGGAGAATGCGTTCCTCAAC- 3'		
TspE4.C2	tspE4.C2 1	5' -GAGTAATGTCGGGGCATTCA – 3'	152	
	TspE3.C2 2	5' -CGCGCCAACAAAGTATTACG – 3'		

Table 7 - List of primers for molecular characterization of phylogenetic groups**. Abbreviations**: F – Forward; R – Reverse; A – Adenine ; T – Thymine; C – Cytosine ; G – Guanine;

2.4.6 Characterization of virulence factor genes in *Escherichia coli* resistant to non β-lactam antibiotics

Characterization of 29 virulence factors divided by 5 pools was determined through PCR, according to Johnson and Stell (57). These virulence factors code for toxins, adhesins, fimbriae, siderophores, among others. Further detail on primer sequence and function of each gene can be found bellow on table 4.

The PCR was initiated by a denaturation step at 95ºC for 12 min, followed by 25 cycles of denaturation at 94ºC for 30s, annealing at 63ºC for 30s and extension at 68ºC for 3min, with a final extension step at 72ºC for 10min. The PCR products were observed by performing an agarose gel electrophoresis and results were validated through the absence of amplification in the negative control (ultra-pure water) and comparison with the weight marker.

Gene		Primer	Sequence	bp	Ref
Pathogenicity-associated island	PAI	RPAi f	5' – GGACATCCTGTTACAGCGCGCA - 3'	930	
		RPAi r	5' – TCGCCACCAATCACAGCCGAAC – 3'		
Siderophores	iutA	AerJ f	5' – GGCTGGACATCATGGGAACTGG – 3'	300	
		AerJ r	5' – CGTCGGGAACGGGTAGAATCG – 3'		
	fyuA	FyuA f'	5' - TGATTAACCCCGCGACGGGAA – 3'	880	
		FyuA r'	5' – CGCAGTAGGCACGATGTTGTA – 3'		
	traT	TraT f	5' – GGTGTGGTGCGATGAGCACAG – 3'	290	
		TraT r	5' – CACGGTTCAGCCATCCCTGAG – 3'		

Gene		Primer	Sequence	bp
Adhesins	fimH	FimH f	5’ – TGCAGAACGGATAAGCCGTGG – 3’	508
		FimH r	5’ – GCAGTCACCTGCCCTCCGGTA – 3’	
	sfaS	SfaS f	5’ – GTGGATACGACGATTACTGTG – 3’	240
		SfaS r	5’ – CCGCCAGCATTCCCTGTATTC – 3’	
	focG	FocG f	5’ – CAGCACAGGCAGTGGATACGA – 3’	360
		FocG r	5’ – GAATGTCGCCTGCCCATTGCT – 3’	
	Afa/dra	Afa f	5’ – GGCAGAGGGCCGGCAACAGGC – 3’	559
		Afa r	5’ – CCCGTAACGCGCCAGCATCTC – 3’	
	Sfa/foc DE	Sfa 1	5’ – CTCCGGAGAACTGGGTGCATCTTAC – 3’	410
		Sfa 2	5’ CGGAGGAGTAATTACAAACCTGGCA– 3’	
	BmaE	bmaE-f	5’ – ATGGCGCTAACTTGCCATGCTG – 3’	507
		bmaE-r	5’ – AGGGGGACATATAGCCCCCTTC – 3’	
	NafE	nfaE-f	5’ – GCTTACTGATTCTGGGATGGA – 3’	557
		nfaE-r	5’ – CGGTGGCCGAGTCATATGCCA – 3’	
	gafD	gafD-f	5’ – TGTTGGACCGTCTCAGGGCTC – 3’	952
		gafD-r	5’ – CTCCCGGAACTCGCTGTTACT – 3’	
	papAH	papA f	5’ – ATGGCAGTGGTGTCTTTTGGTG – 3’	720
		papA r	5’ – CGTCCCACCATACGTGCTCTTC – 3’	
	papC	papC f	5’–GTGGCAGTATGAGTAATGACCGTTA–3’	200
		papC r	5’–ATATCCTTTCTGCAGGGATGCAATA–3’	
	papEF	papEF f	5’–GCAACAGCAACGCTGGTTGCATCAT–3’	336
		papEF r	5’–AGAGAGAGCCACTCTTATACGGACA–3’	
	papG II, III	pG f	5’– CTGTAATTACGGAAGTGATTTCTG – 3’	1070
		pG r	5’– ACTATCCGGCTCCGGATAAACCAT – 3’	
	papG I	pG1”r	5’– TCCAGAAATAGCTCATGTAACCCG – 3’	1190
	papG alleleI	AlleleI-f	5’– TCGTGCTCAGGTCCGGAATTT – 3’	461
		AlleleI-r	5’ – TGGCATCCCCCAACATTATCG – 3’	
	papGallele II	AlleleII-f	5’ – GGGATGAGCGGGCCTTTGAT – 3’	190
		AlleleII-r	5’ – CGGGCCCCCAAGTAACTCG – 3’	

(57)

Gene		Primer	Sequence	bp
	papG allele III	Allele-III-f	5’ – GGCCTGCAATGGATTTACCTGG – 3’	258
		AlleleIII-r	5’ – CCACCAAATGACCATGCCAGAC – 3’	
Toxins	hlyA	Hly-f	5’–AACAAGGATAAGCACTGTTCTGGCT–3’	1177
		Hly-r	5’–ACCATATAAGCGGTCATTCCCGTCA–3’	
	cnf1	Cnf1	5’ –AAGATGGAGTTTCCTATGCAGGAG– 3’	498
		Cnf2	5’–CATTCAGAGTCCTGCCCTCATTATT– 3’	
	Cdtb	Cdt-a1	5’ –AAATCACCAAGAATCATCCAGTTA – 3’	430
		Cdt-a2	5’–AAATCTCCTGCAATCATCCAGTTTA– 3’	
		Cdt-s1	5’–GAAAGTAAATGGAATATAAATGTCCG – 3’	
		Cdt-s2	5’–GAAAATAAATGGAACACACATGTCCG – 3’	
Capsules	kpsMT II	kpsII f	5’ – GCGCATTTGCTGATACTGTTG – 3’	272
		kpsII r	5’ – CATCCAGACGATAAGCATGAGCA – 3’	
	kpsMT III	kpsIII f	5’ – TCCTCTTGCTACTATTCCCCCT – 3’	392
		kpsIII r	5’ – AGGCGTATCCATCCCTCCTAAC – 3’	
	kpsMT K1	K1-f*	5’ – TAGCAAACGTTCTATTGGTGC – 3’	153
	kpsMT K5	K5-f*	5’ – CAGTATCAGCAATCGTTCTGTA – 3’	159
Diverse	ibeA	Ibe10 f	5’ – AGGCAGGTGTGCGCCGCGTAC – 3’	170
		Ibe10 r	5’ – TGGTGCTCCGGCAAACCATGC – 3’	
	cvaC	colV-Cf	5’ – CACACACAAACGGGAGCTGTT – 3’	680
		colV-Cr	5’ – CTTCCCGCAGCATAGTTCCAT – 3’	
	Rfc	Rfc-f	5’ – ATCCATCAGGAGGGGACTGGA – 3’	788
		Rfc-r	5’ – AACCATACCAACCAATGCGAG – 3’	

Table 8 - List of primers used to characterize genes that encode for virulence. **Abbreviations**: F – Forward; R – Reverse; A – Adenine ; T – Thymine; C – Cytosine ; G – Guanine; Ref – Reference

2.4.7 Agarose Gel Electrophoresis

To observe the PCR results, an agarose gel electrophoresis was performed. This procedure consists in the separation of nucleic acids (in this case) through an agarose gel with a defined concentration depending on the resolution we want to observe our results. The more concentrated the gel is the more bands will be separated from each other and longer it will take to run. In this method the DNA is separated through the gel by the action of an electrical field which attracts the DNA that is negatively charged to the positive side, thus separating the PCR products. Since fragments with high molecular weights take longer to run, these will be on the top of the gel, while lower molecular weight fragments will run faster and be on the bottom.

For this experiment, agarose gels of 1,5% and 2% were prepared by dissolving agarose in TAE 1x with heating, after which Midori Green DNA stain, a commercial alternative to ethidium bromide, is added. TAE is a buffer containing Tris, Acetic acid glacial and EDTA.

In each well, 8 ul of each of the PCR products was added along with approximately 1ul of Taq dye (since the Taq used in the PCRs was not previously dyed) and on a separate well, 3ul of MW marker was added. The electrophoresis occurred in a horizontal system, appropriate for nucleic acid visualization.

The conditions in which the race was performed were dependent on the type of PCR performed: Simplex PCRs were run for 30 min at 100V and multiplex PCRs were run for 90min at 85-90V. The results of each electrophoresis were revealed through an UV light.

3 Results and Discussion

3.1 Selection of Gram-negative isolates presenting resistance to both β-lactams and non β-lactams

The isolates presenting resistance to different antibiotic classes were selected from bovine and rabbit meat samples. The types of meat used in this study represent the meat types that are increasing in consumption in Portugal. In the case of bovine meat, this product is typically consumed undercooked, being a possible reservoir of resistant bacteria that may colonize the gastrointestinal tract of the human population, which may lead to infections that may be difficult to treat with common antibiotics.

3.1.1 Selection of Gram-negative isolates presenting resistance to different antibiotic classes from meat samples

In this work, a total of 24 samples were analysed, 21 from bovine meat and 3 from rabbit meat. The inoculation of meat samples, prior and after enrichment, in MacConkey agar allowed, not only the knowledge of the "accurate" density of Gram-negative bacteria present in these samples, but it also permitted the evaluation of the density of isolates with and without resistance to oxyimino-β-lactams or carbapenems in these products. The use of MacConkey agar supplemented with β-lactam antibiotics, allowed the selection of lactose fermenting and non-fermenting bacteria resistant to the antibiotics at the used concentrations, which were representative of the samples. The resistance phenotype was characterized through agar diffusion susceptibility tests for β-lactams and ESBL-producing, quinolone, and carbapenem resistant bacteria were selected through confirmatory phenotypical tests.

There was also an interest in verifying the influence of packaging in the proliferation and colonization of these type of bacteria in meat. Therefore, different types of packaging (Modified atmosphere, vacuum and unpackaged) were analysed. As it is possible to verify in Figure 5**,** the number of samples processed with package and without package were relatively the same (the number of packaged samples is higher since all three rabbit meat samples were packaged). However, when analysing the graph related to the number of isolates, the number of isolates was almost the double in unpackaged samples (30) against the sixteen isolates in the packaged ones (including the rabbit samples). It is important to mention that in three of the packaged bovine meat samples (A4B, A5B and A7B), there was no observed growth in any of the antibiotic-supplemented mediums.

It is known that packaging systems, such as modified atmosphere packaging (MAP) aid in limiting the proliferation of pathogenic and spoilage bacteria as well as increasing shelf life of products, through the injection of different percentages of Oxygen, Nitrogen and Carbon dioxide (58). The above-mentioned results seem to corroborate this, since there was a significative difference in the number of isolates found.

Bacteria were presumptively identified using chromogenic medium, CHROMagar Orientation, and some isolates were identified by using the ID32GN protocol. By observing Figure 6, showing the number and identification of the isolates from bovine and rabbit meat, it is possible to conclude that *Acinetobacter* spp. (8), *Pseudomonas* spp. (8) and *Escherichia coli* (21) were the predominant genera and species detected.

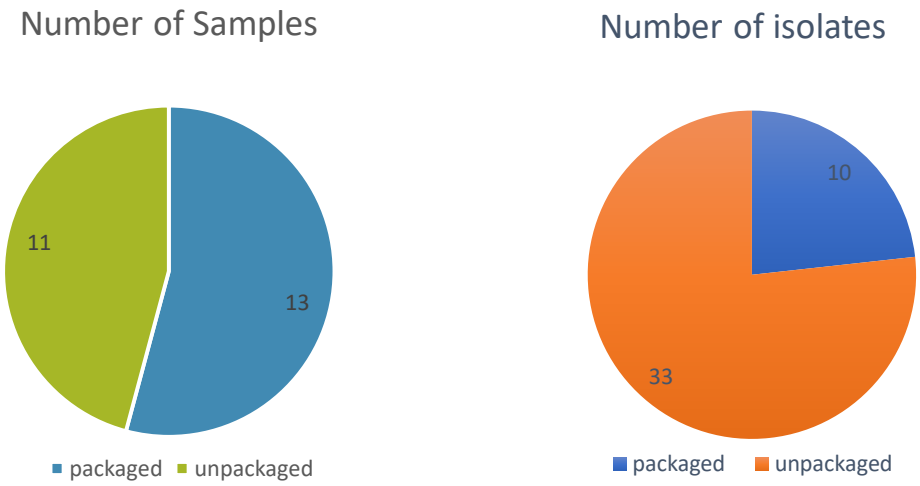

Figure 6 - Distribution of the samples (on the left) and isolates (on the right) through the types of packaging.

Escherichia coli was, by far, the type of bacteria that was present in double of the amount of the other isolates. It was present in thirteen bovine meat samples and in two rabbit meat samples analysed.

The isolates from rabbit meat, A2CCIP and A3CCIP (Table 6), were selected from ciprofloxacin, a non β-lactam antibiotic and kept for molecular characterization of genes related to non β-lactam resistance as well as for a more extensive phenotypical characterization of their resistance.

From the unpackaged samples, thirteen isolates were found, of which eight showed resistance to CIP. These were posteriorly characterized molecularly and phenotypically for non β-lactam resistance. From the other five isolates, one was only resistant to AMP and four showed resistance or intermediate resistance to CAZ or CTX. Latter were submitted to the Combination Disk Test (CDT) and Double-Disk Sinergy Test (DDST), to evaluate the presence of β-lactamases. One of the isolates with resistance to CIP was also tested for the presence of β-lactamases. Positive results for the CDT+DDST methods were kept for molecular characterization of resistance genes.

Isolates from packaged bovine meat (6) were selected in several antibiotics. Two were characterized phenotypically and molecularly regarding non β-lactam resistance since these were resistant to CIP. One was only resistant to AMP and the remaining 3 were

resistant or showed decreased susceptibility to CTX and/or CAZ. With resemblance with the unpackaged isolates, these were tested for the presence of β-lactamases.

Acinetobacter spp. isolates were presumptively identified as belonging to this genus since they were oxidase negative and the color of the isolates in CHROMagar Orientation medium was white. Also, it was possible to visualize the change in color in MacConkey agar from non-fermenting to acid, which is also indicative of this genus, since it is an oxidative user of carbohydrates. All 8 isolates were selected from different samples, 5 from unpackaged meat, 1 from packaged bovine meat and 2 from rabbit meat.

Eight *Pseudomonas* spp. isolates were discovered, 6 belonging to unpackaged meat and 2 from rabbit meat. No growth of these bacteria was observed in packaged bovine meat, since that oxygen absence conditions their growth. Four of these 8 isolates (1 from rabbit and 3 from unpackaged bovine meat) presented reduced susceptibility to meropenem, indicating a possible carbapenemase production. In order to confirm the presence of this type of enzyme a CIM test was performed of which all samples showed an intermediate and inconclusive result (the halos were reduced when compared to the control). Moreover, a CDT was performed to evaluate which carbapenemase was present. Results suggested the presence of metallo-β-lactamases for all the isolates, which were kept for molecular characterization of genes that encode for carbapenemase production.

From the remaining 4 *Pseudomonas* spp. isolates, 1 (A10BCIP1) was resistant to CIP and was molecularly analysed for genes that encode for quinolone resistance, 1 (A10BCTX4) presented the native AmpC phenotype, characteristic of this genus. The other 2 isolates (A11BCTX4 and A1CCTX), showed resistance to other β-lactam antibiotics besides the ones related to an AmpC phenotype and were once again submitted to an Antibiotic Susceptibility test using a Muller Hinton plate supplemented with Cloxacillin, an AmpC inhibitor. If other β-lactamases were being expressed, they would be visible in using this method.

In the samples without packaging it were also presumptively identified 3 isolates belonging to the KESC (*Klebsiella* spp., *Enterobacter* spp., *Serratia* spp. and *Citrobacter* spp.) group, through CHROMagar Orientation medium. Further phenotypical characterization of these isolates was executed relatively to their resistance range.

One *Hafnia alvei* isolate was identified through ID32GN, since it presented a typical β-lactamase phenotype and its color on the chromogenic medium did not aid on a possible identification (mixture of white and blue). The isolate was retrieved from a vacuum packaged bovine meat sample, A6B (matured meat). Confirmatory tests were performed to verify the presence of the β-lactamase.

Gram-negative bacteria producers of AmpC beta-lactamases were also detected in bovine samples. Without neglecting these findings, the isolates presenting these characteristics were preserved for future studies.

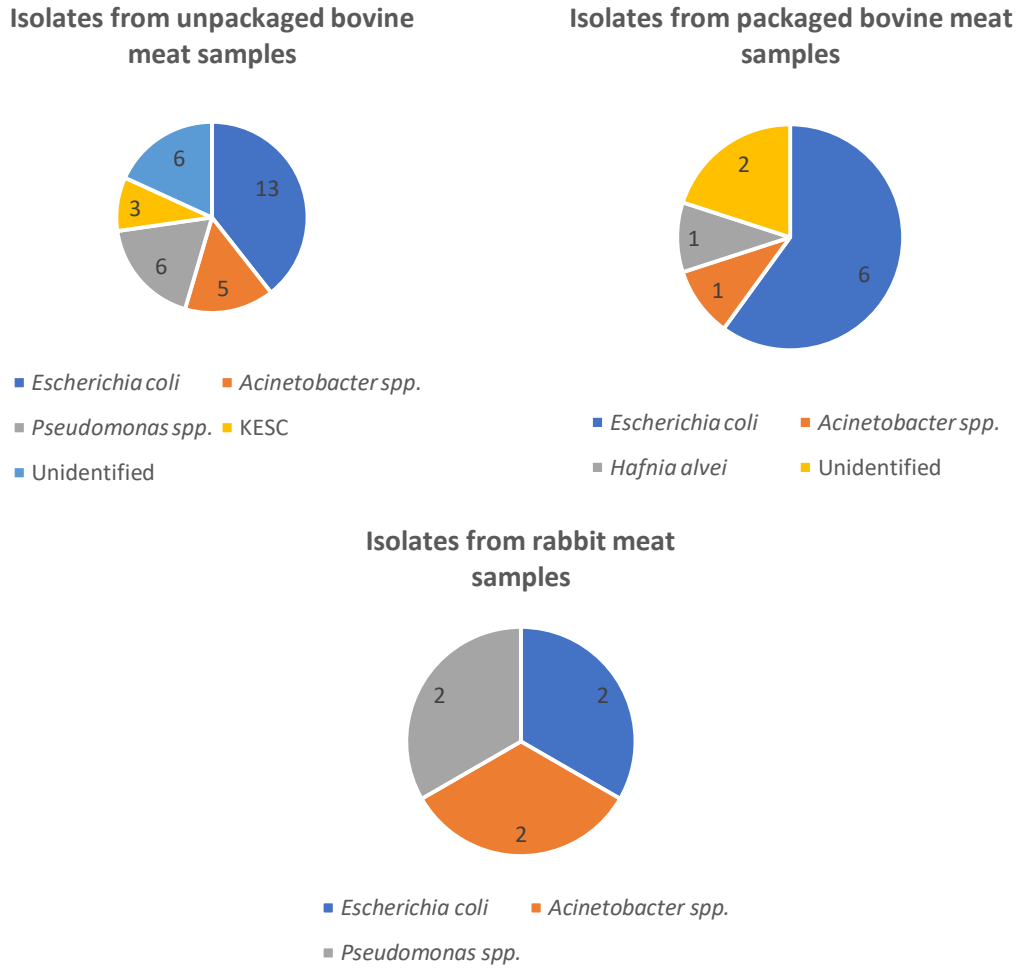

Figure 7 - Distribution of the number of isolates from packaged and unpackaged bovine (top graphs) and rabbit (bottom graph) meat samples

Number	Sample	Isolate	Identification	AMP	AMC	CTX	FOX	CAZ	ATM	MEM	CIP	FEP
1	veal chuck steak	A1B1CTX	*Acinetobacter* spp.	12.5	28	16	12	16	6	27.5	28	21
		A1B3CTX	KESC	6	10	20	27.5	19	26.5	32	20	+
3	Minced Bovine meat	A3BCIP	*Escherichia coli*	6	+	28	18	26	+	+	6	+
		A3BMRP	*Pseudomonas fluorescens*	6	6	13	6	18	6	14	+	+
		A3BCTX1	KESC	12	16	20	11	22.5	17.5	28	30	23
8	Sirloin meat	A8BCIP	*Escherichia coli*	6	22	27	21	+	+	+	17	+
		A8BAMP	*Escherichia coli*	6	20	+	22	+	+	+	+	+
9	Chuck steak	A9BCIP	*Escherichia coli*	6	20	+	+	+	+	+	14	+
		A9BCTX	*Acinetobacter* spp.	14	20	20	15	21	15	25	27	24
		A9BAMP1	KESC	6	16	+	17	+	+	+	+	+
		A9BAMP2	*Escherichia coli*	6	18	29	22	+	29	+	13	28
		A9BMRP	*Pseudomonas* spp.	6	6	9.5	6	22	6	13	+	+
10	Minced Bovine meat	A10BCTX4	*Pseudomonas* spp.	6	6	20	6	30	28	34	34	29
10	Minced Bovine meat	A10BCIP1	*Pseudomonas* spp.	7	24	34	22	30	+	+	20	30

Number	Sample	Isolate	Identification	AMP	AMC	CTX	FOX	CAZ	ATM	MEM	CIP	FEP
10	Minced Bovine meat	A10BCIP3	*Escherichia coli*	9	26	34	25	30	33	33	14	31
		A10BCTX3	*Escherichia coli*	6	19	11	28	19	18	30	26	18
		A10BAMP3	Unidentified	8	23	30	22	21	30	+	28	22
11	Sirloin meat	A11BCIP1	*Escherichia coli*	6	22	20	22	21	+	+	13.5	29
		A11BCTX1	*Pseudomonas* spp.	6	6	14	6	+	6	+	32	26
		A11BCTX4	*Acinetobacter* spp.	12	19.5	20	12	21	18.5	26	28	22
14	Brisket Steer Meat	A14BCTX1	Unidentified	16	23.5	21	14	23	18	25	27.5	24
		A14BAMP4	Unidentified	6	+	+	+	+	+	+	+	+
15	Brisket Meat	A15BAMP2	Unidentified	6	+	+	+	+	+	+	+	+
		A15BCIP	Unidentified	6	13	25	20	+	+	+	+	+
		A15BCTX	*Acinetobacter* spp.	13.5	22	19	13	21	17	24	28	21.5
		A15BCTX2	*Escherichia coli*	6	10.5	21	13.5	19	22	+	+	+
20	Minced meat	A20BCTX3	*Escherichia coli*	7.5	8.5	22	25	20	25	32	34	32
		A20BCTX2	Unidentified	14	7	28	28	25	+	34	+	34
		A20BCIP	*Escherichia coli*	6	23	30	+	+	30	+	14	+
		A20BMRP	*Pseudomonas* spp.	6	6	17	6	25	19	20	+	20

Number	Sample	Isolate	Identification	AMP	AMC	CTX	FOX	CAZ	ATM	MEM	CIP	FEP
21	Veal rib	A21BCTX2	*Escherichia coli*	6	9	22	24.5	18	24	32	33	30
		A21BCTX1	*Acinetobacter* spp.	6	9	24	25	19	25	34	+	30
		A21BCIP	*Escherichia coli*	6	22	30	24	26	30	+	13	30

Table 9 - Antibiotic susceptibility test results regarding unpackaged bovine meat samples. **Abbreviations:** AMP – Ampicillin; AMC – Amoxicillin and Clavulanic Acid; CTX – Cefotaxime; FOX – Cefoxitin; CAZ – Ceftazidime; ATM – Aztreonam; MEM – Meropenem; CIP – Ciprofloxacin; FEP – Cefepime; **Red** color for resistant, **green** for susceptible and **yellow** for intermediate. **Plus sign (+)** – unmeasurable halo (Susceptible)

Number	Sample	Isolate	Identification	AMP	AMC	CTX	FOX	CAZ	ATM	MEM	CIP	FEP
6	Rib Bovine meat, matured	A6B4	*Hafnia alvei*	10	14	12	+	18	+	+	+	+
		A6BCIP	*Escherichia coli*	6	20	+	+	+	+	+	6	+
12	Bovine Round Steak	A12BAMP1	*Escherichia coli*	6	8	21	16	16	26	32	+	30
		A12BCTX2	Unidentified	6	9	24	25.5	21	27	31	32	30
13	Bovine Burger	A13BAMP2	*Escherichia coli*	6	21	30	22	29	30	+	12	28
		A13BCTX3	Unidentified	6	6	25	28	18	+	+	+	+
16	veal Round Meat	A16BCIP3	*Escherichia coli*	6	20	27	22	26	28	28	6	30
		A16BCTX2	*Escherichia coli*	6	10	19.5	25	16	+	+	+	+
17	Bovine Meat Carpaccio	A17BCTX	*Acinetobacter* spp.	6	9.5	22	24	19	26	34	30	30
19	Filet steak	A19BCTX	*Escherichia coli*	7	9	18	25	13	22	30	32	28

Table 10 - Antibiotic susceptibility test regarding packaged bovine meat samples. **Abbreviations:** AMP – Ampicillin; AMC – Amoxicillin and Clavulanic Acid; CTX – Cefotaxime; FOX – Cefoxitin; CAZ – Ceftazidime; ATM – Aztreonam; MEM – Meropenem; CIP – Ciprofloxacin; FEP – Cefepime; **Red** color for resistant, **green** for susceptible and **yellow** for intermediate. **Plus sign (+)** – unmeasurable halo (Susceptible)

Number	Sample	Isolate	Identification	AMP	AMC	CTX	FOX	CAZ	ATM	MEM	CIP	FEP
1	Rabbit meat	A1CCTX	*Pseudomonas* spp.	6	+	14	6	23	6	+	24	24
		A1CMRP	*Pseudomonas* spp.	6	R	18	6	25	15	18	32	27
2	Rabbit hands	A2CCIP	*Escherichia coli*	6	19	19	30	24	30	31	6	28
3	Rabbit fricassee	A3CCIP	*Escherichia coli*	6	19.5	30	25	26	27	28	8.5	30
		A3CCTX	*Acinetobacter* spp.	6	6	15	6	+	25	29	30	28.5
		A3CMRP	*Acinetobacter* spp.	6	11	18.5	6	28	18	22	33	28

Table 11 - Antibiotic susceptibility tests of rabbit meat isolates. **Abbreviations:** AMC – Amoxicillin and clavulanic acid; AMP – Ampicillin; CTX – Cefotaxime; FOX – Cefoxitin; CAZ – Ceftazidime; ATM – Aztreonam; MEM – Meropenem; CIP – Ciprofloxacin; FEP – Cefepime; **Red** color for resistant, **green** for susceptible and **yellow** for intermediate. **Plus sign (+)** – unmeasurable halo (Susceptible)

3.2 Phenotypic characterization of β-lactam and non β-lactam resistance in Gram-negative isolates

A broader phenotypic characterization of the antibiotic resistance was performed, with inclusion of several antibiotics from different classes (carbapenems, quinolones and aminoglycosides and others), for several isolates. Tables 10, 11 and 12 show the associated resistances for each of the tested isolates, for unpackaged and packaged bovine meat samples and rabbit meat, respectively.

As previously mentioned in Chapter 1, antibiotic resistance is defined as the ability of a bacteria to inactivate the action of an antibiotic. Given that there are several resistance patterns for different bacteria, a necessity to create a standardized terminology emerged. Therefore, the Centers for Disease Prevention and Control (CDC) and the European Centre for Disease Prevention and Control (ECDC), classified the several resistance patterns into the following categories: Multidrug Resistant (MDR), extensively drug resistant (XDR) and Pandrug resistant (PDR) (59).

Multidrug resistance is defined as acquired non-susceptibility to at least 1 agent in 3 or more antimicrobial classes. Extensively drug resistant is defined as susceptible to only 1 or 2 antimicrobial categories. Pandrug resistant is associated with bacteria that are non-susceptible to all antimicrobial classes (59).

Observing Tables 9 and 12, from the 23 unpackaged meat isolates kept for further testing, 17 (>50% of the isolates) showed resistance to at least one agent in 3 antibiotic categories, being considered MDR. The same happens in packaged bovine meat samples (Tables 10 and 13). In rabbit meat samples, 100% of the isolates were considered MDR (Tables 11 and 14).

Sample	Isolate	Identification	Product	Selection medium	Associated resistance
A1B	A1B1CTX	*Acinetobacter* spp.	veal chuck steak	CTX	ETP, CAZ10, CPT, NA (I), FOT, F300, F100
	A1B3CTX	KESC		CTX	CAZ10, CPT, PRL30, TZP36, CXM, PRL100, TZP110 (I)
A3B	A3BMRP	*Pseudomonas fluorescens*	Minced Bovine meat	MRP	ETP, EFT, MRP, CAZ10, IMI (I), CPT, DOR, NA, FOT, CXM, C, F300, F100, TGC (I)
	A3BCTX	KESC		CTX	ETP, CAZ10, CPT, PRL30, FOT, CXM, PRL100 (I), TZP36(I), C, F300, F100
	A3BCIP	*Escherichia coli*		CIP	CPT, TE, ENR, NA, C, PRL30

Sample	Isolate	Identification	Product	Selection medium	Associated resistance
A8	A8BCIP	*Escherichia coli*	Bovine sirloin Steak	CIP	CPT, PRL30, ENR, NA, PRL100, SXT
A9B	*A9B5MRP*	*Pseudomonas* spp.	Chuck steak	MRP	ETP, EFT, MRP, CAZ10, IMI (I), CPT, DOR, TZP36 (I), NA, FOT, CXM, C, F300, F100
	A9BAMP1	*KESC*		AMP	PRL30, TE, PRL100 (I)
	A9BCTX	*Acinetobacter* spp.		CTX	ETP, CAZ10, CPT, TZP36 (I), FOT, F300, F100
	A9BAMP2	*Escherichia coli*		AMP	CPT, PRL30, NA, PRL100 (I), SXT, TE
	A9BCIP	*Escherichia coli*		CIP	CPT, PRL30, ENR, NA
A10B	A10BCTX3	*Escherichia coli*	Minced Bovine meat	CTX	CAZ10, CPT, PRL30, TOB, CN, NET, CXM, PRL100, C, SXT, TE
	A10BCTX4	*Pseudomonas* spp.		CTX	ETP, CAZ10, CPT, TZP36, NA, CXM, C, F300, F100, SXT, TE, TGC
	A10BCIP1	*Pseudomonas* spp.		CIP	F30, F100
	A10BCIP3	*Escherichia coli*		CIP	CPT, PRL30, ENR, NA, PRL100, SXT, TE
A11B	A11B4CTX	*Acinetobacter* spp.	Sirloin meat	CTX	ETP, CAZ10, CPT, PRL30, TZP36 (I), FOT, PRL100 (I), C, F300, F100, TE
	A11BCTX1	*Pseudomonas* spp.		CTX	ETP, CAZ10, F300, F100
	A11BCIP	*Escherichia coli*		CIP	CPT, PRL30, ENR, NA, SXT, TE
A15B	A15BCTX2	*Escherichia coli*	Brisket Meat	CTX	CAZ10, CPT, TZP36 (I)
A20B	A20BMRP	*Pseudomonas* spp.	Minced meat	MRP	ETP, MRP, CAZ10, IMI, CPT, DOR, PRL30 (I), TZP 36(I), NA (I), FOT, CXM, F300, F100

	A20BCIP	*Escherichia coli*		CIP	CPT, PRL30, ENR, NA, SXT, TE
A21B	A21B2CTX	*Escherichia coli*	Veal rib	CTX	CAZ10, CPT, PRL30 (I), TZP36
	A21BCIP	*Escherichia coli*		CIP	CPT, PRL30, ENR, NA, PRL100, C, SXT, TE

Table 12 - Phenotypic resistance regarding other antibiotic classes of unpackaged bovine meat samples. The column of associated resistance shows only the antibiotics to which the isolates presented resistance or intermediate resistance. **Abbreviations:** CAZ10 – Ceftazidime 10 µg; DOR - Doripenem; ETP – Ertapenem; IMI – Imipenem; MRP – Meropenem; CPT – Ceftaroline; PRL30 or PRL100 – Piperacillin 30µg or 100µg; ENR – Enrofloxacin; NA – Nalidixic acid; C – Chloramphenicol; SXT – Trimethoprim/Sulfamethoxazole; TE – Tetracycline; CXM – Cefuroxime; FOT – Fosfomycin; F30 or F100 – Nitrofurantoin 30µg or 100µg; TOB – Tobramycin;; TZP36 or 110 – Piperacillin/Tazobactam 36µg or 110µg; (I) – intermediate resistance

Sample	**Isolate**	**Identification**	**Product**	**Selection medium**	**Associated resistance**
A6B	A6B4CTX	*Hafnia alvei*	Rib Bovine meat, matured	CTX	CAZ10, CPT, PRL30 (I), TZP36, CXM, PRL100 (I), TZP110 (I)
	A6BCIP	*Escherichia coli*		CIP	CPT, PRL30, TZP(I), ENR, NA, TOB, CN, PRL100, SXT, TE, C, TZP100**
A12B	A12BAMP	*Escherichia coli*	Bovine Round Steak	AMP	ETP, CAZ10, CPT, NA (I), FOT, CXM, C, F300, F100
A16B	A16B2CTX	*Escherichia coli*	veal Round Meat	CTX	CAZ10, CPT, PRL30, TZP36 (I), CXM, PRL100, TZP110 (I)
	A16BCIP	*Escherichia coli*		CIP	CPT, PRL30, ENR, NA, PRL100, TE
A19B	A19BCTX	*Escherichia coli*	Filet steak	CTX	CAZ10, CPT, PRL30, TZP36, FOT, CXM, PRL100, TZP110 (I)

Table 13 - Phenotypic resistance regarding other antibiotic classes of packaged bovine meat samples. The column of associated resistance shows only the antibiotics to which the isolates presented resistance or intermediate resistance. **Abbreviations**: CAZ10 – Ceftazidime 10 µg; ; CPT – Ceftaroline; PRL30 or PRL100 –

Piperacillin 30µg or 100µg; ENR – Enrofloxacin; NA – Nalidixic acid; C – Chloramphenicol; SXT – Trimethoprim/Sulfamethoxazole; TE – Tetracycline; F30 or F100 – Nitrofurantoin 30µg or 100µg; TOB – Tobramycin;; TZP36 or 110 – Piperacillin/Tazobactam 36µg or 110µg; CXM – Cefuroxime; FOT - Fosfomycin; (I) – intermediate resistance

Sample	Isolate	Identification	Product	Selection medium	Associated resistance
A1C	A1CMRP	*Pseudomonas* spp.	Rabbit meat	MRP	ETP, CAZ10, TZP36 (I), NA, FOT, CXM, C, F300, F100, SXT
	A1CCTX	*Pseudomonas* spp.		CTX	EFT, CAZ10 (I), CPT, NA (I), FOT, CXM, F300, F100
A2C	A2CCIP	*Escherichia coli*	Rabbit hands	CIP	CPT, PRL30, ENR, NA, PRL100, C, SXT, TE
A3C	A3CCTX	*Acinetobacter* spp.	Rabbit fricassee	CTX	ETP, CAZ10, CPT, PRL30 (I), TZP36 (I), NA, FOT, CXM, C, F300, F100, SXT, TE, TGC
	A3CMRP	*Acinetobacter* spp.		MRP	ETP, CAZ10, CPT, DOR (I), PRL30 (I), TZP36 (I), NA, FOT, CXM, PRL100 (I), F300, F100, SXT
	A3CCIP	*Escherichia coli*		CIP	CPT, PRL30, ENR, NA, PRL100, SXT, TE

Table 14 - Phenotypic resistance regarding other antibiotic classes of packaged rabbit meat isolates. The column of associated resistance shows only the antibiotics to which the isolates presented resistance or intermediate resistance. **Abbreviations:** CAZ10 – Ceftazidime 10 µg ; ETP – Ertapenem; EFT - Ceftiofur; DOR – Doripenem; CPT – Ceftaroline; PRL30 or PRL100 – Piperacillin 30µg or 100µg; ENR – Enrofloxacin; NA – Nalidixic acid; FOT – Fosfomycin; CXM – Cefuroxime; C – Chloramphenicol; SXT – Trimethoprim/Sulfamethoxazole; TE – Tetracycline; F30 or F100 – Nitrofurantoin 30µg or 100µg; TZP36 or 110 – Piperacillin/Tazobactam 36µg or 110µg; (I) – intermediate resistance

3.3 Molecular characterization of Gram-negative isolates presenting resistance to β-lactams and non β-lactams

Molecular characterization of Gram-negative bacteria regarding the genes that codify for β-lactam antibiotics resistance was performed for isolates that expressed typical β-lactamase phenotype, as confirmed by phenotypical tests.

For the *Pseudomonas* spp. isolates expressing carbapenem susceptibility reduction a multiplex PCR was performed for the search of typical carbapenemases.

Since that, in this study, a high number of *Escherichia coli* isolates expressing resistance to quinolones such as ciprofloxacin was discovered, this work focused on the search of genes that codified for quinolone resistance. Given the association with colistin use in veterinary and the selection of resistance in humans, PCRs were performed for genes that codify for colistin resistance. In other to evaluate the virulence potential of these isolates, the search of genes that codify for virulence factors was performed as well as for the *Escherichia coli* phylogenetic groups.

3.3.1 Molecular characterization of *Escherichia coli* isolates regarding the genes that codify for quinolone and colistin resistance

Colistin or polymyxin E, has been widely used in veterinary medicine worldwide. Although its use in humans was restricted, the recent increase in multirresistant bacteria has led to its administration as a last resort for complicated infections (55). Reports concerning colistin resistant isolates found in meat, due to their intensive use in veterinary medicine have increased in the last decade (60). Bearing this in mind, it was relevant to analyse the resistance of these isolates to colistin. Through the agar diffusion method, antibiotic susceptibility tests with colistin were performed in order to analyse phenotypical resistance. All samples showed to be susceptible to colistin at the used concentrations (10µg). However, the phenotypical analysis of colistin using these methods is not standardized and is not accepted by EUCAST or CLSI. Moreover, even though the resistance phenotype is not being expressed, it does not mean that the gene encoding for resistance is not present and inactive.

With the discovery of plasmid-mediated colistin resistance genes, the spreading of resistance to several microorganisms has led to concern with the efficacy of colistin not only in veterinary but also in humans. Therefore, the presence of plasmid-mediated genes that encode for colistin resistance was evaluated by PCR (55). As observed in Figure 8, there was no amplification of any of the 5 *mcr* genes used, showing that, for the known transferable colistin resistance genes, these isolates are not a possible vehicle for the passage of this resistance. These results can be corroborated with a recent study by Clemente et al., where bovine meat samples were analysed for the presence of colistin resistant phenotypes and mcr-1 and mcr-2 encoding plasmid-mediated colistin resistance. From the 12 samples processed in that study, zero presented colistin resistance (60).

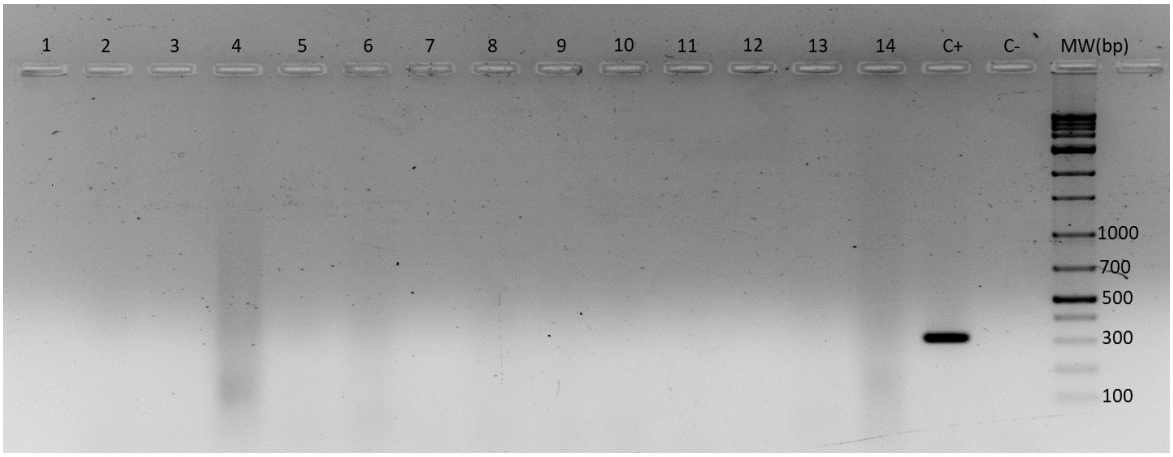

Figure 8 – Agarose gel electrophoresis of PCR products for mcr genes. Lanes 1-11: Bovine meat isolates; 12-14: Rabbit meat isolates; C+ :positive control (mcr-1); C-: negative control (water); MW (molecular weight)

Quinolones are extensively used in veterinary medicine as well as to treat human infections. With such a high rate of quinolones being given to animals, either to treat infections or just as prevention, an increase in the reports of quinolone resistant bacteria have been found (49,61). As mentioned in the first chapter, quinolone resistance can occur either through mutations in chromosomal genes of topoisomerase IV and DNA gyrase subunits *parC* and *gyrA,* respectively, or through acquired plasmid-mediated quinolone resistance (PMQR) genes *qnrA, qnrB, qnrC, qnrD, qnrS* and *aac(6')-Ib-cr* (31). PMQR genes present higher relevance in this study as these can be transferred to other bacteria, either from the same genus or not, by conjugation, transformation and other horizontal gene transfer mechanisms.

The resistance phenotype expressed by 12 *Escherichia coli* and 1 *Pseudomonas* spp.

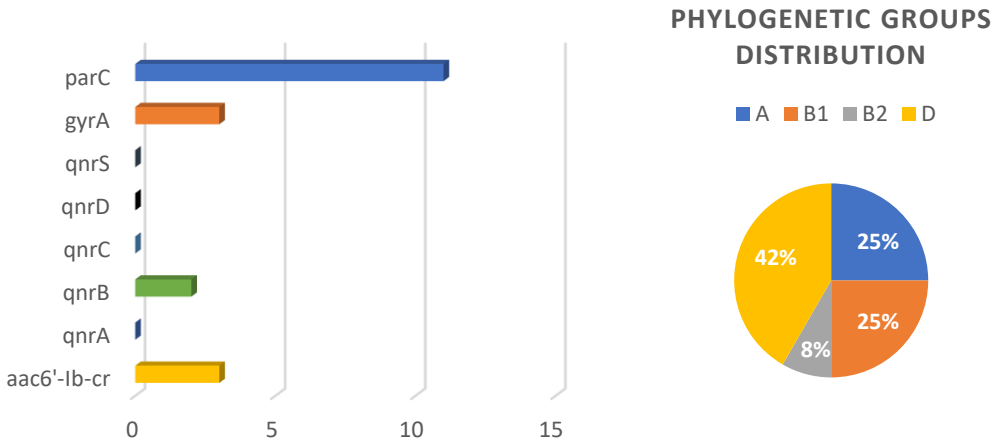

Figure 9 - Molecular characterization of *Escherichia coli* isolates regarding quinolone resistance genes (right) and phylogenetic groups (left)

isolates needed further confirmation by molecular methods, in order to corroborate the observed resistance phenotype. Analyzing Figure 9 and having in mind Table 15, it is possible to observe that 11 of the 13 samples tested, amplified for *parC* gene and 3 of the 11 samples also amplified for *gyrA* gene. Besides containing the *parC* gene, 3 samples also

amplified for the PMQR gene *aac (6')-Ib-cr*, and 2 samples amplified for *qnrB* gene. It is possible for a bacterium to contain more than one resistance gene in its DNA sequence.

Phylogenetic groups A, B1, B2 and D were analyzed by PCR (56). As previously mentioned, B2 and to a lesser extent D groups are associated with virulent *Escherichia coli* and B1 and A belong to commensal ones. Visualizing Figure 8 and Table 12, 5 isolates belong to group D, 3 to group A, 3 to group B1 and 1 to group B2. Both isolates belonging to group D and B2 were isolated from unpackaged bovine meat. These can result either from cross contamination from butcheries workers, cutlery or even from the slaughterhouse (62).

All the isolates belonging to the packaged samples were from groups B1 and A, which are typically commensal. The existence of *Escherichia coli* (although in low density, since all samples needed enrichment) is a proof of fecal contamination, possibly from slaughtering of meat from workers .

Sample	Isolate	Package	Product	Associated resistances	β-lactamases	Non β-lactam resistance genes	Phylogenetic Group	Virulence Factors
A3B	A3BCIP	Unpackaged	Minced meat	CPT, TE, ENR, NA, C, PRL30		qnrB, parC	B2	fimH, PAI, iutA, papC, papG allele II, traT, cvaC
A6B	A6BCIP	Vacuum	Matured meat	CPT, PRL30, TZP*, ENR, NA, TOB, CN, PRL100, SXT, TE, C, TZP100**		parC, gyrA	A	fimH, iutA, papC, traT
A8B	A8BCIP	Unpackaged	Sirloin meat	CPT, PRL30, ENR, NA, PRL100, SXT		Aac(6')-Ib-cr, qnrB, parC	A	fimH, iutA, rfc, traT
A9B	A9BCIP	Unpackaged	Chuck steak	CPT, PRL30, ENR, NA		Aac(6') – Ib-cr, parC	D	fimH, iutA, fyuA, kpsMTII, traT
A9B2	A9BAMP2	Unpackaged	Chuck steak	CPT, PRL30, ENR**, NA, PRL100*, SXT, TE	TEM	gyrA, parC	D	iutA, cvaC, traT
A10B1	A10B1CIP	Unpackaged	Minced meat	F30, F100		parC	--	--
A10B3	A10B3CIP	Unpackaged	Minced meat	CPT, PRL30, ENR, NA, PRL100, SXT, TE		parC	A	fimH, papG allele II, traT
A11B	A11BCIP	Unpackaged	Sirloin meat	CPT, PRL30, ENR, NA, SXT, TE		gyrA, parC	D	fimH, traT
A16B	A16BCIP	MAP	Veal round meat	CPT, PRL30, ENR, NA, PRL100, TE		-	B1	Rfc, traT
A20B	A20BCIP	Unpackaged	Minced meat	CPT, PRL30, ENR, NA, SXT, TE		-	D	FimH, iutA, traT
A21B	A21BCIP	Unpackaged	Veal rib	CPT, PRL30, ENR, NA, PRL100, C, SXT, TE		parC	D	fimH, iutA, traT
A2C	A2CCIP	MAP	Rabbit hands	CPT, PRL30, ENR, NA, PRL100, C, SXT, TE		parC	B1	fimH, traT
A3C	A3CCIP	MAP	Rabbit fricassee	CPT, PRL30, ENR, NA, PRL100, SXT, TE		Aac(6')-Ib-cr, parC	B1	fimH, iutA, traT

Table 15 - Summary of the phenotypic and genotypic resistance characteristics of the *Escherichia coli* and 1 *Pseudomonas* spp. (A10B1) isolates presenting quinolone resistance. **Label**: "-" did not amplify for the present genes; "--" was not evaluated; * intermediate resistance; ** resistant subpopulation. CPT – Ceftaroline; PRL30 or PRL100 – Piperacillin 30µg or 100µg; ENR – Enrofloxacin; NA – Nalidixic acid; C – Chloramphenicol; SXT – Trimethoprim/Sulfamethoxazole; TE – Tetracycline; F30 or F100 – Nitrofurantoin 30µg or 100µg; TOB – Tobramycin; CN – Gentamycin; TZP36 or 110 – Piperacillin/Tazobactam 36µg or 110µg.

3.3.2 Molecular characterization of *Escherichia coli* isolates regarding virulence factors

It was of relevance to study the possibility of these *Escherichia coli* isolates possessing virulence factors (Figure 10) that would enable their colonization and infection in humans. The emergence of isolates with virulence factors and that are resistant to quinolones poses a risk, especially in the YOPI's (Young, Old, Pregnant and Immunosuppressed), which are prone to infections.

The fact that these can be found in cut meat, ready to be cooked and, as previously mentioned, most of the times eaten undercooked, is a simple and alarming colonization method that can ultimately affect public health. Therefore, 29 virulence factors of *Escherichia coli* were analyzed through PCR procedures (57). It is possible to observe through Table 15 that the isolate belonging to phylogenetic group B2 contains the highest number of virulence factors, while B1 group isolates contains the lowest number of them. However, all the *E.coli* isolates contained at least 2 virulence factors, of which traT was present in all of them and fimH was only missing in 1 isolate. traT encodes for a siderophore and fimH for an adhesin. FimH that encodes for a type 1 fimbriae has been reported as common in pathogenic and non-pathogenic *E.coli* (63) and it is essential for the adherence to the host, reinforcing the importance of fecal colonizers in the development of infections (64).

Regarding the virulence factors in *E.coli* isolate belonging to B2, special interest is noted to the presence of PAI factor, that encodes for a pathogenicity associated island, which is a mobile genetic element that harbors virulence factors and can be later transferred to other bacteria (65). Besides PAI, fimH and traT factors, this isolate contains papC and papG

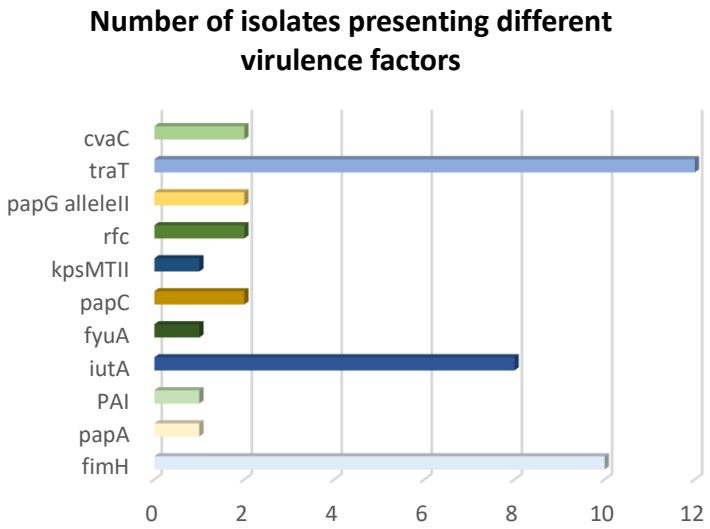

Figure 10 - Graph presenting the number of isolates that obtained amplification for the virulence factors shown. Vertical axis shows virulence genes and horizontal axis shows the number of isolates

genes that encode for P type fimbriae, iutA which is another siderophore (aerobactin receptor) that allows for the proliferation of bacteria in low iron environments such as tissues and fluids of hosts (66) and a cvaC gene that is located, along with traT gene and other virulence factors, in ColV plasmid. The fact that this particular *E.coli* is characterized as virulent (since it belongs to B2 phylogenetic group), possesses several virulence factors, including a pathogenicity island and contains both PMQR genes (qnrB) and chromosomal resistance gene (parC), is of extreme importance since that if ingested, it can lead to infections that cannot be treated by quinolones(56,67).

Overall, these types of bacteria in the gastrointestinal tract of humans, by ingestion of undercooked meat, like bovine meat, can not only pose risk of infection that can be difficult to treat but can also act as a reservoir of PMQR genes for the typical intestinal microflora, posing a risk to humans, specially immunocompromised people.

3.4 Molecular characterization of genes that encode for carbapenem resistance in *Pseudomonas* spp. isolates

Carbapenem resistance is a growing problem worldwide, not only due to the limited treatment options that infected patients have but also to the rapid spreading and transfer of genes from an isolate to another of the same or of a different species/genus (29).

From the samples analyzed, 4 isolates of *Pseudomonas* spp. presented reduced susceptibility to carbapenems. The CIM results were intermediate, existing the possibility of carbapenemase activity at low rates. Given this, the isolates were screened for genes encoding for carbapenem resistance through a multiplex PCR for VIM, IMP, KPC, OXA-48 and NDM. Observing Figure 11, the isolates did not obtain amplification for the tested genes. This result can be due to several factors, such as the production of other types of carbapenemases, since the ones tested were the most common but not the only types reported (29). Another factor that could contribute to the above mentioned result can be due to other mechanisms of resistance, intrinsic to the *Pseudomonas* spp. isolates, that confer this reduction in susceptibility (68). There are several resistance mechanisms described in the literature for the *Pseudomonas* genus, more predominantly about *Pseudomonas aureginosa* such as the overexpression of efflux pumps, low outer membrane permeability, and the presence of an AmpC β-lactamase (69).

The chromosomal gene encoding for AmpC β-lactamase, has been described in *Pseudomonas* spp. isolates being effective against penicillin, and cephalosporines such as ceftazidime, cefotaxime, among others. A characteristic of this β-lactamase is conferring resistance to typical β-lactamase inhibitors, like clavulanic acid. Although these enzymes are susceptible to carbapenems, the association with other resistance mechanisms such as porin loss, seems to contribute to carbapenem resistance (68).

Regarding efflux pumps, there are several in *Pseudomonas* spp. of which 3 have been shown to lead to resistance to several β-lactams and carbapenems, specially reduced susceptibility of meropenem, by exporting the antibiotics to the exterior of the cell (68,69). Like cephalosporinases, the resistance to carbapenems by this method is usually associated with other mechanisms (68).

Low permeability of the outer membrane is the major contributor for carbapenem resistance, since the drugs path of entry to the cell is through porins (29). Loss or modification of outer membrane porin OprD is usually conjugated with presence of AmpC enzyme (68).

For a more accurate explanation for the reduced susceptibility to carbapenems exhibited by the *Pseudomonas* spp. isolates, analysis of these resistance mechanisms must be performed.

Although the presence of *Pseudomonas* spp. isolates in the bovine and rabbit meat samples was in low density (since all the samples were enriched in broth), it is still a case of concern as most of the isolates exhibited reduced carbapenem susceptibility. In the case of bovine meat, a type of food that is eaten undercooked most of the times, the presence of this small number of isolates could lead to a serious infection in immunosuppressed people that would be difficult to treat, due to its resistance phenotype.

Pseudomonas spp. is one of the predominant hospital pathogens, associated with immunosuppressed patients. *Pseudomonas aureginosa, c*an cause respiratory tract, urinary, gastrointestinal and other types of infections (68). These opportunistic pathogens are Gram-negative, non-fermenting, oxidase positive and can grow with limited oxygen, being easily spread through the environment (69).

Carbapenems and quinolones are usually used to treat pseudomonal infections. The fact that these bacteria possess a variety of intrinsic mechanisms of resistance, allied to the possibility of acquiring other genes that encode for resistance, has led to an increase in carbapenem and quinolone resistant *Pseudomonas* spp., as observed in this study, whereas from the 7 *Pseudomonas* spp. isolates, 4 presented reduced carbapenem susceptibility and 1 presented quinolone resistance mechanisms, through the mutation of the topoisomerase IV subunit *parC*.

Relatively to the fact that in Figure 10, 2 of the 5 positive controls did not amplify, this could be related with a possible degradation of the controls through time and not with problems in the PCR method, since there was amplification of the remaining 3 controls, in the correct MW, IMP (232 bp), OXA-48 (438 bp) and VIM (390 bp).

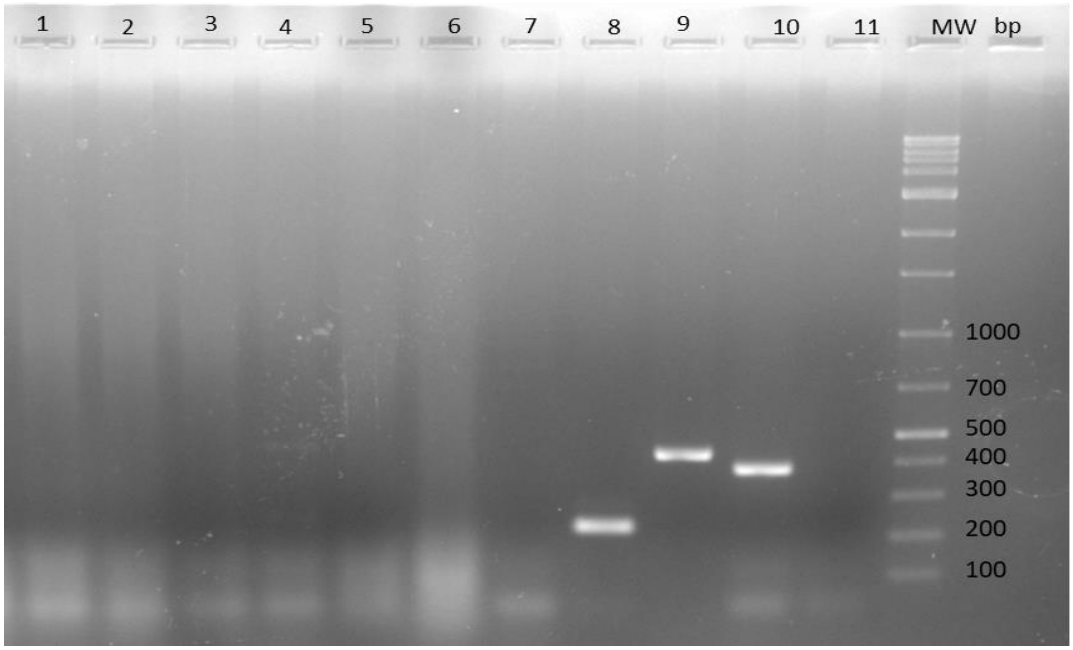

Figure 11 - Agarose Gel Electrophoresis of PCR products for carbapenemase encoding genes. Lanes 1-3: bovine meat isolates; 4: rabbit meat isolates 6: KPC control (798bp); 7: NDM control(621bp); 8: IMP control (232bp); 9: OXA-48 control (438bp); 10: VIM control (390bp); 11: negative control (water); MW: molecular weight

3.5 Molecular characterization of genes that encode for β-lactamases or β-lactam resistance in isolates suspected of being ESBL-producers

Isolates showing a β-lactamase-producing phenotype, confirmed through the DDST with CDT methods, were screened for the presence of β-lactamase encoding genes TEM, SHV, OXA and for the 5 main CTX-M groups. From all the isolates tested only 2 (A9BAMP2 and A10BCTX3, lane 7 and 8, respectively) identified as *Escherichia coli* amplified for TEM β-lactamase (Figure 12) and one of these isolates (A10BCTX3, lane 5) also amplified for CTX-M group 1 (Figure 13).

The isolate that amplified for a TEM β-lactamase does not seem to be an ESBL, since it showed resistance only to AMP, AMC. From this phenotype we determined that this isolate probably contains a TEM-1. This *E.coli* was also resistant to quinolones, as seen in Table 6, presenting both gyrA and parC genes. It belonged to phylogenetic group D, which like B2 group is considered to contain virulent *E.coli* strains. Besides the virulent component, the resistant phenotype also amplified for adhesins and siderophores, necessary for dissemination in humans (56,64).

Relatively to the *Escherichia coli* that amplified for TEM and CTX-M β-lactamase, this isolate presented resistance to several antibiotics, such as AMP, ATM, CTX and FEP, as well as intermediate resistance to CIP and CAZ. There was a clear synergy observed between

cefotaxime and clavulanic acid in the AST, indicative of the presence of an ESBL, which was further confirmed through the DDST+CDT.

As analyzed in the section 3.2 , both *Escherichia coli* isolates showed a multirresistant phenotype, since resistance to at least one drug from 3 different classes of antibiotics was observed.

The presence of fecal contamination indicators such as these bacteria in meat puts in risk the health of meat consumers, by enabling the colonization of the gastrointestinal tract by virulent bacteria that can cause a diverse number of infections. Allied to this, the fact that these isolates possess a multidrug resistance phenotype decreases the treatment options in case of an infection. Both isolates were found in unpackaged bovine meat samples, which highlights, once more, the need of packaging in controlling the type and number of bacteria in these products (58).

The low number of ESBL-producing bacteria found in these samples (1 isolate) is not surprising given that there are few reports of these bacteria in bovine meat and it is considerably higher in poultry and swine (61).

In both Figure 12 and 13, faint amplification bands can be observed which can or cannot be corresponded with the primers used. This slight amplification was not considered and was thought to be a problem with PCR reaction mixes such as impurities in water, high magnesium chloride concentration , DNA purity and high dNTP concentration (70,71). New PCR protocols should be performed in order to achieve single amplification bands for the different samples to further confirm these results.

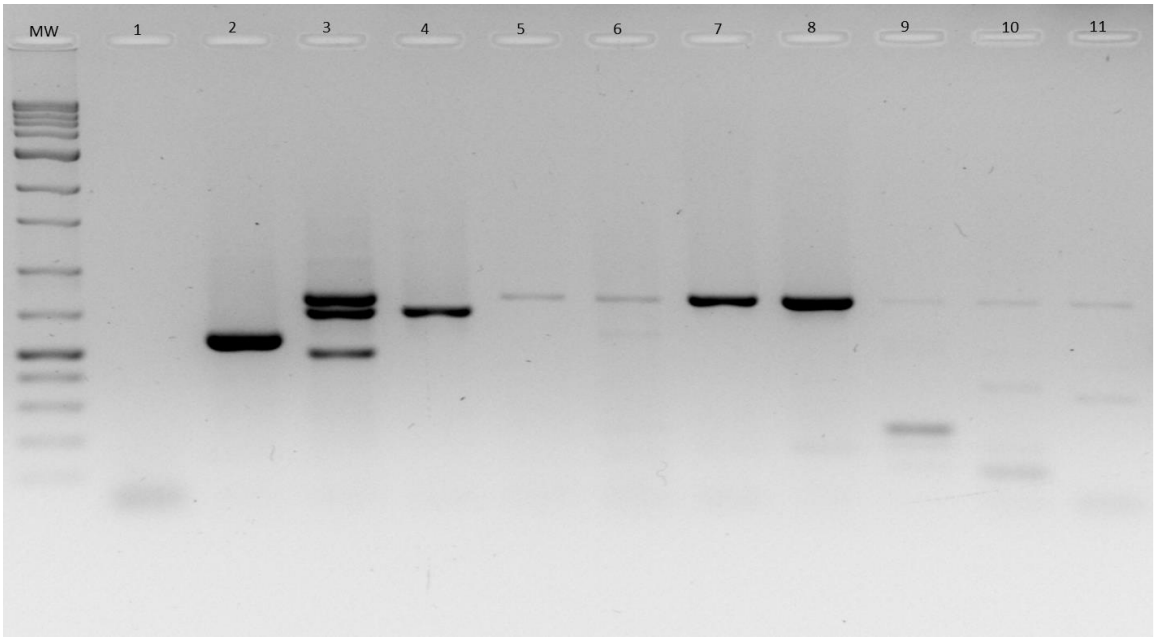

Figure 12 - Agarose gel electrophoresis of the multiplex PCR products for TEM, SHV and OXA genes. **Lanes** 1: negative control(water) ; 2: OXA(564bp); 3: TEM (800bp) and SHV(713bp); 4: SHV(713bp); 5-11: bovine meat isolates; MW – Molecular weight (bp)

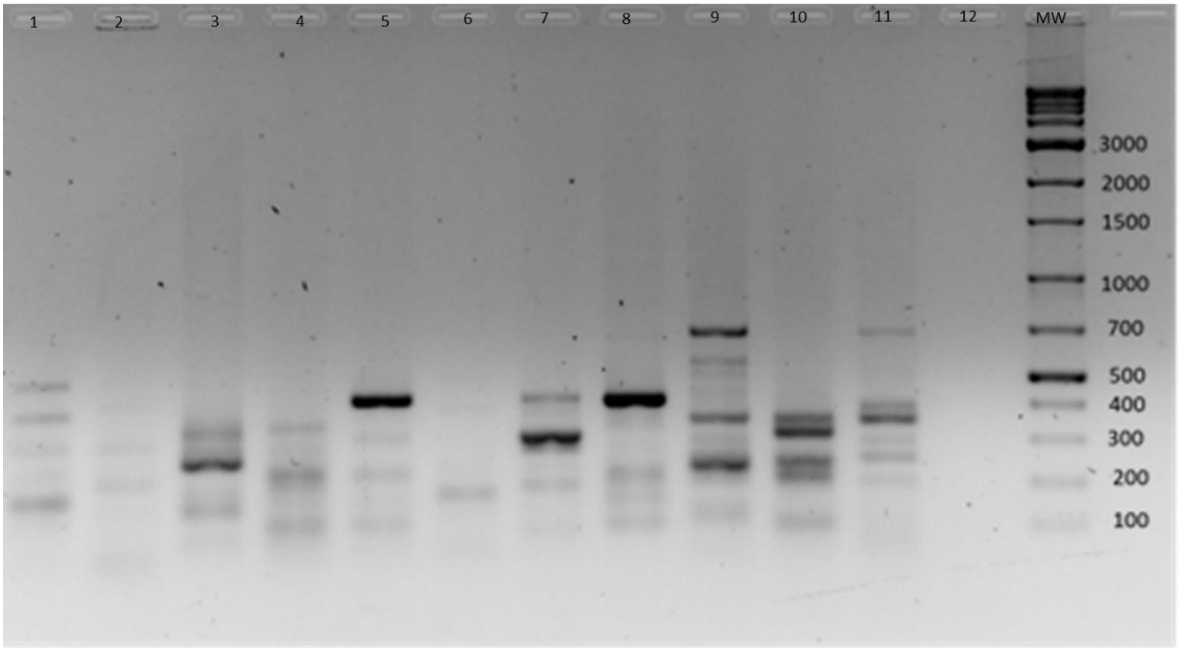

Figure 13 - Agarose gel electrophoresis of the multiplex PCR products for CTX-M group 1, 2, 8, 9 and 25 genes. **Lanes** 1-7: bovine meat isolates; 8: CTX-M G1 control (415bp); 9: CTX-M group 2 control(552bp); 10: CTX-M group 9 control (205bp): 11: CTX-M group 8 control (666bp); 12: negative control (water); MW- molecular weight (bp)

4 Conclusions

In this work, several retail bovine and rabbit meat samples were analysed in order to evaluate the presence of antibiotic resistant bacteria. From the results observed we could find that:

- Unpackaged bovine meat samples contained the double of the quantity of antibiotic resistant bacteria when compared with packaged samples, demonstrating the need for packaging in order to control the proliferation of bacteria in these products or better hygienic conditions in retail;

- Presence of spoilage microorganisms such as *Pseudomonas* spp. and *Acinetobacter* spp. in samples with multirresistant characteristics. Several *Pseudomonas* spp. isolates showed reduced susceptibility to carbapenems, last line antibiotics, proving the ability to cause serious infections with limited chances of treatment in humans;

- Presence of fecal contamination indicators (*Escherichia coli*) in most of the meat samples, indicating contamination of the products with either human or animal fecal matter. This could have occurred either during slaughtering or anywhere in the transport chain to the retail market as well as by retail workers;

- *Escherichia coli* isolates with quinolone-resistant genes in meat with multirresistant and virulence characteristics, showing that not only these bacteria have the capacity to infect a host but may also act as reservoirs of quinolone resistance genes for endogenous bacteria;

- *Escherichia coli* producer of TEM β-lactamase and type CTX-M ESBL, with multirresistant phenotype, within unpackaged bovine meat samples demonstrating meat as a possible dissemination route of these bacteria with implications on human health;

- *Escherichia coli* producer of TEM β-lactamase, probably a TEM-1, and both chromosomal and plasmid-mediated quinolone resistance genes in unpackaged bovine meat sample, with virulence characteristics, demonstrating the capacity of these bacteria to acquire and possibly transfer resistance genes.

The results highlight meat as a possible dissemination pathway of antibiotic multirresistant isolates within human population and confirms that packaging systems influence the transmission of these bacteria by decreasing its proliferation. These will

not only enhance shelf-life of the products but also diminish colonization possibilities, due to low bacterial population. Nevertheless, a stricter quality control of these products, regarding antibiotic resistance should be employed, in order to control the dissemination of antibiotic resistant bacteria that can reach population.

5 Future Perspectives

In this study, it was possible to observe the presence of antibiotic resistance from faecal colonization indicator bacteria and other spoilage bacteria. However, further work should be considered in order to characterize all the isolates found throughout the study, such as:

- Species identification of presumptively identified isolates: some of the isolates found in the samples were not identified relatively to their species, due to the discontinuation of the ID32GN. Other methods such as PCR of the 16S ribosomal RNA could be performed to achieve a better identification of such isolates;

- Analysis of some of the isolates profile in terms of phenotypical resistance to other antibiotic classes;

- Characterization of genes that codify for AmpC phenotype: Molecular confirmation of the AmpC phenotype was not performed due to lack of primers. Further confirmation with accurate primers for the genes that encode for AmpC enzyme should be carried out;

- Conjugation procedures in samples that showed the presence of β-lactamases and fluoroquinolone resistance genes mediated through plasmids: Conjugation method using a well characterized receptor strain should be conducted to analyse if the bacteria possess the ability to transfer the plasmid-mediated resistance genes to others.

- Analysis of cutting surfaces, manipulators hands and grinding machines in order to correlate the resistance phenotypes found with the contamination surfaces: since that it is possible that butchers do not hygienize their hands properly; the cutting surfaces may not be washed during extensive work as well as the grinding machines, which can lead to the proliferation of bacteria and contamination of meat with antibiotic resistant bacteria.

- Analysis of other bovine meat samples, from different origins to obtain a broader view of typical meat microbiota and resistance patterns;

- Analysis of a larger number of rabbit and bovine meat samples (with and without packaging), to achieve a more significant result with an appropriate populational size (n=30).

The above-mentioned steps combined would give a more precise and complete view of the results obtained, highlighting the importance of a strict quality control of retail meat regarding antibiotic resistance.

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