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*Master Course in Food and Beverages Innovation and Management*

## **Study of the incidence of the resistances to carbapenems along some bovine and swine supply chains of the Marche Region**

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## **ACRONIMS AND ABBREVIATIONS**

ABS Absorbance

AR Antibiotic-resistance

ARG Antibiotic resistance gene

BLA  $\beta$ -lactamase

CPE Carbapenemase producing Enterobacteriaceae

CEG Carbapenem encoding gene

CLSI Clinical and Laboratory standard institute

ddPCR Droplet Digital PCR

D3A Dipartimento di Scienze Agrarie, Alimentari ed Ambientali  
(UNIVPM)

DISVA Dipartimento di Scienze della Vita e dell'Ambiente (UNIVPM)

DO Optic density

GES Guiana extended spectrum

HGT Horizontal gene transfer

KPC Klebsiella pneumoniae carbapenemase

LB Luria Bertani

MDR Multidrug resistance

MH Muller Hinton

MHII Muller Hinton II

MHT Modified Hodge Test

MIC Minimal Inhibitory Concentration

NDM New Delhi metallo-beta-lactamase

OMP Outer membrane protein

OXA Carbapenem-hydrolysing oxacillinase

PBP Penicillin binding protein

UFC Colony forming unit

VIM Verona integron-encoded metallo-beta-lactamase

## **1. INTRODUCTION**

### **1.1. Antibiotics**

Antibiotics are antimicrobial substances which can act as bacteriostatic, interfering with the growth of microorganisms and preventing them from dividing, or as bactericidal, killing microorganisms directly. They are used to treat or prevent infections in humans and animals. Production of antibiotics by microorganisms is a naturally occurring event, but not all antibiotics are produced by bacteria or fungi. Some antibiotics are partially or completely produced by chemical synthesis in specialized laboratories. Antibiotics can be classified in different groups based on their mechanisms of action and chemical structure (Geisla et al., 2012). The discovery, commercialization, and administration of antimicrobial compounds to treat infections can be considered a revolution in terms of modern medicine. Indeed, antibiotics have gained tremendous importance for the development of complex medical approaches.

### **1.2 Antibiotic Resistance**

Antibiotic resistance is the ability of bacteria to resist the action of an antimicrobial agents. Acquired antibiotic resistance is the major concern linked to the overuse and misuse of antibiotics. Indeed, bacteria are characterized by a substantial genetic plasticity that allows them to respond to different environmental conditions that pose a threat to them, including the presence of antibiotic molecules. Bacteria had to develop mechanisms to coexist with competing microorganisms and therefore to survive the effect of the antibiotic molecule produced by them, thus acquiring an intrinsic resistance that allows them to survive and multiply (Munita et al., 2016). Unfortunately, the marked increase in antimicrobial resistance among common bacterial pathogens is now threatening the importance of antibiotics in our modern medicine. The World Health Organization has named antibiotic resistance as one of the three most important public health threats of the 21st century (WHO; 2014). Infections caused by multi-drug

resistant organisms (MDR) are responsible for a high rate of mortality. The Centers for Disease Control and Prevention estimates that at least 23,000 people die each year in the United States from an infection caused by an antibiotic-resistant microorganism (Centers for Disease Control and Prevention, 2013). Additionally, according to a recent report, antibiotic resistance is estimated to cause approximately 300 million premature deaths by 2050. (Jim O'Neill, 2015). This situation is aggravated by the lack of a robust antibiotic pipeline, resulting in the onset of infections that are becoming untreatable and leaving doctors with no viable alternative to treat infected patients. In order to understand the problem of antimicrobial resistance, it is useful to discuss a relevant concept. Antimicrobial resistance appeared early in evolution and it is the result of the interaction of many microorganisms with their environment. Most antimicrobial compounds are mainly produced by different microorganisms in a natural way, and, in order to survive, co-resident bacteria have evolved mechanisms to overcome their action. Thus, these microorganisms are often considered to be “intrinsically” resistant to one or more antimicrobials. However, when discussing the antimicrobial resistance, bacteria carrying intrinsic determinants of resistance are not considered the main focus of the problem (Jose et al., 2016). Rather, the main issue is related to microorganisms that express “acquired resistance” in a bacterial population that was originally susceptible to the antimicrobial compound. The development of acquired resistance can be the result of mutations in chromosomal genes or due to the acquisition of external genetic determinants of resistance, likely obtained from intrinsically resistant microorganisms present in the environment. Another important consideration to make is related to the fact that the concept of antimicrobial resistance in clinical practice is a very complex and relative phenomenon. The creation of clinical susceptibility breakpoints (sensitive, intermediate and resistant) is mainly based on the *in vitro* activity of an antibiotic against a bacterial strain, combined with some pharmacological parameters. Therefore, the clinical scenario and the availability of treatment options may vary the interpretation of susceptibility patterns in the treatment of antibiotic resistant bacteria.

### 1.2.1 Genetic bases of antibiotic resistance

In general, the main mechanisms of antimicrobial resistance are: modification of the antimicrobial target site, decreased cell permeability to the antibiotic, activation of efflux mechanism to extrude the toxic molecules (efflux pump) and modification of metabolic pathways via modulation of regulatory networks. Bacteria evolve rapidly not only by mutation and rapid multiplication, but also by acquisition of DNA encoding advantageous features. Acquisition of exogenous DNA material through horizontal gene transfer (HGT) is one of the most important drivers of bacterial evolution and it is frequently responsible for the development of antimicrobial resistance in different bacterial species. The term “horizontal” is related to the transmission of genetic material between unrelated strains, not by inheritance. The genetic exchanges occur within and between bacterial species, and involve mobile genetic elements, such as plasmids, integrons, transposons, insertion sequences, and phage-related elements frequently harbouring resistance genes (Lilley et al., 2000) (Figure 1).

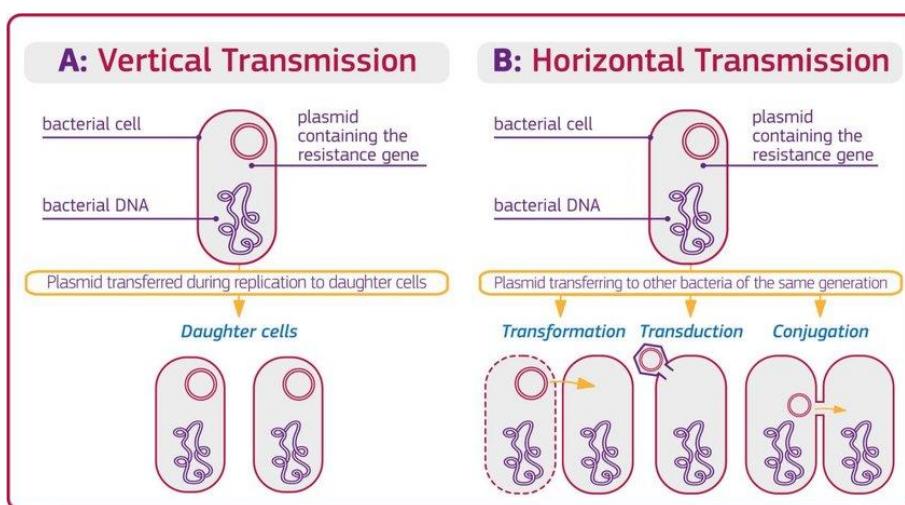


Figure 1: Mechanisms of antibiotic resistance genes transmission (Navarro et al., 2018).

Therefore, the transfer of genetic material (plasmids or genomic sequences) among bacteria is achieved through three different mechanisms: conjugation, transformation

and transduction (Shannon, 2015) (Figure 2). The identification of gene transfer related to both plasmids and viruses and the recognition of transposable elements, provided the stepping stones to our current picture of gene flux and so, the importance of mobile genetic elements (Thomas, 2000).

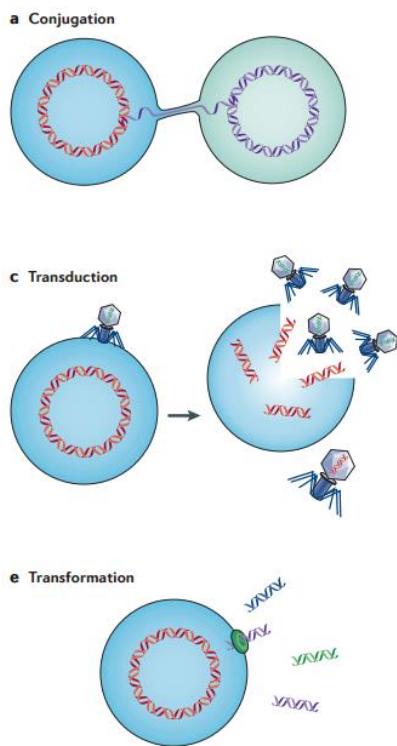


Figure 2: Bacterial strategies for the acquisition of external genetic material (Shannon et al., 2015)

- Transformation (incorporation of naked DNA): gene transfer results from the uptake by a recipient cell of naked DNA from a donor cell. Certain bacteria can take up exogenous DNA that can be incorporated into the recipient's chromosome. Transformation is perhaps the simplest type of HGT, but only a handful of clinically relevant bacterial species are able to "naturally" incorporate naked DNA (Johnston et al., 2014). For natural transformation to occur, bacterial cells must first develop a regulated physiological condition called "competence". The steps involved in this process include:

- Release of extracellular DNA in the environment.
- Stability of extracellular DNA in the environment.
- Uptake of DNA into the bacterial cell.
- Recombination of exogenous DNA with the host genome.

The proportion of bacteria that develop competence in a bacterial population might range from near zero to almost 100% because growth environments and factors that regulate competence development are correlated and vary between bacterial species and strains (Cohan et al., 1991) (Figure 3):

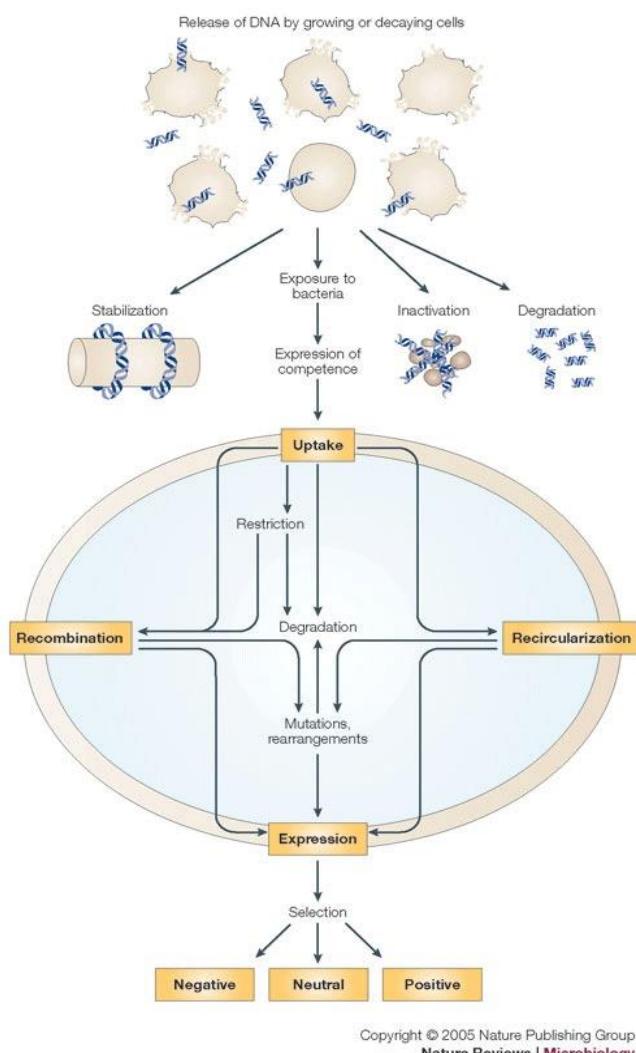


Figure 3: The natural transformation of recipient bacteria and selection of transformants (Thomas et al., 1998).

- Transduction (phage mediated): a bacteriophage acts as vector and “mobilize” bacterial genes from one bacterial cell to another. There are two types of transduction: generalized, in which a random piece of the host DNA is transferred; and specialized, in which only restricted parts of the bacterial chromosome can be transferred by a temperate phage.
- Conjugation (bacterial “sex”): the transfer of DNA fragment, mostly a plasmid occur through cell-to-cell contact thanks to a sexual type IV pilum. Resistance genes transfer by conjugation occurs at high rates also in the microbiota of human gastrointestinal tract under antibiotic treatment (Shannon et al., 2015).

### **1.2.2. Reservoirs of antibiotic-resistances**

Food animals, fish, and vegetables are considered large reservoirs of antibiotic-resistant bacteria, as the food production chain is an ecosystem composed of different ecological niches, where large amounts of antibiotics are used and numerous bacteria co-exist (Acar, 2006).

Due to the worldwide emergence of antibiotic resistance (AR), antibiotics are now an “endangered species”(O'Neill, 2015; WHO, 2015). An increasing number of treatment failures have been reported in patients with infections caused by resistant bacteria. The problem is that antibiotics normally used against bacteria are no more effective, so it becomes necessary to use other, so-called “last resort” antibiotic that are often more expensive and/or toxic (O'Neill, 2015). The use of antibiotics in animals has contributed to the rise of the global challenge of AR (FAO, 2015). In fact antibiotics are administered not only for therapeutic use, but also as metaphylactics in the intensive production farm and aquaculture, where the identification of an affected animal require treatment of the whole herd, and as prophylactics where sub-therapeutic doses are administered to contrast opportunistic infections developing in animals weakened by growth in stress conditions (FAO, 2015). In addition, another cause of the spread of antibiotic resistances is the extensive use of antibiotics as growth promoters (FAO, 2015). The agricultural environment in fact plays an important role in the spread of antibiotic residues in the

environment due to the fact that these medicines are often used as additives and biocides in crops. Consequently, adjacent natural environments consisting of water, soil and plants are environmental niches to consider in the dynamics of antimicrobial resistance (Taubeset al., 2008; Drlica et al., 2008). The selective pressure exerted on bacterial communities by widely discharged antibiotic residues in the environment contributes strongly to the emergence in numerous niches of antibiotic resistant bacteria. Consequently, since vegetables are grown in the soil, these bacteria can reach the food chain (Vakulenko et al., 2003). Acquired antibiotic resistance is also frequent among wild animal isolates which represents an area of concern (Kohanski et al., 2007). It should also be noted that bacterial resistance to antibiotics can be related to soil and native aquatic microorganisms with the ability to produce antimicrobial compounds (Dwyer et al., 2007; Kohanski et al., 2008). Several other factors contribute to antibiotic resistance like important anthropogenic actions such as international travel and global food trade (Espeli et al., 2004; Sugino et al., 1977). The human hand represents a classic example of a vehicle for the transmission of antibiotic-resistant bacteria, because it can easily become contaminated by environmental surfaces close to patients in hospitals or farm animals (Gellert et al., 1977; Hooper et al., 2003). Antibiotic-resistant bacteria, along the food chain, may reach humans in two different ways:

- Indirectly: through consumption of contaminated food or food derived products.
- Directly: through direct contact with infected animals or other biological substances (blood, urine, feces, saliva) (Chang et al., 2015).

In this regard the direct interaction of humans with the animal-ecosystem is essential to zoonotic transmission of antibiotic-resistant bacteria and antibiotic resistant genes (ARGs) from food animals-associated reservoirs to humans. Consequently, the World Health Organization is strongly committed to make people aware of the problem of antibiotic resistance, in particular through the promotion of good hygiene practices (Hooper et al., 2003; Rubinstein, 2001). There is an urgent need to clarify the correlation between the antibiotic use, the environment and natural bacterial communities, since microorganisms can be transmitted from an ecosystem to another with consequence

for human health (Lu et al., 2001). Different settings can constitute reservoirs of AR and ARGs, playing important roles in the spread of resistant bacteria to humans.

### **1.3. Older and new carbapenems antibiotics**

Carbapenems play a very important role in our fight against antibiotic resistant bacteria. In relation to the many hundreds of different  $\beta$ -lactams, carbapenems possess the widest spectrum of activity and greatest efficacy against Gram-positive and Gram-negative bacteria. As a result, they are often used as "last resort antibiotic" for serious infections caused by of antibiotic resistant strains (Bradley et al., 1999; Paterson et al., 2005; Torres et al., 2007). Unfortunately, the recent increase in multidrug-resistant (MDR) pathogens seriously threatens also this class of antibiotics (Queenan et al., 2007). Several recent studies clearly show that resistance to carbapenems is increasing throughout the world (Chouchani et al., 2011; Gopalakrishnan et al., 2010; Patel et al., 2011; Rossi, 2001). To find solutions to the increasing problem of antibiotic resistance bacteria,  $\beta$ -lactamase inhibition begins to acquire the most important role. In the late 1960s, in fact, when bacterial  $\beta$ -lactamases began to threaten the use of penicillin, the search for  $\beta$ -lactamase inhibitors began (Cole, 1980; Rolinson, 1991). In 1976 the first  $\beta$ -lactamase inhibitors were discovered; these were the olivanic acids a natural product produced by the Gram-positive bacterium *Streptomyces clavuligerus*. The olivanic acids possess a "carbapenemic backbone" and act as broad-spectrum  $\beta$ -lactamases (Rolinson, 1991) but unfortunately this compound is characterized by a chemical instability and poor penetration in the bacterial cell (Reading et al., 1984). After, two effective  $\beta$ -lactamase inhibitors were discovered: clavulanic acid and thienamycin. Clavulanic acid is produced by *Streptomyces clavuligerus* and clinically used in combination with  $\beta$ -lactams (Brown et al., 1976); thienamycin is a natural product isolated from *Streptomyces cattleya* (Albers-Schonberg et al., 1976). Thienamycin was the first discovered carbapenem and all carbapenems can be considered as derivatives of this compound.

### **1.3.1. Carbapenems structure and properties**

Carbapenems are antibiotics similar in structure to penicillins. They consists of a  $\beta$ -lactam ring fused with a five-membered ring with a double bond between C-2 and C-3 and Carbon at C-1 instead of sulfur (Figure 4). Carbapenems have a hydroxyethyl side chain linked to the  $\beta$ -lactam ring instead of acylamine substituent as in penicillins and cephalosporins; the hydroxyethyl side chain has a key role for carbapenem activity and resistance to hydrolysis by  $\beta$ -lactamases (Kahan et al., 1979). Thienamycin demonstrated a wide broad-spectrum antibacterial and  $\beta$ -lactamase inhibitory activity (Kropp et al., 1976; Kropp et al., 1979). Unfortunately, thienamycin was unstable in an aqueous solution, was sensible to hydrolysis of the mild base (above pH 8.0) and highly reactive to nucleophiles (Kahan et al., 1976). Given the chemical instability of thienamycin, its derivatives with greater stability were developed. The N-formimidoyl derivative, named imipenem (Miyadera et al., 1983) and other closely related carbapenems, such as panipenem, were later identified and proved to be more stable derivatives of thienamycin. In 1985, imipenem (originally called MK0787) became the first carbapenem available for the treatment of complex microbial infections. Imipenem, like its parent, thienamycin, has also shown a high affinity for PBP (Penicillin Binding Proteins) and stability against  $\beta$ -lactamases (Hashizume et al., 1984). However, both imipenem and panipenem are susceptible to deactivation by dehydropeptidase I (DHP-I), present at the edge of the human renal brush (Graham et al., 1987; Hikida et al., 1992). More recently, carbapenems with a broader spectrum, meropenem, biapenem, ertapenem and doripenem have been discovered and introduced in human therapy (Hashihayata et al., 2002; Kang et al., 1999; Lee et al., 2003). An important advance was the addition of a methyl group to the 1- $\beta$  position. This modification seems to be protective against the hydrolysis of DHP-I (Fukasawa et al., 1992). Several carbapenems were identified with this modification over the next 2 decades and they are similar to the carbapenems currently available, with a 1- $\beta$ -methyl and a pyrrolidine ring at C-2 (Imada et al., 1980).

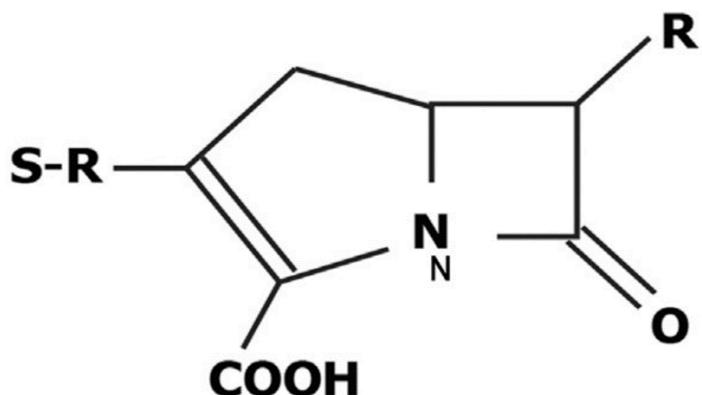


Figure 4: Chemical structure of carbapenem clinically available (Alizadeh et al., 2018)

### 1.3.2. Pharmacokinetics of carbapenems

Carbapenems are not able to easily enter into the bacterial cell (Martínez, 2008). In general, carbapenems are able to cross the membrane of Gram-negative bacteria through outer membrane proteins (OMP), also called porins. Once they have crossed the periplasmic space, the carbapenems bind to the Penicillin Binding Proteins (PBPs) (Figure 5) (Hashizume et al., 1984) that are enzymes (i.e. transglycolase, transpeptidase and carboxypeptidase) that catalyze the formation of 3-4 crosslinks between stem peptides of peptidoglycan in the cell wall of bacteria. Consequently, carbapenems act as inhibitors of the PBP transpeptidase activity, by acylating their active-site serine. The effectiveness of carbapenems consist of their ability to bind to several different PBPs (Hashizume et al., 1984). Since cell wall formation is a dynamic process, with the occurrence of peptidoglycan formation and autolysis simultaneously, when PBPs are inhibited, autolysis can continues (van Dam et al., 2009). The result of these processes is the weakening of the peptidoglycan structure and the consequent cell lysis due to osmotic pressure.

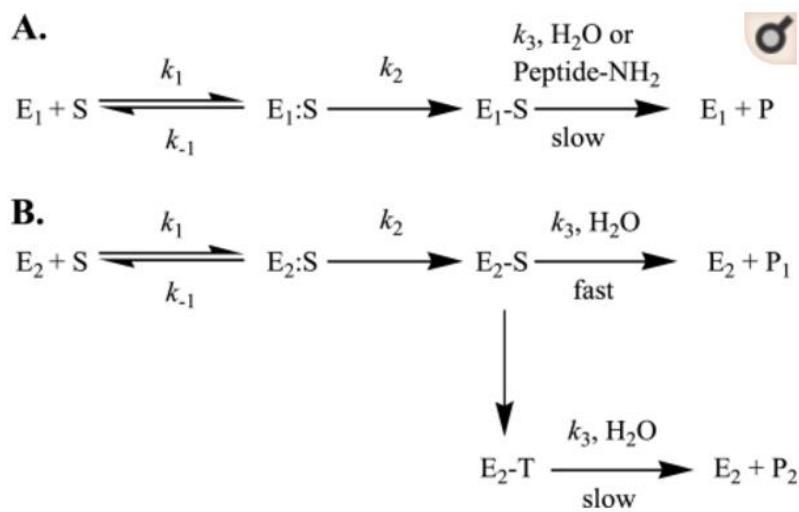


Figure 5: (A) Enzymatic scheme for  $\beta$ -lactam inhibition of PBPs. (Krisztina et al., 2011).

### 1.3.3. Resistance to carbapenems

A common mechanism of resistance to  $\beta$ -lactams is based on the action of  $\beta$ -lactamase enzymes which have the ability to hydrolyze these antibiotics. Specifically,  $\beta$ -lactamases are bacterial enzymes that inactivate  $\beta$ -lactams by opening the amide bond of the  $\beta$ -lactam ring.  $\beta$ -lactamases were first described in the early 1940s. However, there is evidence of their existence for thousands of years (D'Costa, 2011). After penicillin became widely available, resistant *Staphylococcus aureus* strains, producers of a penicillinase encoded by conjugative plasmids, were discovered. This resulted in the rapid spread of the resistance and of the infections caused by penicillin-resistant *S. aureus* (Bush, 2013). For this reason, the discovery and development of new  $\beta$ -lactam compounds with a broader spectrum of activity and a lower susceptibility to penicillinases was encouraged. However, in the 1960s a new  $\beta$ -lactamase encoded by plasmids capable of hydrolyze ampicillin was found among gram-negative bacteria (Paterson, 2005). From that moment on, unfortunately, the development of new generations of  $\beta$ -lactams has been systematically followed by the rapid appearance of enzymes able to degrade any new compound, in a process that is an excellent example

of adaptive bacterial evolution. The genes encoding  $\beta$ -lactamases are generally called *bla*, followed by the name of the specific enzyme (e.g. *bla<sub>KPC</sub>*) and have been found on the chromosome or located in MGE as part of the accessory gene pool of the bacterial genome. To date, more than 1000 different  $\beta$ -lactamases have been described and many others will probably continue to be reported as part of the normal process of bacterial evolution. Two main classification schemes have been proposed to group this large number of enzymes. First, the Ambler classification based on the amino acid sequence similarity,  $\beta$ -lactamases are divided into 4 groups A, B, C and D. Second, the Bush-Jacoby classification that divides  $\beta$ -lactamases into 4 categories according to their biochemical function, mainly related on the specificity of the substrate (Bush, 2013; Bush, 2010). The most common resistance mechanism to carbapenem is based on the production of specific enzyme called: carbapenemase, able to hydrolyze antibiotic molecule. However, carbapenem resistance can also be mediated by the activation of efflux mechanisms to extrude the toxic molecule, via specific proteins called efflux pumps.

### **1.3.4 Carbapenemase-producing *Enterobacteriaceae***

The *Enterobacteriaceae* family is composed of almost 100 different species of Gram-negative bacteria that colonize the intestine of human and other vertebrates as well as natural environments. They are ubiquitous microorganisms that can also be found in the soil, water and vegetable. *Enterobacteriaceae* are carriers of different genes encoding carbapenemases. In this way the carbapenemase-producing *Enterobacteriaceae* (CPE) show resistance to many  $\beta$ -lactams antibiotics (Tzouvelekis et al., 2012; Paterson et al., 2015; Fischer et al., 2017). The spread of CPE has recently become a matter of concern in public health. The rise in carbapenem resistance has been attributed to the rapid spread of plasmid-borne genetic determinants encoding  $\beta$ -lactamases (US Centers for Disease Control and Prevention, 2015). The first identification of CPE took place almost 20 years ago (USA, 1996) and became public knowledge only in 2001 (Yigit et al., 2001). *Enterobacteriaceae* are carriers of different subclasses of genes encoding

carbapenemases but the emerging global problems in *Enterobacteriaceae* are mainly associated five main genes:

- Class A β-Serine lactamase (*Klebsiella pneumoniae* carbapenemase KPC and GES) which has become endemic in parts of the Americas, southern Europe, Israel and China.
- Metallo-β-lactamase include three main families, IMP (metal-lactamase imipenemase), VIM (Verona integron-borne metal-lactamase) and, more recently, NDM variants (New Delhi metal-lactamase). There are several other acquired Class B carbapenemases that, although having not acquired global relevance, they are a problem in certain countries, in particular SPM (Sao Paulo metal-lactamase), which was found in *Pseudomonas aeruginosa* strains throughout Brazil.
- Class D Serine β-lactamase are highly diverse reflecting the diversity that exists within the entire Class. Four variants (OXA-23-like, OXA -40-like, OXA -58-like and OXA-143-like) are spread almost exclusively in *Acinetobacter* spp., while OXA-48-like enzymes are cause of growing concern in enterobacteria.

### **1.3.5 Reservoirs of carbapenemase-producing *Enterobacteriaceae* and of transferable carbapenem resistances**

In the last decade, the rapid and global dissemination of infections caused by CPE have been first found mainly in hospitals and healthcare institutions, becoming a serious problem since these outbreaks are often associated with high mortality rates due to an inadequate and limited existence of alternative treatments (Nordmann et al., 2011; Paterson et al., 2015; Rossolini, 2015; Grundmann et al., 2017; Zhang et al., 2017). But scientific studies underline the presence of carbapenemase-producers, and carbapenemase-encoding genes (CEG) also in animals, environment and food. European infection control experts from 13/38 countries reported inter-regional spread of CPE or an endemic situation in 2015, compared with 6/38 countries in 2013 (Magiorakos, 2013) The current opinion is that the acquired carbapenemases are a primary pressing public

health threat in terms of AR (Tzouvelekiset et al., 2012; EFSA BIOHAZ Panel, 2013; Woodford et al., 2014; Paterson et al., 2015). To evaluate the effective risk, occurrence and prevalence of zoonotic CRE in animal species a systematic search of the literature and extracted data about CPE in livestock, seafood, companion animals, food items, and exposed humans was performed.

The occurrence of CPE in livestock and sea food has been reported in African, American, Asian and European countries, mostly in studies performed after 2010 (Figure 6) (Nandi, 2013). Carbapenemase-encoding gene in CPE isolates from livestock or sea food items were first described in China (Qiao et al., 2017). The following carbapenemase genes were detected:

- Verona integron-encoded metallo Beta lactamase ( $bla_{VIM}$ )
- *K. pneumoniae* carbapenemase ( $bla_{KPC}$ )
- New Delhi metallo  $\beta$  lactamases ( $bla_{NDM}$ )
- Carbapenem-hydrolysing oxacillinase ( $bla_{OXA}$ )
- Imipenem carbapenemase ( $bla_{IMP}$ )

(Yaici et al., 2017)

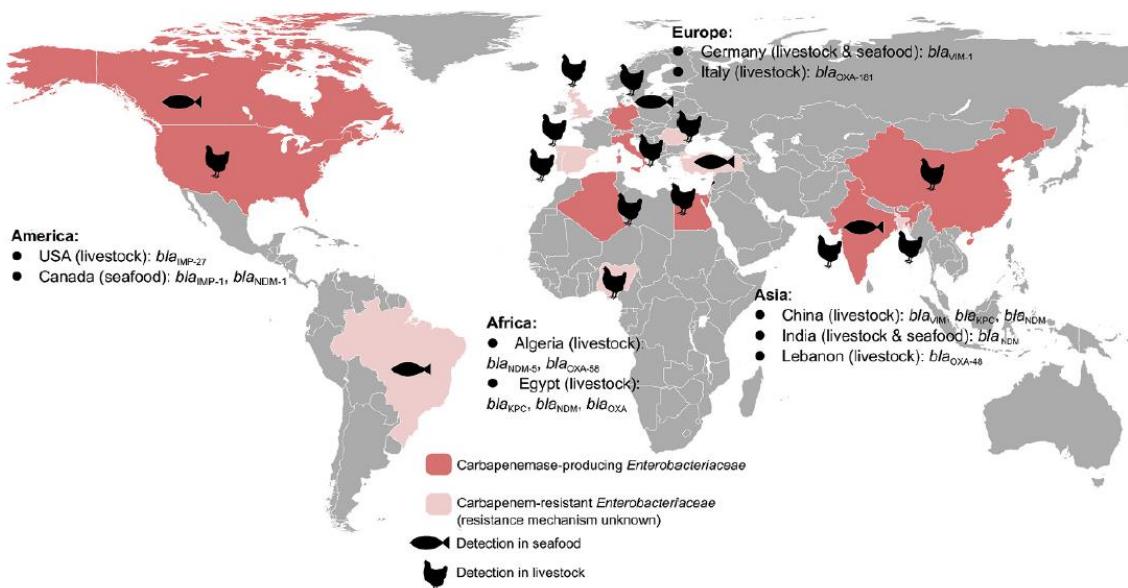


Figure 6. Global distribution of carbapenem-resistant and carbapenemase-producing *Enterobacteriaceae* in livestock and seafood (Kock et al., 2018).

Carbapenemases were associated with the following genera and species of *Enterobacteriaceae*: *Citrobacter* spp. (IMP, NDM), *Cronobacter sakazakii* (NDM), *Enterobacter* spp. (IMP) *E. coli* (NDM, VIM, OXA, IMP), *Klebsiella oxytoca* (IMP, NDM), *K. pneumoniae* (KPC, NDM, OXA) *Morganella* spp. (IMP), *Proteus* spp. (IMP) and *Salmonella* spp. (VIM, KPC, NDM). Despite the use of carbapenems is prohibited in food producing animals in all countries (OIE, 2015; Webbet et al., 2016), carbapenemase-producing *Enterobacteriaceae* are isolated from poultry (meat), chicken, poultry or fowl (meat), pig (meat), cows or raw milk, cattle and various type of seafood all around the world (Wang et al., 2017).

CPE in companion animals and wildlife have been reported in different countries (Figure 7). CPE isolates harbouring *bla*<sub>OXA-48</sub>, *bla*<sub>NDM-1</sub>, *bla*<sub>NDM-5</sub>, *bla*<sub>NDM-9</sub>, or *bla*<sub>VIM-1</sub> were detected in animals like dogs, cats (Yousfi et al., 2016; Schmiedel, 2014), horses (Yousfi et al., 2017), pet birds (Yousfi et al., 2017), swallows (Wang et al., 2005), wild boars (Bachiri et al., 2017), wild storks (Bouaziz et al., 2017), silver and yellow-legged gulls (Dolejska et al., 2016, Vittecoq et al., 2017) and in a black kite (Vittecoq et al., 2017). CEG were found in the following bacterial species isolated from companion animals and wildlife: *E. coli* (*bla*<sub>OXA-48</sub>, *bla*<sub>NDM-1</sub>, *bla*<sub>NDM-5</sub>, *bla*<sub>NDM-9</sub>, *bla*<sub>IMP-4</sub>, *bla*<sub>VIM-1</sub>, *bla*<sub>KPC-2</sub>), *Klebsiella* spp. (*bla*<sub>OXA-48</sub>, *bla*<sub>VIM-1</sub>, *bla*<sub>IMP-4</sub>, *bla*<sub>IMP-26</sub>), *Enterobacter* spp. (*bla*<sub>OXA-48</sub>, *bla*<sub>IMP-4</sub>), *Salmonella* spp. (*bla*<sub>IMP-4</sub>, *bla*<sub>NDM-1</sub>), *Proteus* spp. (*bla*<sub>IMP-4</sub>), *Kluyvera* spp. (*bla*<sub>IMP-4</sub>), and *Citrobacter* spp. (*bla*<sub>IMP-4</sub>, *bla*<sub>IMP-38</sub>).

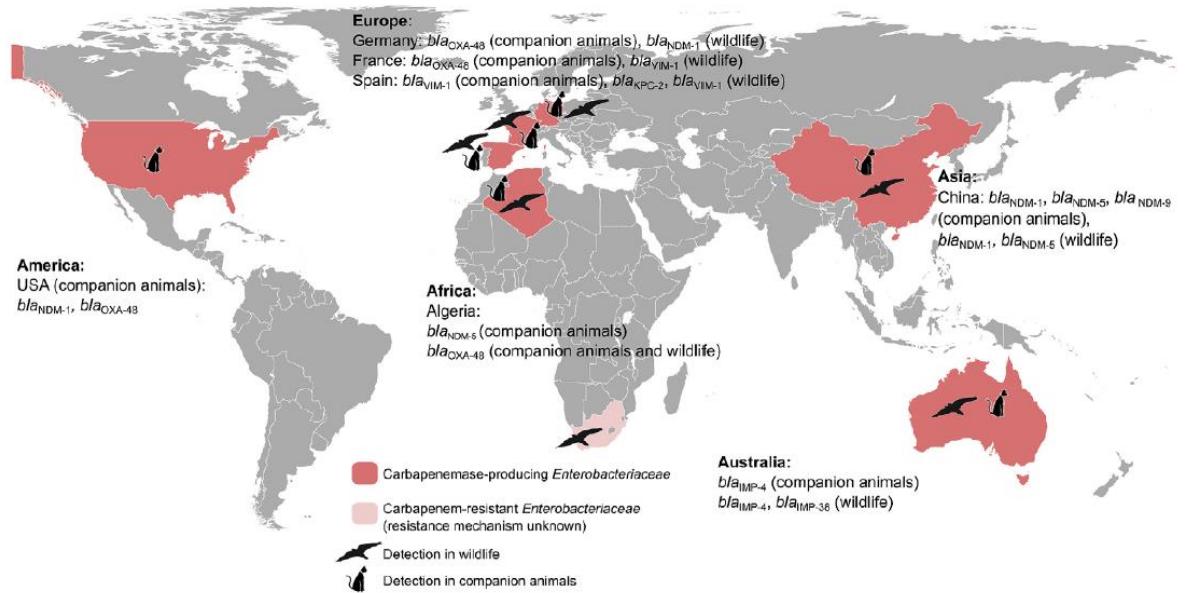


Figure 7: Global distribution of carbapenem-resistant and carbapenemase-producing *Enterobacteriaceae* in companion animals and wildlife (Kock et al., 2018).

In Europe, EARSN data for 2010 show that resistance rates in *K. pneumoniae* are higher than 5% only in some countries. Specifically, KPC β-lactamases were first observed in USA (Yigit et al., 2001) but are nowadays widely diffused worldwide, particularly in Greece (Tsakris et al., 2008) and in Italy (Canton et al., 2012). VIM carbapenemase was firstly described in Italy (Lauretti et al., 1999), whereas NDM enzyme was firstly isolated in Sweden, then in the UK and at the end worldwide (Kumarasamy et al., 2010; Poirel et al., 2010; Yong et al., 2009). OXA-48 have been identified mostly in the Mediterranean and southern European countries with a rapid spread in France and Turkey (Nordmann, 2014; Poirel et al., 2012). Although a number of bacterial species can harbor carbapenemase genes, they are most commonly described in *Klebsiella pneumoniae* isolates, which can carry different resistance determinants such as *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub>, *bla*<sub>NDM</sub>, *bla*<sub>OXA-48</sub> and *bla*<sub>IMP</sub>, linked to transmissible plasmids. The spread of CPE has dramatically increased in Italy since 2009. In fact in 2009 the percentage of invasive isolates of *K. pneumoniae* resistant to carbapenems was 1.3%, rising to more than 10% in 2010 and reaching the rate of 34.3% in 2013 (European Centre for Disease Prevention,

2010, European Centre for Disease Prevention, 2014). Recently, Monaco et al. (2014) pointed out that colistin resistance has emerged in Italy as an important topic in carbapenem-resistant *K. pneumoniae* (Monaco et al., 2014). After a number of different outbreaks in different hospitals in Emilia-Romagna region (Italy), caused by *K. pneumoniae* harboring KPC the Infectious Risk Unit of the *Agenzia Sanitaria e Sociale Regionale* (Gagliotti et al., 2011), develop guidelines to prevent other similar spread of CPE in the future. However, the prevalence of CPE among livestock seemed to be lower (<1%) in European countries (The Netherlands, Switzerland, UK), respect to other, like Asia (China: 15% in milk samples, India: 1–3% in piglets, Lebanon: 2.5% in fowl) and Algeria (6% in milk samples, 26% in chickens). Among wildlife, two studies found a high prevalence of CPE among silver gulls and yellow-legged gulls in Australia (15.9%) and France (19.4%), and wild boars in Algeria (Bachiri et al., 2017, Bouaziz et al., 2017; Vittecoq et al., 2017). In companion animals, 2.4–4% of cats, 1.5–2.6% of dogs, and 4% of horses tested in Algeria (Yousfi et al., 2016; Yousfiet al., 2017), as well as 0.6% of the dogs in France and Spain carried CRE (Melo, 2017, González-Torralba, 2016). Ceccarrelli et al. 2017, found that blaOXA-48-like genes detected in samples from European livestock were present in the (non-enterobacterial) genus *Shewanella* and were not associated with mobile genetic elements, suggesting a limited public health relevance. In contrast, cases of plasmid-encoded carbapenemases, such as VIM-1 in *E. coli* and *Salmonella* from German broilers and pigs or OXA-48-like in *E. coli* from Italian pigs may be of relevance for humans, as OXA-48 and VIM-1 predominate among CPE isolated from human clinical infections in Germany and other European countries (Grundmann et al. 2017; Kaase et al., 2015). In contrast to Europe, evidence was found for the dissemination of CPE among livestock in China, India, and Algeria (Yaici, 2016, Qiao et al. 2017, Yao et al., 2016, Wang et al., 2017, Wang et al., 2017, Wang et al., 2015). In conclusion looking at a global distribution of CPE among humans and animals, India, Northern Africa, and China can be considered endemic regions (Nordmann, 2014). Transmission of multidrug-resistant bacteria in households was found for MRSA (Meticillin resistant *Staphylococcus aureus*) and extended spectrum beta lactamase (ESBL) producing *E. coli* and might also be anticipated for CPE (David et al., 2010, Ljungquist et al. 2016). Hence, the emergence of

CRE in dogs, cats, and horses and the rather high prevalence detected in Northern Africa are cause for concern (Yousfi et al., 2016, Yousfi et al., 2017). This highlights the need to plan systematic screenings using modern molecular techniques and culture-based methods in combination. Transmission between animals and humans in both direction using this combined approach has so far been poorly investigated, but seems to be required for an evidence-based public health risk assessment.

## 1.5. Aim of the thesis

This thesis was carried out within the frame of a national project funded by the Cariverona Foundation titled “Dissemination of resistance to carbapenem antibiotics: search for environmental reservoirs”. The aim of the present thesis is to contribute to evaluate the presence and distribution of carbapenem resistances among food-producing animals with the scope of identifying possible non-human reservoirs of such resistances. In detail, the distribution of carbapenemase-producing *Enterobacteriaceae* (CPE) and carbapenem resistance genes (CRGs) have been evaluated through periodic sampling of products of animal origin from swine and bovine food chains as meat, salami, and cheese and also feces and feed. The sampling have been carried out in three different farms located in the Marche region. The samples have been analyzed by using culture-dependent methods to enumerate and isolate CPE. In addition, the microbial DNA have been extracted directly from the samples and it has been analyzed to verify the presence of the most common CRGs ( $bla_{KPC}$ ,  $bla_{VIM}$ ,  $bla_{IMP}$ ,  $bla_{NDM}$ ,  $bla_{OXA}$ ) using molecular techniques based on ddPCR (Droplet digital Polymerase Chain Reaction) that have been developed by the University of Verona which is involved in the project. Thanks to the collaboration with the “Dipartimento di Scienze Della Vita e dell’Ambiente-DiSVA” it was possible to carry out further assays to confirm the presence of CPE as the Hodge test and a quantitative assay based on the Minimum Inhibitory Concentration (MIC). The results of this research will contribute in determining safety aspects in terms of incidence of resistance genes to carbapenems and the presence of

potentially pathogenic bacteria for humans that could be carried by food producing animals.

## **2.0. Materials and methods**

### **2.1. Sampling**

A total of 41 samples were randomly withdrawn from three different livestock farms of pigs and bovine (one organic farm and two conventional farms) located in the province of Ancona (Marche region) in the period between November 2018 and March 2019. In detail, 17 samples of feces, 5 of feed, 2 of salami, 2 of cheese and 15 of meat have been sampled. The samples from the biological farm are listed in Table 1 whereas the samples from the conventional farms are listed in Table 2.

Table 1: List of samples obtained from an organic livestock farm

<b>SAMPLES</b>	<b>CODE</b>	<b>PERIOD OF SAMPLING</b>
Pig feces 1	FS1	26/11/2018
Beef feces 1	FB1	26/11/2018
Pig meat 1	CS1	26/11/2018
Beef meat 1	CB1	26/11/2018
cheese 1	FRV1	26/11/2018
Salami 1	SAL1	26/11/2018
Feedstuff 1	SF1	26/11/2018
Hay 1	F1	26/11/2018
cheese 4	FRB4	11/02/2019
Beef meat 4	CB4	11/02/2019
Salami 4	SAL4	11/02/2019
Beef feces 4	FB4	11/02/2019
Pig feces 4	FS4	11/02/2019
Pig feces 8 (1)	FS8(1)	12/03/2019
Pig feces 8 (2)	FS8(2)	12/03/2019
Pig meat 8 (1)	CS8(1)	12/03/2019
Pig meat 8 (2)	CS8(2)	12/03/2019

Table 2: List of samples obtained from the two conventional livestock farms (one for pigs and another for bovine)

SAMPLES	CODE	PERIOD OF SAMPLING
Beef feces 2	FB2	05/12/2018
Beef feces 3	FB3	11/12/2018
Beef meat 2	CB2	05/12/2018
Beef meat 3	CB3	11/12/2018
Beef feed 2	MB2	05/12/2018
Pig feces 5(1)	FS5(1)	25/02/2019
Pig feces 5(2)	FS5(2)	25/02/2019
Pig feces 5(3)	FS5(3)	25/02/2019
Pig feed 5	MS5	25/02/2019
Pig meat 5(1)	CS5(1)	25/02/2019
Pig meat 5(2)	CS5(2)	25/02/2019
Pig meat 6(1)	CS6(1)	06/03/2019
Pig meat 6(2)	CS6(2)	06/03/2019
Pig feces 6(1)	FS6(1)	06/03/2019
Pig feces 6(2)	FS6(2)	06/03/2019
Beef meat 7	CB7	06/03/2019
Beef feed 7	MB7	06/03/2019
Beef feces 7	FB7	06/03/2019
Pig feces 9(1)	FS9(1)	15/03/2019
Pig feces 9(2)	FS9(2)	15/03/2019
Pig meat 10(1)	CS10(1)	18/03/2019
Pig meat 10(2)	CS10(2)	18/03/2019
Beef feces 11	FB11	19/03/2019
Beef meat 11	CB11	19/03/2019

About 60g of all the feces samples were obtained from a mix of 3-5 stool samples. Meat samples of about 100 g was taken directly to the slaughterhouse. 30g of hay and 100g of feedstuff were taken directly from animal's mangen and silos. About 100 g of each sample of cheese and salami were taken from the company's sales counter.

Sampling was always carried out under sterile conditions, the samples were immediately stored at 4 ° C and processed within 24 hours.

## **2.2. Media**

Two different culture media were used for the isolation of *Enterobacteriaceae*: the MacConkey Agar for the viable counts, and the Luria Bertani Broth medium for the enrichment in carbapenem resistant *Enterobacteriaceae* of the homogenates.

### **2.2.1. MacConkey Agar**

MacConkey agar is a selective and differential media used for the isolation and differentiation of gram-negative enteric bacteria lactose fermenting. Colony morphology on MacConkey Agar is shown in Figure 8.

Pancreatic digestion of gelatin and peptones (meat and casein) provide the essential nutrients, vitamins and nitrogenous factors required for growth of microorganisms. Lactose monohydrate is the fermentable source of carbohydrate. The selective action of this medium is attributed to crystal violet and bile salts, which are inhibitory to most species of gram-positive bacteria. Sodium chloride maintains the osmotic balance in the medium. Neutral red is a pH indicator that turns red at a pH below 6.8 and is colourless at any pH greater than 6.8. Agar is the solidifying agent.

The lyophilized medium (the composition is indicated in Table 3) was diluted in water according to the indications given on the package (Oxoid Limited, United Kingdom) and subsequently sterilized in an autoclave at 121 ° C for 15 minutes. Ertapenem at concentration 0.12 µg/mL has been added to the sterilized media for the isolation of *Enterobacteriaceae* resistant to ertapenem.

Table 3: MacConkey Agar composition (Oxoid Limited, United Kingdom).

Typical Formula	g/L
Peptone	20.0 g/L
Lactose	10.0 g/L
Bile salts	1.5 g/L
Sodium chloride	5.0 g/L
Neutral red	0.03 g/L
Crystal violet	0.001 g/L
Agar	15.0 g/L
pH 7.1 ± 0.2 a 25°C	

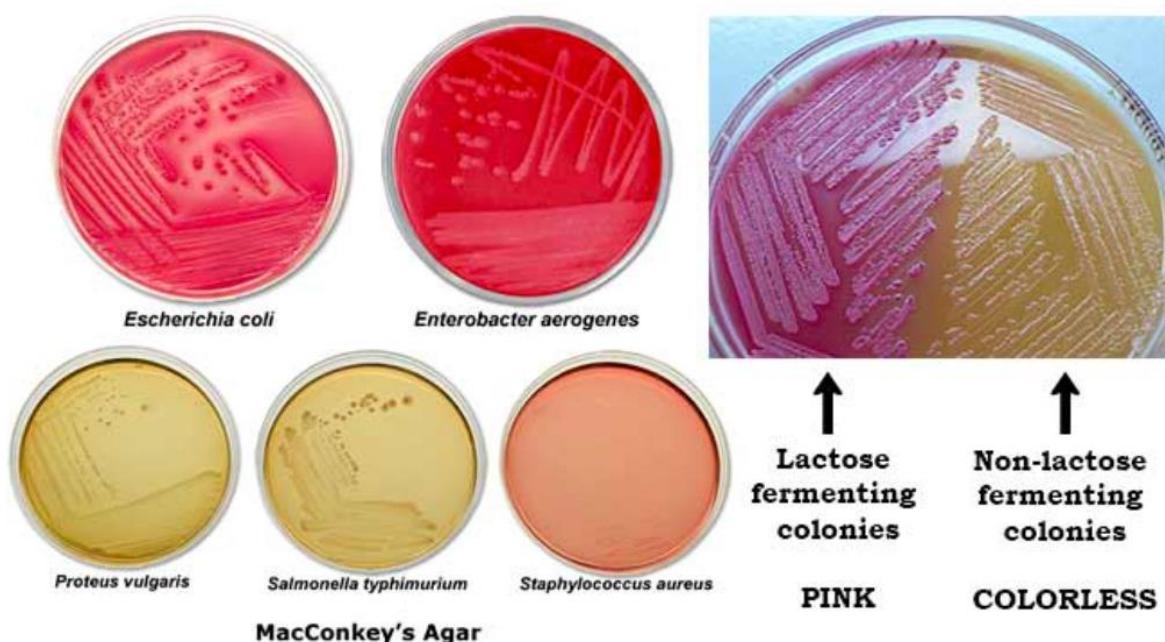


Figure 8: Colony morphology on MacConkey Agar.

### 2.2.2. LB (Luria-Bertani) broth

LB Broth is a rich liquid medium suitable for the growth of various bacterial species. It has been used for the purpose of increasing the number of bacteria within the homogenates for subsequent plating in MacConkey agar containing ertapenem (0.12 µg / mL). In this way it could be possible to increase the chances of selecting carbapenem-

resistant *Enterobacteriaceae*. The medium was prepared with the ingredients reported in Table 4. After sterilization ten mL aliquots of broth supplemented with ertapenem at a final concentration of 0.12 µg/mL were distributed in 15 mL tubes and inoculated with 100 µL of homogenate.

Table 4: Composition of LB Broth.

Ingredients	g/L
Tryptone	10 g/L
yeast extract	5 g/L
NaCl	10 g/L
pH 7± 0.2 a 25°C	

### 2.2.3. Mueller Hinton II broth and Mueller Hinton Agar

For the Hodge test and the MIC determination were used Mueller Hinton Agar (MH Agar, Sigma-Aldrich, Spruce Street, St. Louis) and Mueller Hinton II broth (MHII Broth, Sigma-Aldrich) respectively. These two media are routinely used for antimicrobial susceptibility tests and the composition is listed in Table 5 and Table 6. MHII Broth medium contains a final concentration of Ca<sup>2+</sup> ions equal to 20-25 mg/L and Mg<sup>2+</sup> ions equal to 10-12.5 mg/L.

Table 5: Composition of Mueller Hinton Agar (MH Agar, Sigma-Aldrich, Spruce Street, St. Louis).

Ingredients	g/L
Acid Hydrolysate of Casein	17.5 g/L
Bovine heart infusion	2.0 g/L
Soluble Starch	1.5 g/L
Agar	17.0 g/L
pH 7.3 ± 0.2 a 25°C	

Table 6: Composition of Mueller Hinton II broth (MHII Broth, Sigma-Aldrich, Spruce Street, St. Louis).

Ingredients	g/L
Acid Hydrolysate of Casein	17.5 g/L
Beef extract	3.0 g/L
Starch	1.5 g/L
pH 7.3 ± 0.2 a 25°C	

Mueller Hinton Media contains Beef Extract or Bovine heart infusion, Acid Hydrolysate of Casein, Starch and Agar (in Mueller Hinton Agar). Beef Extract and Acid Hydrolysate of Casein provide nitrogen, vitamins, carbon, amino acids, sulphur and other essential nutrients. Starch is added to absorb any toxic metabolites produced. Starch hydrolysis yields dextrose, which serves as a source of energy.

### 2.3. *Enterobacteriaceae* viable counts

The protocol reported in Figure 9 was used to perform the viable counts and the isolation of *Enterobacteriaceae* from the samples under study.

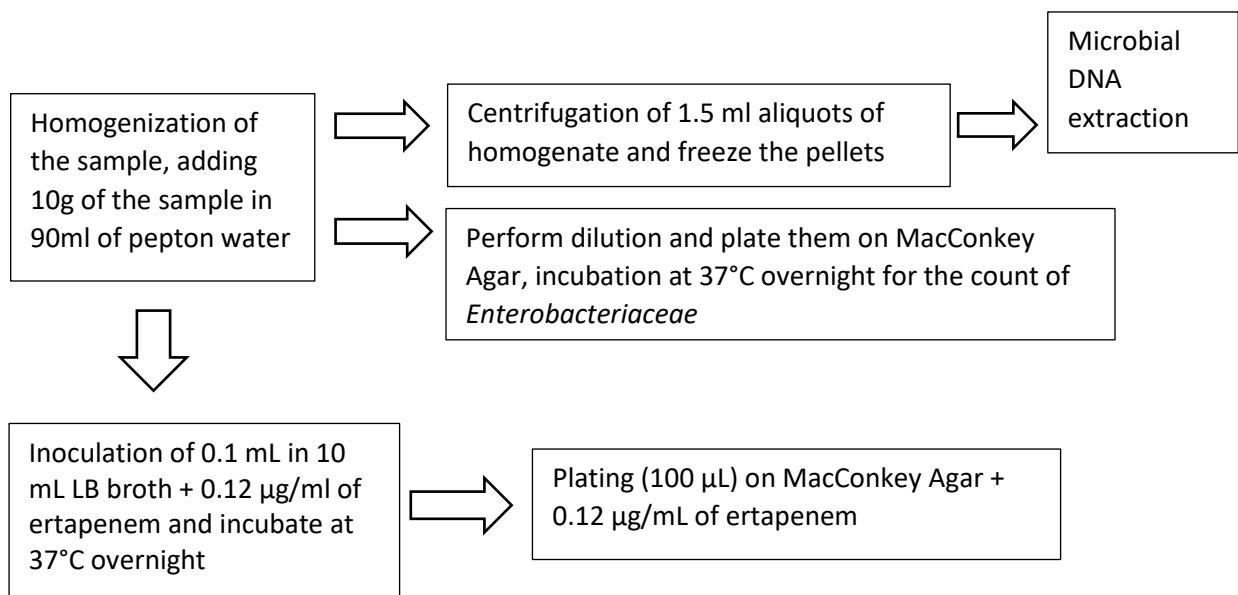


Figure 9: Analytical steps for enumeration and isolation of total *Enterobacteriaceae* and *Enterobacteriaceae* resistant to ertapenem

In detail 10 g of each sample (meat, feces, cheese, salami, feed) was homogenized in 90 ml of sterilized peptone water (10.0 g/l peptone). The homogenates were obtained by using the Stomacher® 400 Circulator (International PBI, Milan, Italy) by setting the program at 260 rpm for 2 minutes. Successive serial dilutions were prepared from the homogenate ( $10^{-1}$ dilution) up to dilution  $10^{-3}$  for the meat, salami and cheese samples, and up to  $10^{-5}$  for feces and feed samples. From each dilution 100 µL were inoculated onto a plate containing agar medium (MacConkey) using the spread plate method. The plates were incubated at 37 ° C overnight and then the enumeration of the colonies have been performed.

Plates with a number of colonies from 30 to 300 were considered for viable counting. Indeed, the number of Colony Forming Unit (CFU)/ g of sample was subsequently determined by multiplying the number of colonies counted in the plate (30-300 colonies) by the dilution factor of the same Petri dish. At the same time 1.5 mL aliquots of each homogenate were centrifuged and the pellets were freezed at -20 ° C for the subsequent extraction of microbial DNA.

In parallel, from the homogenate, 100 µL were taken and inoculate onto 10 mL of LB broth previously supplemented with ertapenem (0.12 µg/mL). The tubes were then incubated at 37° C overnight. The following day, the LB tubes that showed cloudiness were used to make serial dilutions, up to  $10^{-4}$ , and then from each dilution 100 µl have been spread on MacConkey + ertapenem 0.12 µg / mL. Plates were incubated at 37 ° C overnight.

The isolation of the presumptive resistant *Enterobacteriaceae* was carried out by taking morphologically different colonies from the MacConkey agar plates with antibiotic and the isolation was done using the streak plate method. These isolates were then used for the Hodge test and for the determination of the MIC.

## 2.4. Modified Hodge test

Modified Hodge test (MHT) was performed to confirm carbapenemase production (resistance to carbapenems) by isolates grown on MacConkey agar supplemented with ertapenem. Specifically, this test is very sensitive for *Enterobacteriaceae* producers of class A and class D carbapenemase. A dilution of *Escherichia coli* ATCC 25922 was prepared to achieve the concentration of  $10^8$  UFC/mL which corresponds to the optical density (DO) of 0.1 at 625 nm. The dilution was streaked as lawn on to a MH agar plate. A 10 µg ertapenem disk was placed, in the center of the test area. Test organisms were streaked in a straight line from the edge of the disk to the edge of the plate. The third ray was streaked with a resistant strain to ertapenem, producing carbapenemase. The plate was incubated overnight at 37°C for 16-24 hours. Quality control of the carbapenem disks were performed according to CLSI guidelines. MHA plate were analysed at the intersection of the streaked strain and the inhibition halo of *E. coli* strain ATCC®25922:

- MHT negative test: no enhanced growth of the *E. coli* ATCC 25922 at the intersection with the tested strain and no modification of the zone of inhibition of *E. coli* around the ertapenem disk (Figure 10).

-MHT positive test: enhanced growth (leaf-like indentation of the inhibition zone) of the *E. coli* ATCC 25922 at the intersection with the tested strain.

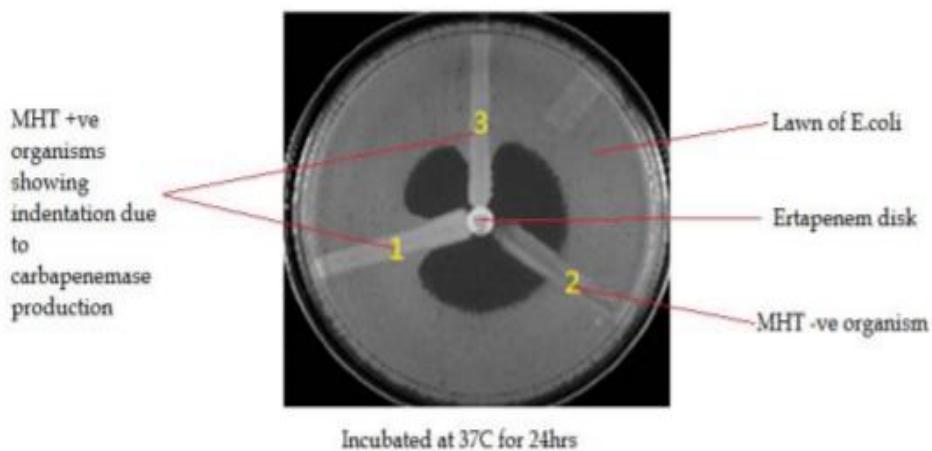


Figure 10: The MHT performed on a Muller Hinton Agar plate. (1) MHT positive result (2) MHT negative result; and (3) a clinical isolate, positive result (Amjad et al., 2011).

## 2.5. Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) determination provides the lowest concentration (in microgram/ml) of an antibiotic required to inhibit growth of a given bacterial strain. In this study this method was used for testing carbapenem susceptibility of the isolates.

This procedure has been performed according to the standard procedures of the "Clinical and Laboratory Standards Institute" (CLSI) using the broth microdilution method (Figure 11). Microplate used for this test are composed by 96 wells, in which antibiotic to be tested were diluted in MHII broth. In each horizontal row a different strain is inoculated, while in each column there are scalar concentrations of the antibiotic to be tested. In our case, the ertapenem concentrations ranged from 8 to 0.0075 µg/mL.

The antibiotic ertapenem was diluted in MHII broth at a final concentration of 16 µg/mL (at double of the highest concentration to be tested) from a stock solution of 10 mg/mL. Multi-channel pipette was used to inoculate 50µL of MHII in each well, with the exception of the first column. Subsequently 50µL of the antibiotic solution containing 16 µg/mL of ertapenem were inoculated in each well of the first and second column and serial two-fold dilutions were performed all over the microplate. The wells of the last column were used as growth control wells, therefore they contained antibiotic-free broth only. After microdilutions of the antibiotic a standardized inoculum of each bacterial culture was prepared. When 50µL of this suspension is inoculated into the broth of each well, the final concentration of bacteria should be approximately  $5 \times 10^5$  UFC/mL. The colonies grown in plate were diluted in to MHII Broth until they reached an optical density at 625 nm ( $DO_{625}$ ) of about 0.1, corresponding to a bacterial concentration of about  $1 \times 10^8$  UFC/mL. This cell suspension was further diluted to 1:100

by placing 50 µL in 5 mL of MHII Broth. Once the desired concentration of  $1 \times 10^6$  UFC/mL was obtained, 50 µL of the cell suspension was placed in the wells that already contained 50 µL of MHII Broth + ertapenem, in each well the final concentration obtained was equal to  $5 \times 10^5$  UFC/mL. Also a reference strain was inoculated (*E. coli* ATCC® 25922) in the microplate as control strain. The plate was finally incubated at 37° C overnight. The following day, after verifying that the control strain ATCC25922 showed a MIC within the expected MIC range, the plate was examined to determination of MIC results for tested isolates. The lowest concentration of antibiotic that inhibits growth of the strain was considered the MIC value of the strain for the tested antibiotic.

Carbapenem breakpoints reported by CLSI or EUCAST were used to MIC results interpretation. For ertapenem, the resistance breakpoint for *Enterobacteriaceae* is equal to a MIC  $>1$  µg/mL (source EUCAST). So strains able to grow in wells with ertapenem concentration  $>1$  µg/mL are resistant to ertapenem. Resistant or intermediate isolates (i.e. showing a MIC greater than or equal to 1µg/mL respectively against ertapenem) were further evaluated for the presence of carbapenemase encoding genes.



Figure 11: microtiter used for MIC determination.

## **2.6. Direct extraction of total microbial DNA from each samples**

The total microbial DNA extraction have been performed from each pellet obtained as indicated in Figure 8. In detail, 1.5 mL of each homogenate were placed in eppendorf tubes and then centrifuged at maximum speed for 5 minutes. The supernatants have been discarded and each pellet was stored at -20 ° C. For the extraction of the microbial DNA the molecular kit "E.Z.N.A.® Soil DNA Kit" from Omega Bio-tek was used, following the manufactures instructions. In detail:

1. Add 725 µL SLX-Mlus Buffer to the pellet. Vortex at maximum speed for 3-5 minutes to lyse samples.
  2. Spin at 500 x g for 5 seconds to remove drops of liquid from the lid.
  3. Add 72 µL DS Buffer. Vortex to mix thoroughly.
  4. Incubate at 70°C for 10 minutes. Briefly vortex the tube once during incubation.
  5. Centrifuge at 10,000 x g for 5 minutes at room temperature.
  6. Transfer 400 µL supernatant into a new 1.5 mL microcentrifuge tube (not provided).
  7. Add 135 µL chilled P2 Buffer. Vortex to mix thoroughly.
  8. Let sit on ice for 3 minutes.
  9. Centrifuge at maximum speed ( $\geq$ 13,000 x g) for 1 minute.
  10. Carefully transfer the supernatant to a new 1.5 mL microcentrifuge tube.
  11. Add 200 µL HTR Reagent. Vortex to mix thoroughly.
- Note: Completely resuspend HTR Reagent by shaking the bottle before use. It may be necessary to cut the end of the pipette tip to aspirate and dispense HTR Reagent.
12. Let sit at room temperature for 2 minutes.
  13. Centrifuge at maximum speed for 1 minute.
  14. Transfer cleared supernatant (~500 µL) to a new 1.5 mL microcentrifuge tube.

Note: If supernatant still has a dark color from the soil, repeat Steps 12-14 for a second HTR Reagent step. This will require additional HTR Reagent that can be purchased separately.

15. Add an equal volume XP1 Buffer. Vortex to mix thoroughly.
16. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube (provided).
17. Transfer up to 700 µL sample from Step 16 to the HiBind® DNA Mini Column.
18. Centrifuge at 10,000 x g for 1 minute at room temperature.
19. Discard the filtrate and reuse the Collection Tube.
20. Repeat Steps 18-20 until all the lysate from Step 16 has passed through the HiBind® DNA Mini Column.
21. Add 500 µL HBC Buffer.

Note: HBC Buffer must be diluted with 100% isopropanol before use.
22. Centrifuge at 10,000 x g for 1 minute.
23. Discard the filtrate and the Collection Tube.
24. Transfer the HiBind® DNA Mini Column into a new 2 mL Collection Tube.
25. Add 700 µL DNA Wash Buffer.
26. Centrifuge at 10,000 x g for 1 minute.
27. Discard the filtrate and reuse the Collection Tube.
28. Repeat Steps 25-27 for a second DNA Wash Buffer wash step.
29. Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes at room temperature.

Note: This step is critical in removing residual ethanol that may interfere with downstream applications.
30. Transfer the HiBind® DNA Mini Column into a clean 1.5 mL microcentrifuge tube.

31. Add 50-100  $\mu$ L Elution Buffer heated to 70°C directly onto the center of HiBind® matrix.
32. Let sit at room temperature for 1-2 minutes.
33. Centrifuge at maximum speed for 1 minute.
34. Take the filtrate from Step 33 and place onto the center of the same HiBind® DNA Mini Column used in the procedure.
35. Let sit at room temperature for 1 minute.
36. Centrifuge at maximum speed for 1 minute.
37. Store eluted DNA at -20°C.

## **2.7. Quantification of DNA extracted by spectrophotometer**

The concentration of DNA extracted from each sample was determined by spectrophotometer using the UV-1800 Spectrophotometer (Shimadzu Corporation, Japan). For spectrophotometer analysis, 5  $\mu$ L of each DNA was mixed with 495  $\mu$ L of sterile deionized water in a quartz cuvette to obtain a 1:100 dilution. The spectrophotometer reading was carried out at a wavelength of 260 nm. For the calculation of the DNA concentration the following formula was used:

$$1: 50 \text{ } \mu\text{g mL}^{-1} = A_{260} : Y \text{ } \mu\text{g mL}^{-1}$$

Where:

$$Y \text{ } \mu\text{g mL}^{-1} \times 100 \text{ (dilution factor)} = \text{DNA concentration in each sample in } \mu\text{g mL}^{-1}$$

### 3. Results and Discussions

#### 3.1. *Enterobacteriaceae* viable counts

The enumeration of the *Enterobacteriaceae* was performed on MacConkey agar plates without the addition of antibiotic. In the Tables 7 and 8 are reported the results of the viable counts of *Enterobacteriaceae* performed on raw meat, fecal, feed and food samples from organic and conventional livestock farm, respectively. The results are expressed as log colony forming units (log CFU) per gram of the sample ± standard deviation. Moreover, in each table is reported the concentration of the total microbial DNA extracted directly from each sample.

Table 7: Results of the viable counts of *Enterobacteriaceae* and of the concentration of the microbial DNA extracted directly from the samples obtained from the organic livestock farms with the concentration.

SAMPLES	CODE	PERIOD OF SAMPLING	log CFU/g	DNA ng/ µL
Pig feces 1	FS1	26/11/2018	6.54±0.02	55
Beef feces 1	FB1	26/11/2018	3±0.0	29.5
Pig meat 1	CS1	26/11/2018	3.5±0.09	64.5
Beef meat 1	CB1	26/11/2018	1.54±0.03	10
Cheese 1	FRV1	26/11/2018	<1±0.0	15
Salami 1	SAL1	26/11/2018	<1±0.0	77.5
Feedstuff 1	SF1	26/11/2018	5.73±0.04	22
Hay 1	F1	26/11/2018	4.65±0.02	165
cheese 4	FRB4	11/02/2019	<1±0.0	15
Beef meat 4	CB4	11/02/2019	5.42±0.01	16.5
Salami 4	SAL4	11/02/2019	<1±0.0	10
Beef feces 4	FB4	11/02/2019	5.65±0.04	110
Pig feces 4	FS4	11/02/2019	5.73±0.14	138
Pig feces 8 (1)	FS8(1)	12/03/2019	6.98±0.03	120
Pig feces 8 (2)	FS8(2)	12/03/2019	5.09±0.12	71
Pig meat 8 (1)	CS8(1)	12/03/2019	1±0.0	50
Pig meat 8 (2)	CS8(2)	12/03/2019	<1±0.0	95

Table 8: Results of the viable counts of *Enterobacteriaceae* and of the concentration of the microbial DNA extracted directly from the samples obtained from two conventional livestock farms

SAMPLES	CODE	PERIOD OF SAMPLING	log CFU/g	DNA ng/ µL
Beef feces 2	FB2	05/12/2018	5.71±0.05	23
Beef feces 3	FB3	11/12/2018	6.71±0.02	137.5
Beef meat 2	CB2	05/12/2018	<1±0.0	108.5
Beef meat 3	CB3	11/12/2018	3.48±0.02	461.5
Beef feed 2	MB2	05/12/2018	2±0.0	61
Pig feces 5(1)	FS5(1)	25/02/2019	7.93±0.01	123.5
Pig feces 5(2)	FS5(2)	25/02/2019	6.52±0.04	280
Pig feces 5(3)	FS5(3)	25/02/2019	6.15±0.04	85
Pig feed 5	MS5	25/02/2019	4.73±0.03	15
Pig meat 5(1)	CS5(1)	25/02/2019	1±0.0	40
Pig meat 5(2)	CS5(2)	25/02/2019	2±0.0	160
Pig meat 6(1)	CS6(1)	06/03/2019	4.10±0.02	8
Pig meat 6(2)	CS6(2)	06/03/2019	4.9±0.01	110
Pig feces 6(1)	FS6(1)	06/03/2019	8.01±0.01	79
Pig feces 6(2)	FS6(2)	06/03/2019	4.74±0.03	52
Beef meat 7	CB7	06/03/2019	1±0.0	22.5
Beef feed 7	MB7	06/03/2019	6.19±0.06	15
Beef feces 7	FB7	06/03/2019	6.15±0.04	26.5
Pig feces 9(1)	FS9(1)	15/03/2019	6.83±0.0	130
Pig feces 9(2)	FS9(2)	15/03/2019	6.80±0.01	58.5
Pig meat 10(1)	CS10(1)	18/03/2019	2.15±0.21	28
Pig meat 10(2)	CS10(2)	18/03/2019	2.66±0.26	53
Beef feces 11	FB11	19/03/2019	5.98±0.15	42
Beef meat 11	CB11	19/03/2019	3.26±0.12	155

The results showed a great variability among the analysed samples. The samples of pig and bovine feed were characterized by relatively high loads of *Enterobacteriaceae* ranging from 5 to 6 CFU/g. These values were similar to those found in the literature for hay (4-6 log CFU/g) used as feed for cattle (Jacobson et al., 2002) and for wheat feed (about 4 log CFU/g) used in pig farming (Burns et al., 2015). Regarding the fecal samples, relatively high load of *Enterobacteriaceae* was detected, ranging from 5 to 7 log CFU/g.

These data were in line with those reported in the literature for human feces with values comprised between 5 and 9 log CFU/g (Grall et al., 2017).

By analyzing the meat samples, values ranging from 2 to 5 log CFU/g were found. This was probably due to *Enterobacteriaceae* contamination between meat and environment or between meat and the intestinal content of the animals at the time of slaughtering. Indeed, the meat and, in particular, the muscle of the slaughtered animal is sterile, but during the processes of skinning and evisceration, it may come into contact with the skin and with the content of the gastrointestinal tract that could lead to an increase in the bacterial load, in particular *Enterobacteriaceae* (Bellucco et al., 2015). Therefore, these processes were considered key points for *Enterobacteriaceae* contamination.

Fermented products such as salami and cheese were characterized by low loads of *Enterobacteriaceae*. In a few samples, such as SAL1, SAL4 and FRV4, *Enterobacteriaceae* were not detected by the viable counts (< 1 log CFU/g). For salami, bacterial growth may be inhibited by different factors including the addition of salt and ripening which reduce the water activity, and even by the addition of starter culture. In the cheese samples the presence of *Enterobacteriaceae* was probably inhibited by the competition with other microorganisms present in the milk itself or added as starter cultures. These transformation processes justify the low load of *Enterobacteriaceae*.

### **3.2. Microbial DNA quantification**

The quantification results of microbial DNA extracted directly from the samples (Tables 8 and 9) revealed a great variability among them, ranging from a minimum of 8 ng/µL for a DNA extracted from pig meat sample [CS6(1)] to a maximum of 461.5 ng/µL for a DNA extracted from the beef meat sample (CB3). The microbial DNA extracted from the samples with low viable counts or with viable counts below the detection limit (< 1 log CFU/g) may derive from microorganisms other than *Enterobacteriaceae*, from unculturable cells or even from dead but not lysed cells. The quantity of the DNA extracted from the fecal samples showed a high variability with the concentration

comprised between 23 and 280 ng/ $\mu$ L. Similarly, the DNA extracted from the meat and the feed samples showed values ranging from 8 to 461.5 ng/ $\mu$ L and from 15 to 161 ng/ $\mu$ L, respectively.

### **3.3. Detection of carbapenemase gene from samples**

These DNA extracts have been subjected to Droplet Digital PCR (ddPCR), which is a high sensitive PCR method based on the partitioning of DNA into droplets. This technique has been developed and used by the University of Verona that is a partner of the project for analyzing the presence of the most CRG ( $bla_{KPC}$ ,  $bla_{OXA}$ ,  $bla_{VIM}$ ,  $bla_{NDM}$ ,  $bla_{IMP}$ ) within the samples under study. Up to now, only the fecal samples have been analyzed for the presence of three out of five genes ( $bla_{KPC}$ ,  $bla_{OXA}$ ,  $bla_{NDM}$ ). This culture-independent approach was combined with culture-dependent approach aimed to identify genes which may spread in environment and distribute the carbapenem resistance among different reservoirs.

Intriguingly, ddPCR was able to detect the  $bla_{KPC}$  gene in a sole sample of pig feces [FS8(1)] obtained from the organic farm. Despite the very low incidence of the genes under study within the analyzed fecal samples, this data confirm the usefulness of ddPCR as a very sensitive method for the detection of carbapenem resistances. Furthermore, it is of note that the  $bla_{KPC}$  gene have been detected within a fecal samples obtained from organic farm, thus indicating that the AR genes may spread among environments irrespective of the use of antibiotics. The source of this gene is unknown, but it may spread and contaminate meat or processed foods thus posing a risk for consumers. Although raw meat is generally cooked before consumption, the DNA might not be degraded by heat-processing due to its stability on high temperatures. Therefore, after consumption of the contaminated meat, the genes may be transferred via transformation to the consumer's intestinal microbiota thus constituting the so called "resistome". This gene transfer may also involve pathogenic microorganisms thus representing a serious risk to the consumer.

### 3.4. Isolation and characterization of carbapenemase-producing *Enterobacteriaceae*

The phenotypic confirmation of carbapenem resistance in *Enterobacteriaceae* is one of the major problems, since there is no single phenotypical test suitable for all the possible cases, so the use of a combined approach seems to be the best solution. After the enrichment on LB medium, to isolate the possible carbapenem resistant *Enterobacteriaceae*, the samples were spread on MacConkey agar plates supplemented with ertapenem (0.125 mg/mL). Colonies with different morphologies were selected, isolated, purified and further characterized by modified Hodge test (MHT) and the Minimum Inhibitory Concentration (MIC) determination. The results are reported in Tables 9 and 10.

Table 9: Results of the modified Hodge test and of the Minimum Inhibitory Concentration (MIC) determination performed on the isolates from the samples of the organic livestock farm.

SAMPLES	ISOLATES CODE	HODGE TEST	MIC µg/ml
Pig Feces 1	FS1	-	n.d.
Beef Feces 1	FB1	-	n.d.
Pig meat 1	CS1	-	<0.25
Beef meat 1	CB1	-	n.d.
cheese 1	FRV1	-	n.d.
Salami 1	SAL1	-	n.d.
Feedstuff 1	SF1	-	<0.25
Hay 1	F1	-	<0.25
cheese 4	FRB4	-	<0.25
Beef meat 4	CB4	-	n.d.
Salami 4	SAL4	-	n.d.
Beef Feces 4	FB4	-	8
Pig Feces 4	FS4	-	<0.25
Pig Feces 8 (1)	FS8(1)	-	n.d.
Pig Feces 8 (2)	FS8(2)	-	n.d.
Pig meat 8 (1)	CS8(1)	-	<0.25
Pig meat 8 (2)	CS8(2)	-	n.d.

n.d = not determined; - negative

Table 10: Results of modified Hodge test and Minimum Inhibitory Concentration determination performed on the isolates from the samples of two conventional livestock farms.

SAMPLES	ISOLATES CODE	HODGE TEST	MIC $\mu\text{g}/\text{ml}$
Beef feces 2	FB2	-	<0.25
Beef feces 3	FB3	-	<0.25
Beef meat 2	CB2	-	n.d.
Beef meat 3	CB3	-	<0.25
Beef feed 2	MB2	-	n.d.
Pig feces 5(1)	FS5(1)	-	n.d.
Pig feces 5(2)	FS5(2)	-	n.d.
Pig feces 5(3)	FS5(3)	-	n.d.
Pig feed 5	MS5	-	n.d.
Pig meat 5(1)	CS5(1)	-	n.d.
Pig meat 5(2)	CS5(2)	-	n.d.
Pig meat 6(1)	CS6(1)	-	n.d.
Pig meat 6(2)	CS6(2)	-	<0.25
Pig feces 6(1)	FS6(1)	-	n.d.
Pig feces 6(2)	FS6(2)	-	n.d.
Beef meat 7	CB7	-	n.d.
Beef feed 7	MB7	-	<0.25
Beef feces 7	FB7	-	<0.25
Pig feces 9(1)	FS9(1)	-	n.d.
Pig feces 9(2)	FS9(2)	-	n.d.
Pig meat 10(1)	CS10(1)	-	n.d.
Pig meat 10(2)	CS10(2)	-	n.d.
Beef feces 11	FB11	-	n.d.
Beef meat 11	CB11	-	n.d.

n.d = not determined; -negative

As reported in Tables 9 and 10, all the isolates resulted negative on the MHT, showing no ertapenem resistance among the isolates. According to CLSI (2017) breakpoints, the *Enterobacteriaceae* are considered resistant to ertapenem when the MIC value is  $>1 \mu\text{g}/\text{mL}$ . In the present study, the MIC determination results demonstrated that most of the samples were sensitive to ertapenem with MIC values below  $0.25 \mu\text{g}/\text{mL}$  except for

the FB4 sample that showed a MIC of 8 µg/mL, thus showing the resistance to ertapenem.

Although the determination of the MIC on the isolates and the analyses of ddPCR on microbial DNA extracted directly from samples is still ongoing, an overall low incidence of the carbapenem resistances have been detected among the samples under study. Indeed, only 1 (beef feces, sample FB4) out of 16 isolates from fecal samples showed a MIC higher than 1 µg/ml and only 1 [pig feces, sample FS8(1)] out of 16 samples of feces was found positive for the presence of *bla<sub>KPC</sub>* gene by culture-independent ddPCR method. These data are in line with the few studies available in literature on the distribution of resistances to carbapenems in livestocks. Fisher et al. (2012) performed a study in German pig farm holding 4100 pigs, where potential carriers of Extended-spectrum beta lactamase (ESBL) microorganisms collected from fecal samples were grown and further isolated on MacConkey agar plates supplemented with 1mg/L of cefotaxime. The obtained isolates (221) were subjected to disk diffusion method and further characterized for the presence of beta-lactamase encoding genes. The results showed the presence of *bla<sub>VIM</sub>* gene among different isolates. This study underlines the fact that the occurrence of ESBL in food producing animals is becoming an important worldwide concern. Zurflue et al. (2016) collected 83 fecal samples from pigs and 160 fecal samples from cattle from two major slaughterhouses in Switzerland. The isolates from 2 pig and 27 cattle fecal samples, resulted to have the reduced susceptibility to ertapenem, so they were further subcultured on Triple Sugar Iron agar used for differentiation of *Enterobacteriaceae*. The production of carbapenemases was checked using the RAPIDEC® CARBA NP test which finally showed only seven isolates, all from cattle feces, resulted positive. The isolates were further subjected to molecular screening for the presence of carbapenemase genes. The results showed five isolates ascribed to *Aeromonas veronii* harbouring *bla<sub>MIS</sub>*, 1 isolate ascribed to *Pseudomonas alcaligens* harbouring *bla<sub>PAM-1</sub>* and 1 isolate ascribed to *Stenotrophomonas maltophilia* containing *bla<sub>L1</sub>* and *bla<sub>L2</sub>* genes. Hamza et al. (2016) investigated the occurrence of carbapenem resistant *Klebsiella pneumoniae* in 100 samples of broiler chickens

(including internal organs), 20 samples of drinking water and 49 fecal samples of workers which had the direct contact with chickens from an Egyptian farm. Disk diffusion method was used to test the susceptibility of *K. pneumoniae* isolates on carbapenem. Furthermore, the DNA extracted from the isolates was screened for the presence of carbapenems resistance genes (*bla<sub>KPC</sub>*, *bla<sub>OXA-48</sub>*, *bla<sub>NDM</sub>*). Among 35 *K. pneumoniae* strains isolated from poultry, 15 resulted *bla<sub>NDM</sub>* positive and 11 of them resulted also *bla<sub>KPC</sub>*, *bla<sub>OXA-48</sub>* and *bla<sub>NDM</sub>* positive. Three isolates from drinking water resulted positive for *bla<sub>KPC</sub>* and *bla<sub>NDM</sub>* genes. All *K. pneumoniae* isolates obtained from the farm workers were positive for at least one of the tested carbapenem resistance genes. More recently, the study of Pulls et al. (2017) highlighted that only 2 out of 7850 *E. coli* isolates from 2160 pig fecal samples obtained from a farm in Italy were found positive for the presence of *bla<sub>OXA-48</sub>* gene.

## Conclusion

The aim of the present thesis was to contribute to a screening of CPE occurrence in pigs and bovine livestocks in the Marche region. The investigation takes into consideration the complete food supply chain, thus including the feed, raw meat, fecal and processed food (salami and cheese) samples. The culture-dependent methods applied to the 41 samples under study resulted in only 1 isolate from beef feces (sample FB4) showing a MIC value > 1 µg/mL and precisely of 8 µg/mL. Then, the DNA was extracted directly from the fecal samples and screened by ddPCR for the presence of the genes encoding resistance to carbapenems. Only one pig fecal sample [FS8(1)] from the organic livestock resulted positive for the presence of *bla<sub>KPC</sub>* gene. Overall, due to the low incidence of CPE and CRGs, the supply chains of bovine and swine in the Marche region object of the present study cannot be considered a reservoir of CPE. Nonetheless, the source of these gene and isolate is unknown and they may spread among the livestocks making it more difficult to treat infected animals. The CRGs may also contaminate meats or processed foods which may become the vehicles of carbapenem resistant genes into the human gut. In this way, the human gut microbiota would represent a reservoir of

antibiotic resistance that could also potentially be transmitted to other pathogenic bacteria, making it difficult, or sometimes impossible, to eradicate the infections caused by these resistant bacteria. The molecular screening will be expanded to all the samples analyzed in the present thesis, to obtain a complete picture of the resistances in the supply chains under study. Finally, the present study aims to underline the importance of such investigations to identify reservoirs of antibiotic resistances and to understand the mechanisms involved in the spread of resistance in order to implement specific strategies to counteract resistances spreading. In general, this study underlines the need to raise public awareness about the responsible use of antibiotics, especially when used in both human and animal therapy.

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