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Risk Analysis of the transmission of antimicrobial resistant Escherichia coli in pork food chain: a "farm-to-fork" perspective

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Abstract

Antimicrobial resistance is a public health risk that needs to be faced in a One Health perspective, including humans, animals, and environmental health. Food production chain has been identified as a possible route of transmission of AMR bacteria to humans. The most critical phenomenon is related to Critically Important Antimicrobial's (CIA) resistance such as β-lactams antibiotics (cephalosporin of 3rd, 4th generation, carbapenem, monobactams and penicillins), quinolones, aminoglycosides, polymyxin and glycylcyclines.

The same pigs were analysed along the entire food producing chain steps sampling pigs' feces, then carcasses and finally pork food products (fresh meat, fermented and seasoned product). *Escherichia coli (E. coli)* were isolated from samples collected, and AMR and MDR profiles were evaluated:

1) The ability of *E. coli* to produce ESBL and AmpC β-lactamases was evaluated both phenotypically by disk diffusion method and genotypically by the research of the most common ESBL and AmpC plasmidic related genes;
2) MDR to 17 antimicrobial molecules were evaluated on the same isolates using Minimal Inhibitory Concentration;
3) All the strains that showed the same AMR in different stages of the food producton chain of the same pig or of pigs originating from the same farm were considered;
4) Phylogenetic analysis were performed through Enterobacterial Repetitive Intragenic Consensus (ERIC-PCR). Phylogenetic similarities between the strains identified were considered in order to evaluate the possible "farm-to-fork" transmission of AMR bacteria;
5) Average Nucleotide Identity (ANI) was used to genomically identify the strains considered and confirm the phylogenetically relation found by ERIC-PCR.

Results showed that on 243 pork food chain fully or partially analysed the *E. coli* AMR profiles from feces, through carcasses, fresh meat to fermented and seasoned meat product, only in one case showed that resistant bacteria were phylogenetically similar from farm-to-fork (isolates in feces, carcasses and fresh meat have more than 95% of genomic similarities). Frequent similarities were shown in resistant *E. coli* isolates from carcasses and fresh meat or fermented product and in one case, bacteria isolated from fresh meat and fermented product were genomically similar.

In conclusion, results showed that the transmission directly from farm-to-fork is possible but not frequent. However, the food producing environment and cross contamination can play an important role in the dissemination of AMR in fact antimicrobial resistant bacteria were easily found in the different stages of the food chain.

New drugs are needed, and metal-based compounds represent a valid scaffold to explore new antibiotic classes. In this study we choose to investigate gallium(III) complexes for their potential antimicrobial activity against different strains of *Klebsiella pneumoniae*, *E. coli* and *Pseudomonas aeruginosa* which have developed different type of resistance mechanism including the production of β-lactamases (NDM-1, ESBL or AmpC). The compounds were active against resistant Gram-negative strain with minimal inhibitory concentration in the μM range, while no cytotoxicity was detected in eukaryotic cells.

Further studies are needed to improve risk communication to consumers and access to clear and reliable information and health concerns on pork food labels.

Chapter 1. General introduction

Antimicrobial resistance: a global health risk

Antimicrobial resistance (AMR) is one of the leading public health risks of the 21st centaury. An increasing prevalence of bacteria, viruses, parasites, and fungi hard-to-treat infections because of no long susceptibility to the most common drug used is threatening human health (Prestinaci et al., 2015). Bacterial AMR is the most urgent issue nowadays. Scientifical reports argue that this phenomenon is killing 700.000 people every year and could kill 10 million people by 2050, lots more than other deadly diseases such as cancer, tetanus, road traffic accidents, diabetes, and cholera (O'Neil, 2016).

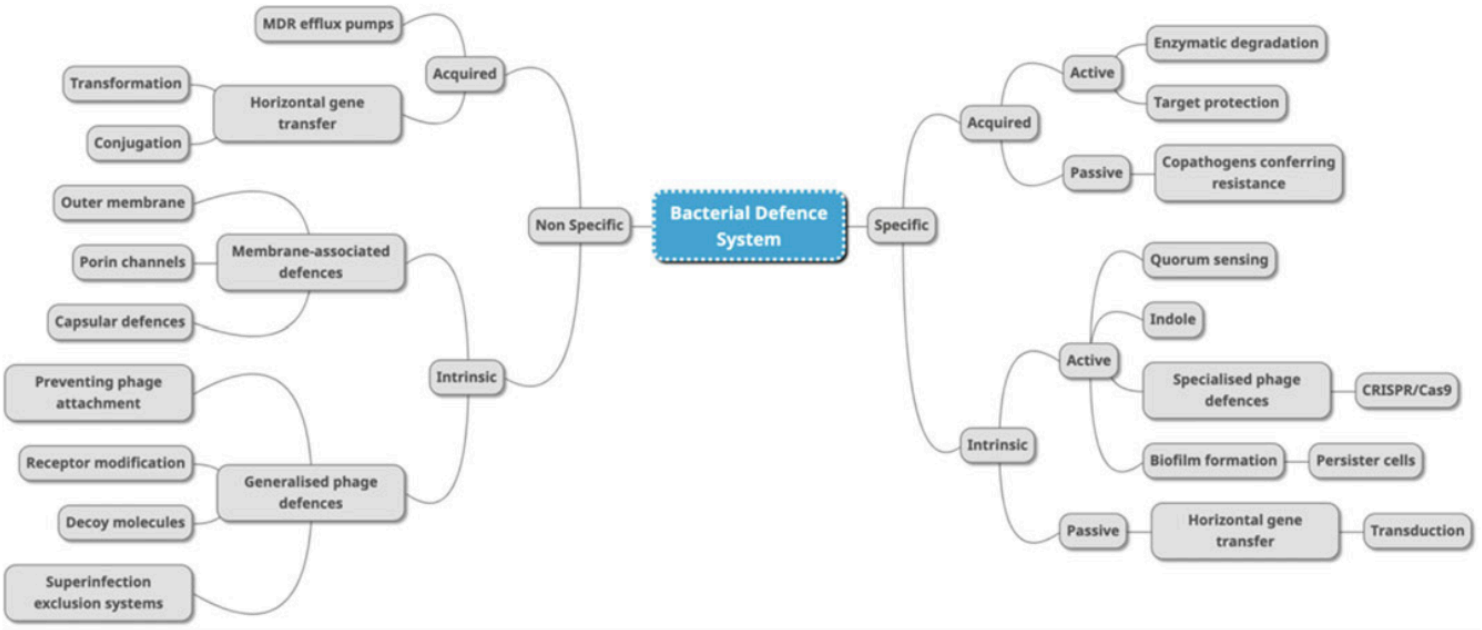
Sir. Alexander Fleming, after discovering penicillin, understood that the AMR phenomenon in 1945 was going to warn future generations on the unavoidable consequences of antibiotic ineffectiveness (Austriam and Gold, 1964).

AMR means that a therapeutical approach through antibiotics can be less effective, up to become ineffective, against the microorganisms it targets (Annunziato, 2019). It is a natural evolutionary bacterial phenomenon that consist in random changes in microorganisms' genes. Those mutations produce new abilities that can be offspring during reproduction or horizontally through the exchange of mobile genetic elements (Mozhayskiy and Tagkopoulos, 2012). New bacterial lineage descended become then more common than parental cells because of new helpful trait for the survival and reproduction of the bacterium (Laxminarayan et al., 2013).

The ability of bacteria to resist to antimicrobial effect can be compared to the role of immune system. In fact, immune system is a medium that defend multicellular and multi-organs system in eukaryotes and, despite it is less complex in prokaryotes cell, can still guarantee defensive response. The comparison is a metaphorical concept to draw parallels about two distinct

mechanisms of defenses. The immune system response to pathogen-mediated, toxin-mediated or a combination of those diseases (Abedon, 2012; Bikard and Marraffini, 2012; Shabbir et al., 2019). If antimicrobials are viewed as being toxin-mediated infections to microbes, it is possible to evaluate AMR as the result of a serial of defensive mechanisms adding the ability of microorganisms to exchange that develop within bacterial communities through generations (Thomas, 2020).

Figure 1. Bacterial defense mechanisms (Thomas, 2020)



This process is rapid in bacterial cells because of their ability to reproduce up to every 20 minutes. That is why ampicillin, developed only half a century ago, it is now widely tolerated by many strains of microorganisms (Thomas, 2020).

During 20th century it was not a serious problem because of the possibility of counting on new alternative molecules and in this period the continuous discover of new molecular classes allowed not to identify necessarily the AMR as a human health risk. Nowadays the number of new

available antimicrobial molecule is dramatically decreased creating a risk that frightens the world globally (Laxminarayan et al., 2013; Uddin et al., 2021).

The selection of antimicrobial resistant microorganisms crucially involves humans' antibiotic consumption, their inappropriate prescribing, poor adherence to prescribed therapy, use of counterfeit and low-quality antibiotics and poor hygiene practices in hospitals (Majumder et al., 2020).

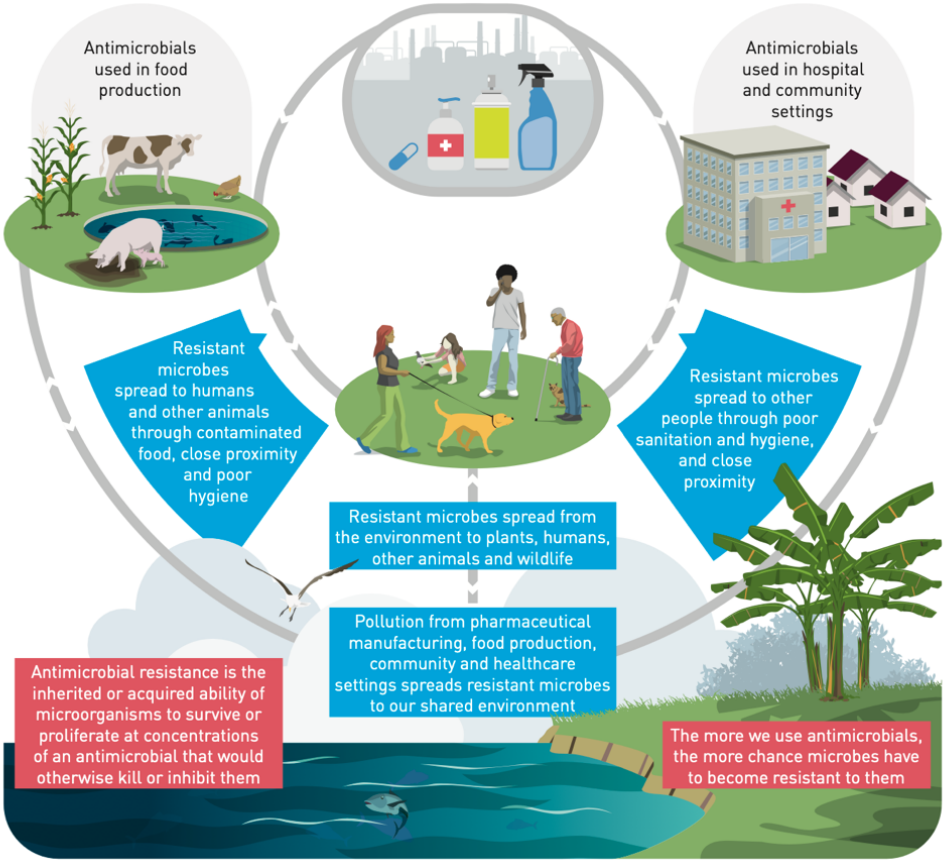
Simultaneously, the extensive use of antimicrobials in livestock production can further promote the growth of resistant microorganisms (Economou and Gousia, 2015). Globally, the majority of antimicrobials are administered to animals rather than consumed by humans. In Europe, for example, countries sales of veterinary antimicrobial agents marketed mainly for food producing animals are ranked between 100 and 200 mg/Population Corrected Unit (PCU); this groups includes Italy with 173.5 mg/PCU which equals to 689.3 tons of veterinary antimicrobials sales (European Medicines Agency, 2022). Antimicrobials are used in farm animals to treat sick animals firstly but, particularly in the past, they were used such as preventive treatments to avoid the spread of infectious diseases and were used as growth promotors and feed efficiency contributing (van Boeckel et al., 2015).

AMR is a global threat with a multinational epidemiology highlighting that resistant microorganisms have no boundaries due to increasing mobility and globalization. Half a century ago methicillin-resistant variant of *Staphylococcus aureus* (MRSA) spread in Europe in about two decades, although in the 20th century a carbapenem-resistant strain of *Klebsiella spp.* spread in only 5 years from the United States to Israel, reaching the United Kingdom, Italy, and Colombia (McKenna, 2013). That is why collaborative actions plan must be globally set up considering that the actions of one country can have impact on the entire world and that no country can face this hazard alone (Cecchini et al., 2015).

Antimicrobial Resistance: One Health approach

One Health is defined as "*an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems. It recognizes the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and inter-dependent. The approach mobilizes multiple sectors, disciplines, and communities at varying levels of society to work together to foster well-being and tackle threats to health and ecosystems, while addressing the collective need for clean water, energy and air, safe and nutritious food, taking action on climate changes and contributing to sustainable development*" (WHO, 2023).

Figure 2. Antimicrobial Resistance Diffusion (UNEP, n.d.)



AMR is an ecological problem since wherever antimicrobials are used, there are reservoirs of resistance; in fact, bacteria and their mobile genetic elements spread easily in humans, animals

and environments affecting each other sector (Holmes et al., 2016; Huijbers et al., 2015; Marti et al.,2014). For those reasons AMR needs an ONE health approach considering the world as multidisciplinary collaboration and joint actions (Mcewen and Collignon, 2018). It is known that human health is the most considered factor for preserving the antimicrobials efficacy, but it is also necessary to adequately take care of animals and environment to have good results on this phenomenon (Mcewen and Collignon, 2019). Mutual dependency of animals and humans all along history and evolution hesitate in the creation of the same environment and the same infectious diseases in these species. In the ancient cultures animals were seen as flock of God, metempsychosis. The transformation from humans to animals was known from India and Africa and they see cattle as being part of their society due to their myths of creation (Zinsstag et al., 2012). The first integrated public health system including veterinarians and doctors was created in China from 11th century to 13th century by the Zhou Dynasty and at the end of 19th century the close interaction between these two sectors was need due to the advent of cellular pathology and microbiology. Rudolf Virchow was the main promotor of this integrated and comparative approach to better carry out medical investigations (Zinsstag et al., 2005). The veterinarian Calvin Schwabe finally created the expression "One Medicine" to describe the cohesion of human and animal medicine; he recognizes how veterinary medicine benefits human health both directly and indirectly considering that the 75% of human infectious diseases in recent decades are zoonotic (Robinson et al., 2016; So et al., 2015).

Antimicrobial classes used are the same for human, animals, and environment. Only a few molecules are reserved to humans (e.g., isoniazid to treat tuberculosis or other infection for which animals are culled rather than treated) and, because of human toxicity, a few molecules are reserved to animals (e.g., flavophospholipol, ionophores) (Aslam et al., 2021). In fact, antimicrobials used in humans to treat clinical infections in individual patients, are used in

veterinary medicine in a wide range of animal species (for domestic mammals, birds, farmed fish, honeybees etc.) and are used in environment for treatments in horticulture and sometimes as prophylaxis treatment of bacterial infection (van Boeckel et al., 2015). Moreover, lot of differences are evident in antimicrobials use in veterinary medicine: while the use in domestic animal is basically comparable to the human clinical use, in food-producing animals there is a metaphylactic administration of antimicrobials including both therapeutic and prophylactic use with mass treatment. Growth promotion is the most controversial use of antimicrobials in food producing animals because of its consequences due to long-term, low-dose mass medication. In the past, this antimicrobial use was justified by economic necessity, but it promotes the selection of antimicrobial resistant microorganisms ("Kucers' The Use of Antibiotics," 2017). For those reasons, authorities banned the use of antimicrobials in animals as growth promoters in lots of countries such as in the European Union (EU), the United States and Canada (Collignon and Mcewen, 2019).

The third One Health pillar is the health of the environment threatened by ever-increasing human population growth causing climate changes, increasing pollution, and depletion of the earth's resources. That is the reason why environmental health must be included in a One Health perspective (Robinson et al., 2016). In AMR phenomenon, environmental health usually received less attention than human and animal health. Despite this it is an important reservoirs of antimicrobial resistant bacteria (UNEP, n.d.). Environments with high ecological variables such as water and soil, harbour a great genetic pool that is incredibility higher than humans and animals' ones and the possibility for bacteria to acquire AMR related genes increases (Forsberg et al., 2012; Rinke et al., 2013). It is well known that AMR genes are frequently associated with human and animals' infections meaning that these are the most important environments for resistance evolution under a selective antimicrobial pressure. At the same time, it can be assumed

that many recent AMR genes originate from not yet sequenced environmental species (Rinke et al., 2013; Schulz et al., 2017). The evolution of an AMR is a single and irreversible event creating a new genotyping resistance challenge difficult to predict. Transmission of the already existing AMRs is the next important hazard and prevention through an essential correct use of antimicrobial molecules (Larsson and Flach, 2021). The environment is reached by antimicrobials because of humans and animals' excretions that represent the largest way to release antimicrobials in the environment (Wang et al., 2020). Other route of antimicrobial introduction in the environment are incorrect antimicrobials disposal, contamination in aquaculture or plant and through antimicrobial production wastes (Cabello et al., 2016).Frequently, the antibiotic concentration in the environment is lower than minimal inhibitory concentrations (MICs) not having any effect on antimicrobial resistant strain (Gullberg et al., 2011). Despite those elements, it is difficult to understand and predict how much pollution is the cause of the spread of antimicrobial resistant bacteria in the environment or if the selective environmental pression plays an important role in the AMR transmission to human and animals (Yang et al., 2014). The certainty is that untreated hospital wastewater and pollution from antibiotic manufacturing selected for multi-resistant *Escherichia coli (E. coli)* in different controlled exposure studies (Kraupner et al., 2021). Despite the concentration of antimicrobial molecule in solid and semisolid media (sediments, soils, and sewage sludge) seems to be higher than in aqueous ones, it is important to take into account the bioavailability of antimicrobial molecules in those media. In fact, it can happen that, despite the high concentration of antimicrobial molecule a high number of sensible strains can be found. Finally, the composition of the soil media can affect the bioavailability of antimicrobials in the environment (Wang et al., 2020).

The environmental pollution by antimicrobials is considered a hazard of AMR development in the environment but the spread of resistant bacterial contamination by human and animal manure

is equally impactful. In fact, it led to physical contact and gene exchange between human/animals and environmental microbiota. Many intestinal bacteria usually carry mobile genetic elements such as plasmids, integrative conjugative elements, insertion sequences, transposons or integrons that facilitate this phenomenon, sometimes with the involvement of pathogens (Gillings et al., 2017). Wild animals that populate wild environment encountered various resistances strain and genetic elements becoming themselves source of spread of AMR in the environment (Arnold et al., 2016). AMR commensal gut microorganisms have been found in wild mammals, birds, reptiles, and fish with variable prevalence of resistance patterns (Cabello et al., 2013; Wheeler et al., 2012). The current scenario of the anthropogenic transformation of the landscape forces wildlife to have frequent contact with humans and their livestock, thereby increasing the risk of antimicrobial resistance transmission through different populations. The dramatic increase in the contact between domestic and wild animal species should be monitored (Rega et al., 2022; Torres et al., 2020). Not only wild environment is involved in the dissemination of AMR: food environment for example can be a source of resistant bacteria due to contamination of working surfaces. Moreover, the use of fecal contaminated water can be an additional food-borne risk factor. Raw vegetables, raw meat, ready to eat products and other type of food can be a source of exposure to *Salmonella* spp., enterohaemorrhagic *E. coli* and *Campylobacter jejuni* due to contaminated products. Foods can carry resistant strain with diverse mobile genetic elements that can be a transmission route of AMR to consumer through the food consumption. Finally, the role of selective agents in environmental transmissionism is not completely clear. Biotic and abiotic factors, such as temperature, oxygen pressure, nutrients, predation, and competition with other species are involved in the ability of environmental transmission for both resistant and non-resistant strains (Flach et al., 2017).

Chapter 2. Aim of the study

Commensal bacteria in animals can be a reservoir of AMR and, at the same time, they can spread this phenomenon. Despite this, the role of longitudinal transmission of those bacteria from livestock to human by means of meat products is still limitedly understood (Lugsomya et al., 2018). The present study identifies and analyses by a risk analyses approach, the potential issue of AMR transmission through the pork food chain in a farm-to-fork perspective. Pigs' feces, carcasses and pork food products (fresh meat, fermented and seasoned product) of the same pig were sampled along all the food producing chain. Each sample was processed in order to isolate *E. coli*. Each *E. coli* strain was analysed to evaluate the AMR profile focusing on:

- ESBL and AmpC resistance;
- resistance to 17 antimicrobials belonging to β-lactams, aminoglycosides, polimixin, sulphonamides, quinolones and glycylcyclin classes highlighting Multi Drug Resistance (MDR) pattern.

AMR of bacteria isolated from feces was compared to the ones found in carcasses and meat products isolates of the same pig or group of pigs. When AMR similarities were found, phylogenetical analysis were performed and in some cases sequencing analyses were executed. Data obtained allowed to understand possible phylogenetic connections between bacteria found in the different stages of the food chain.

At the same time, the synthesis of new molecules was performed with the aim of inhibit β-lactams enzyme activity and restore the activity of cephalosporin and carbapenems against resistant bacteria.

Chapter 3. Hazard identification

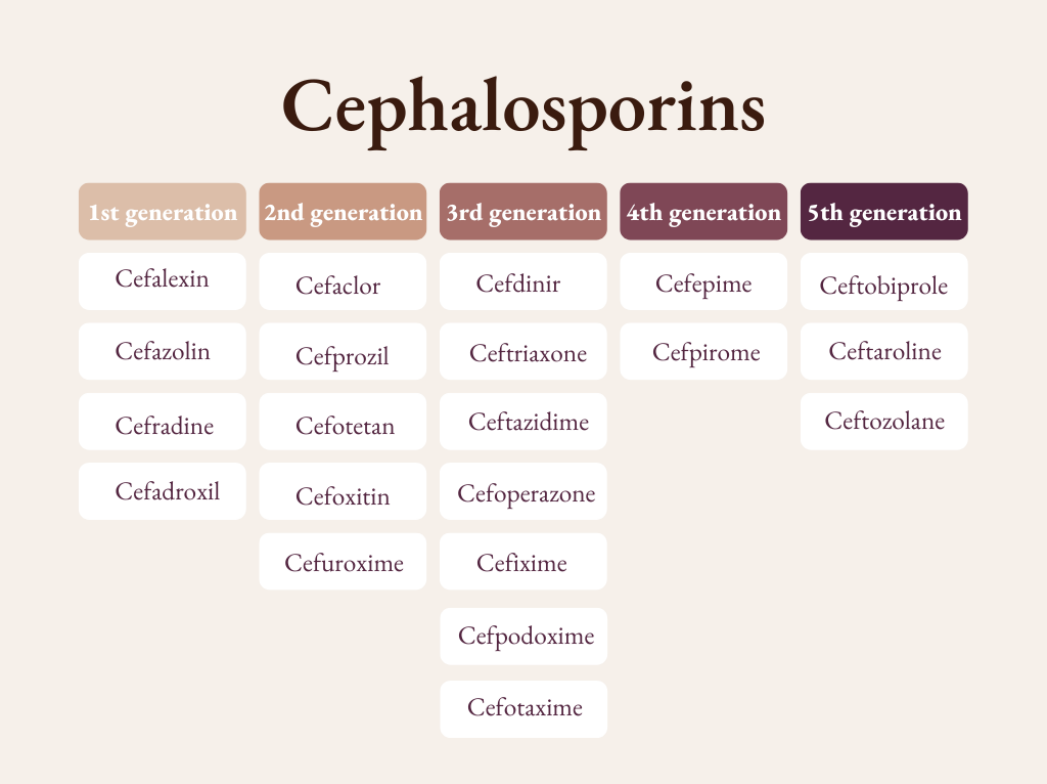
Hazard identification will predominately be a qualitative process. Hazards can be identified from relevant data sources. Information on hazards can be obtained from scientific literature, from databases and through solicitation of opinions of experts.

The purpose is to describe the AMR hazard of concern providing information on the susceptibility or AMR of related microorganisms (Caffrey et al., 2019).

Antimicrobial resistance to β-lactams antimicrobials- cephalosporin

Resistance to 3rd and 4th generation cephalosporin is a One Health issue that involved last generation molecule that were used in animal medicine both as therapeutic, prophylactic treatment and for growth promotion purposes in some countries. Recently, such molecules have become widely used in treatments of hard-to-treat infections in human medicine. 3rd and 4th generation cephalosporin are broad-spectrum β-lactams antimicrobials and resistance to those molecules is becoming common among *E. coli* and *Klebsiella pneumoniae* of severe human infections (Rawat and Nair, 2010). Cephalosporin belongs to β-Lactams antibiotics with penicillins, monobactams and carbapenems and are classified in 5 generation as showed in figure 3 (Bush and Bradford, 2016).

Figure 3. Cephalosporin classification



β-lactams action is based on their ability to interact with bacterial cell wall; particularly, the antimicrobial molecule binds carboxypeptidases and transpeptidases enzyme also called Penicillin Binding Protein (PBPs) that catalyze the D-alanyl D-alanine cross linkages of the peptidoglycan wall interfering with bacterial cell walls synthesis (Mainardi et al., 2005). The antimicrobial activity can also be conveyed by other target enzyme inhibiting L,D-transpeptidases (Ldts), a family of enzymes that form alternative peptide cross-links in bacterial peptidoglycan, particularly important in mycobacteria. As with PBPs, this enzyme is involved in the peptidoglycan transpeptidation (Miyamoto et al., 2020). Other families of protein are targeted by some β-lactams, including some serine and cysteine proteases (Mora-Ochomogo and Lohans, 2021).

A wide variety of β-lactam antibiotics has been discovered as product of nature or have been synthetized in last decades (Hamed et al., 2012). Cephalosporins are structurally related to the penicillins, with variations in chemical structure that lead to interfere with the catalysis of some serine β-lactamases (Salahuddin et al., 2018).Drug production have been based on the acylamido substituent at C7 of the cephalosporin core that is similar to penicillin one in 1st and 2ndgeneration cephalosporins but tend to have bulky C7 acylamido side chains containing a planar oxyimino group in 3rd and 4th generation cephalosporins (e.g., ceftazidime, cefotaxime). This characteristic structurally interferes with some β-lactamase degradation (Mora-Ochomogo and Lohans, 2021). Resistance to β-lactams arose as Darwinian selection, favoring bacteria able to resist to antimicrobial action. In some bacteria, as *Enterococcus* spp., this AMR is intrinsic, because of insensitivity of their PBPs. In other bacteria it may be acquire through spontaneous mutations or DNA transfer. Those variations cause the ability of bacteria to produce β-lactamases, enzyme that hydrolase the β-lactam ring of β-lactams antimicrobials rendering the antibiotic unable to target PBPs (Munita and Arias, 2016). At the same time, aspecific resistance mechanisms can occur such as impermeability or efflux or target modifications (Reygaert, 2018).

β-lactamases are frequently encoded on plasmids and can be readily disseminated through horizontal gene transfer (Ozgumus et al., 2008). Some β-lactamases are sufficient to confer resistance to β-lactam antibiotics alone, while enzymes with lower levels of activity offer sufficient protection when combined with other resistance mechanisms (e.g., decreased porin production, increased production of efflux pumps) (Pages et al., 2009).

Ambler classification and the Bush-Jacoby Medeiros classification are the most common way to classify β-lactamases and are based on molecular and functional classification respectively. Ambler classification divides β-lactamases on protein homology in four classes: β-lactamases of

classes A, C and D are serine β-lactamases (SBLs) and the class B enzymes are metal β-lactamases (MBLs) (Bush and Jacoby, 2010).

- Class A enzymes are identified as TEM-1 and SHV-1; they typically degrade penicillin but can also give low level protection against 1st generation cephalosporins (Bush, 2018).

  The clinical use of 3rd generation cephalosporins led to the ability of bacteria to produce variant of TEM and SHV enzyme producing class A extended-spectrum β-lactamases (ESBLs) (Zarfel et al., 2011); those enzymes belong to the CTX-M family too (Woerther et al., 2013). At the same time the use of carbapenem antibiotics led to the creation of class A enzymes with carbapenemase activity as members of the KPC and GES families (van Duin and Doi, 2017).

  Nowadays this class of SBLs is of great clinical relevance because the most used β-lactams are susceptible of the action of these enzymes. Clavulanic acid, sulbactam and the most recent avibactam and vaborbactam are molecules able to inhibit SBLs activity; despite this, inhibitor-resistant variants are common (Bush, 2018; Ehmann et al., 2012; Tooke et al., 2019). While Class A β-lactamases can easily degrade 1st and 2nd generation cephalosporin, the bulky C7 oxyimino-containing side chains belonged to 3rd and 4th generation cephalosporins (e.g., ceftazidime) is not so sterically compatible with the enzyme (Trehan et al., 2002). Despite this, other mechanisms have been developed to actively degrade those cephalosporins too (Tamma et al., 2021);

- Class C enzymes are the most commonly associated with early generations cephalosporins resistance, while 3rd and 4th generations and cephamycins are slowly degraded (Gupta et al., 2014). This class give resistance to penicillin too while oxacillin is considered an inhibitor. Those enzymes are not able to destroy carbapenem. They are identified as AmpC and belonged to several groups such as CMY, ACC, FOX. Together

with the basic mechanisms, additional mutations in regulatory protein genes that are involved in peptidoglycan synthesis have been described in resistant bacteria (Konaklieva, 2005). Resistance to the most inhibitor molecules made this class of enzyme therapeutically risky (Gupta et al., 2014);

- Class D enzymes were not so common and not so clinically relevant but nowadays plamid-mediated variants has widely been identified (Dabos et al., 2018). The first type identified were penicillinases and embodied selective action on penicillin, different from the ones targeted by other SBLs (Bush, 2018). Nowadays the activity has been extended to the other type of β-lactams antimicrobials particularly in relation to OXA enzyme that made their activity similar to ESBL one. The most notable characteristic is their carbapenemase activity and their resistance to β-lactamase inhibitors such as clavulanic acid and sulbactam and partially avibactam (Tooke et al., 2019);
- Class B enzyme is characterized by metal β-lactamases (MBLs) whose action is dependent on zinc ions while previous enzyme interact with β-lactams forming a covalent complex with a serine residue (Mojica et al., 2016). Those enzymes are widely described in next subchapter.

To protect molecules to β-lactamases action, they are frequently administrated with a β-lactamase inhibitor (BLI). Clavulanic acid has been the first clinically used BLI that can inhibit antimicrobial catalysis through the acylation of a nucleophilic serine in the SBL active site that, with following reaction (Douafer et al., 2019; Al-Tamimi et al., 2019; van den Akker and Bonomo, 2018).

The mechanism allowing the interaction of β-lactam antibiotics with SBLs is similar to the one used to interact with PBPs. It starts with an initial substrate binding where SBL activate an active site serine residue of the β-lactam ring to nucleophilic attack. This binding is more stable with

PBPs than SBLs so that the diacylation of such complex can easily be catalyzed by hydrolyzation (King et al., 2014).

When less therapeutical options are identified for ESBL bacteria, carbapenems are the last resources antimicrobials. Consequently, the increasing of carbapenem usage increase AMR phenomenon against those molecules (Papp-Wallace et al., 2011).

In many countries, resistance to cephalosporin is common in *E. coli* and *Klebsiella pneumoniae* related to severe infections in human (Tilahun et al., 2021) becoming a serious public health treat. World Organization for Animal Health (WHOA) reported that patients infected by ESBL and carbapenem resistant microorganisms have a 2-fold increased mortality (WHO, 2022a; Talebi Bezmin Abadi et al., 2019). β-lactams resistance has been reported in *Enterobacteriaceae* as co-selected by other antimicrobials resistances (Murray et al., 2022). Moreover, clonal and horizontal gene transfer facilitate the dissemination of genetic elements related to antimicrobial resistance (von Wintersdorff et al., 2016).

Multi drug resistances to Critically Important Antimicrobials

All antimicrobials used in human medicine are categorized in either "Critically important" (CIA), "Highly important", or "Important" following specific criteria. The further prioritized molecules are the Critically Important because:

- Criterion 1 (C1): "*The antimicrobial class is the sole, or one of limited available therapies, to treat serious bacterial infections in people*" (WHO, 2018). The reduction of efficiency of this molecule due to AMR has a significant impact on human health, especially for people with life-threatening infections. This criterion does not consider the likelihood that these pathogens may be transmitted, or have been transmitted, from non-human sources to humans (Scott et al., 2019).

- Criterion 2 (C2): *"The antimicrobial class is used to treat infections in people caused by either:*

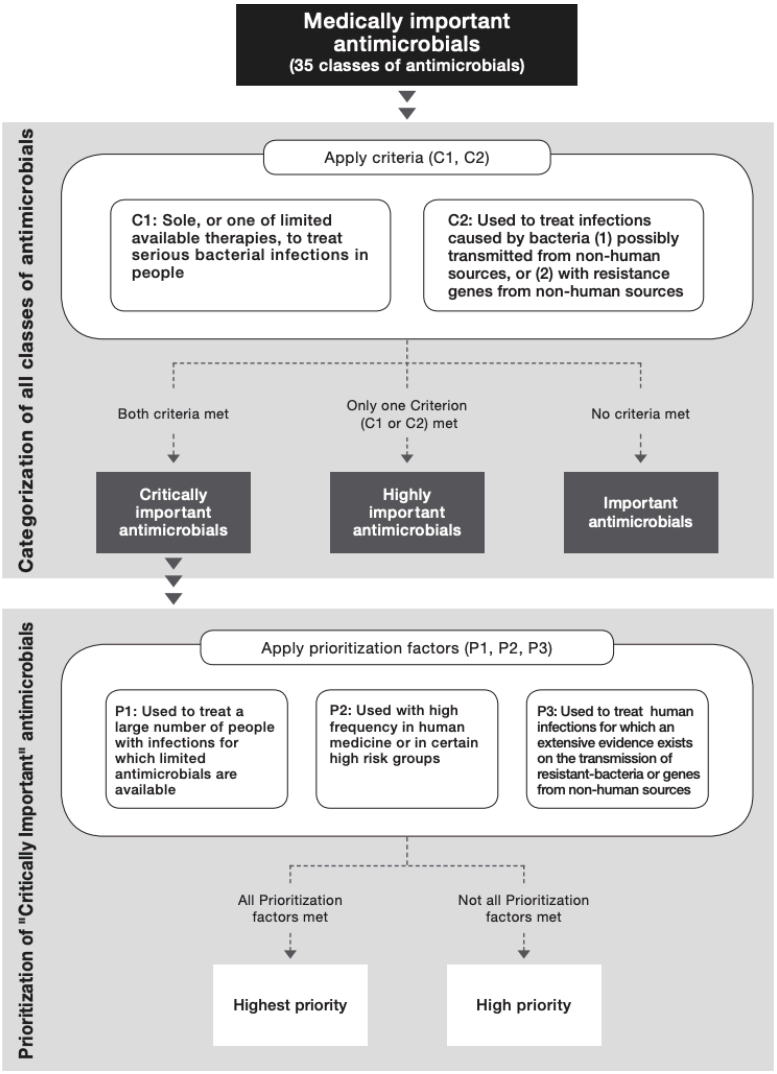
    1) bacteria that may be transmitted to humans from non-human sources.

    2) bacteria that may acquire resistance genes from non-human sources".

    (WHO, 2018). This criterion takes into account the possibility of bacteria to be transmitted to humans by a non-human source making a relation with the necessity to manage non-human use of antimicrobials. Both pathogens and commensal bacteria may also be transmitted (Scott et al., 2019).

World Organization for Animal Health (WOAH) created an additional CIA list of antimicrobials that are important in veterinary medicine (Scott et al., 2019).

Figure 4. Critically Impotant Antimicrobial-classification criteria (OIE, 2007)



This list completes the humans one and allows the identification of the antimicrobials used in human medicine. The human, animal and plant sectors have a shared responsibility to prevent or minimize antimicrobial resistance selective pressures on both human and non-human pathogens (OIE, 2007).

Resistance to carbapenem

Carbapenems are β-lactams antimicrobials that are used in human medicine as last line treatments for hard-to-treat human infections. They are rarely used in veterinary medicine and only in individual treatments of food producing animals. Consequently, there is a very low selective pressure for the development of AMR against those antimicrobials but in recent years resistant *E. coli* have been isolated from animals worldwide (Köck et al., 2018; Woodford et al., 2014). It is interesting to understand how those resistances spread in animals' world, considering the possibility of transmission through human and environment. In addition, the fact that penicillins select for carbapenem resistance too can be a predisposing factor (Poirel et al., 2014).

Carbapenemases are classified with the other β-lactamases described before. Particularly:

- Class A included ESBL and *Klebsiella pneumoniae* carbapenemase (KPC) enzymes;
- Class B included metallo-β-lactamases (MBLs) that has a wide range of action on β lactams antibiotic (except monobactams). They are divided in three subclasses (B1, B2, B3) and the most widespread is B1 subclasses including Verona integron-encoded MBL (VIM), imipenemase (IMP) and New Delhi MBL (NDM);
- Class C: are classified as cephalosporinases and not carbapenemases;
- Class D: are OXA-type β-lactamases that select for infections really hard to trat, resisting to β-lactamase inhibitors.

Carbapenemases hydrolyze carbapenems and most of other β-lactams such as penicillin, cephalosporin and monobactams.

VIM-1 has been the first carbapenemanases detected in *E. coli* isolate from pigs in Germany (Fischer et al., 2017). In United States and China, India and Algeria other carbapenemases were firstly found in dogs, cats, pigs, cattle, poultry and fish *E. coli*: NDM-1 and NDM-5 (Liu et al., 2017; Shaheen et al., 2013; Y. Wang et al., 2017).

IMP-4 carbapenemases was found in *E. coli* isolates from silver gulls in Australia (Dolejska et al., 2016) and OXA-48 carbapenemase has been found in Germany, France, Lebanon, Algeria, and the United States in dogs, cats, and chickens despite it is the most common in human enterobacterial isolates (Melo et al., 2017; Schmiedel et al., 2014). A variant of this enzyme is OXA-181 that is increasingly reported in humans and in *E. coli* isolated from pigs in Italy (Pulss et al., 2017). KPC is the most commonly identified carbapenamases in humans and it is rarely found in *E. coli* isolated from animals, only some cases in wild birds in Africa and in Psittaciformes in Brazil (ben Yahia et al., 2020).

Carbapenemases has molecular inhibitors such as EDTA that can work as an ion chelator. They are not inhibited by ESBL inhibitors molecule (Quale and Spelman, 2022).

Resistance to quinolones and fluoroquinolones

Quinolones and fluoroquinolones are classified as CIAs to treat infections in both humans and animals. They can have bactericidal action against all bacteria. The AMR against these drugs is due to genes mutations in DNA gyrase and topoisomerase IV, that are the quinolones targets. In *E. coli* the first target enzyme is gyrase that consist of two GyrA subunits and two GyrB subunits. The second target is Topoisomerase IV formed by two ParC and two ParE subunits. The majority of mutations found in quinolones chromosomal resistance-determing region are

between Ala67 and Gln107 at codons 83 and 87 in GyrA (Hopkins et al., 2005). Resistance to quinolones is conferred by single mutations in the *gyrA* gene and resistance to fluoroquinolones requires further mutations in *gyrA* and/or *parC* gene (the most common are at 80 and 84) (Hopkins et al., 2005). The AMR against quinolones has been identified as carried by plasmid resistance genes. There are plasmid-mediated quinolone resistance (PMQR) and synthetize for Qnr-like proteins that protect bacterial DNA from drug binding. Different Qnr-like proteins have been identified: QnrA, QnrB, QnrC, QnrD, and QnrS. The AAC(6')-Ib-cr acetyltransferase is an enzyme that conferee a high resistance to fluoroquinolones and is related to a plasmid gene. Finally, QepA and OqxAB are active efflux pump whose synthesis is related to plasmid gene elements. Although, those mechanisms do not conferee a high resistance to quinolones and fluoroquinolones but only a reduction in susceptibility (Jacoby et al., 2014).

PMQRs has been identified in food producing animals including poultry, cattle, and pigs and chromosomal gene mutations (Rega et al., 2022; Veldman et al., 2011)have been identified in wild food producing animals (wild boars) that are not subjected to antimicrobials treatments; this suggests the role of environment in the spread of AMR (Rega et al., 2022).

Moreover gene *aac(6')Ib-cr* was identified in combination with $bla_{\text{CTX-M}}$ ESBL gene (Ewers et al., 2014; Timofte et al., 2016). The PMQR gene *oqxAB* was identified in *E. coli* isolates from food-producing animals conferring resistance not only to quinolones but also to trimethoprim and chloramphenicol (Hansen et al., 2007).

Resistance to aminoglycosides

Aminoglycosides are antimicrobial class often used in combination with β-lactams against hard-to-treat bacterial infections both in humans and animals, including food-producing animals. Streptomycin, gentamicin, kanamycin, neomycin, and paromomycin are the most frequently used

molecules in veterinary medicine and amikacin is reserved for the treatment of infections in pets and horses (Poirel et al., 2018).

Interfering with translation, aminoglycosides are able to inhibit both Gram-negative and -positive bacteria. The using of these molecules globally, led to the development of AMR phenomenon. The resistance is related to mutations 16S RNA and/or the S5 and S12 ribosomal proteins, that particularly affects bacteria with a reduced number of 16S RNA encoding operons (O'Sullivan et al., 2018). Another mechanism that is involved is the methylation (conveyed by ArmA, RmtA/B/C/D/E/F/G/H, and NmpA) of residues G1405 and A1408 of site A of the 16S RNA, conferring high-level resistance to amikacin, tobramycin, gentamicin, and netilmicin. Related resistance genes in combination with sulfonamide related resistance genes, are carried by transposon Tn*1548* favoring AMR diffusion.

ArmA was firstly reported in *E. coli* isolated from pigs in 2005 in Spain, while the first report RmtB was found in China in 2007 with a prevalence of 32% in healthy pigs. In 2000s, ArmA and RmtB were found in other food producing animals particularly poultry and bovine (associate with mastitis). The other gene variants are less common although RmtE was recently found in calves, RmtA in pandas in China (Poirel et al., 2018).

Resistance to aminoglycosides is given by the deactivation of the antimicrobial molecule too. Aminoglycoside-modifying enzymes (AMEs) are acetyltransferases, nucleotidyltransferases, and phosphotransferases. Lots of variants of acetyltransferases have been reported, although AAC(3)-II/IV and AAC(6)-Ib are the most frequent in *E. coli* from different hosts.

In Gram-negative bacteria the nucleotidyltransferases ANT(2") and ANT(3") that are encoded by *aadB* and *aadA* respectively, are most commonly found particularly in *E. coli* isolated from pets, wild animals, and food-producing animals worldwide (Ramirez and Tolmasky, 2010; Xiao and Hu, 2012).

Among phosphotransferases, APH(6)-Ia and APH(6)-Id encoded by the *strA* and *strB* genes, respectively, are most commonly found in *E. coli* isolated from wild rabbits, cattle, poultry, and swine worldwide (Poirel et al., 2018).

Resistance to sulfonamides and trimethoprim

Sulfonamides and trimethoprim are synthetic antimicrobial agents that inhibit different steps in the folic acid synthesis pathway. They have bacteriostatic action but are used in combination for bactericidal synergic effect on susceptible microorganisms: the combination of the two molecule is named "potentiated" sulfonamide. Due to their frequent use in animals and humans, microorganisms acquired resistance mechanisms emerged mainly due to mutations in genes encoding target enzyme dihydropteroate synthase or dihydrofolate reductase.

Acquired resistance mechanisms have been frequently identified, mainly due to mutational modifications in genes encoding the target enzymes, or the acquisition of *sul* genes encoding dihydropteorate synthetases that are insensitive to sulfonamides or *dfr* genes encoding dihydrofolate reductases that are insensitive to trimethoprim (Duijkeren et al., 2018).

In *E. coli* from food-producing and companion animals, sulfonamide resistance is mediated by any of the following three *sul* genes: *sul1*, *sul2*, or *sul3*. The *sul1* gene is particularly widespread because it is part of the 3'-conserved segment of class 1 integrons. As such, the *sul1* gene is often found together with other antimicrobial resistance genes (ARGs) that are located on gene cassettes in the variable part of class 1 integrons. *sul1* and *sul2* genes are present in *E. coli* from healthy and diseased food-producing animals, companion animals, and wildlife all over the world found in combination with streptomycin resistances (Ojo et al., 2016).

The gene *sul3* was first described in 2003 in *E. coli* isolates from pigs in Switzerland (Poirel et al., 2018). Several reports described the *sul3* gene to be linked to other resistance genes, such as the

macrolide resistance gene *mef*(B), and to unusual class 1 integrons (Gillings, 2017).

Trimethoprim resistance is vehicle by numerous *dfr* genes detected in *Enterobacteriaceae* and other Gram-negative bacteria. Based on their sizes and structures, they have been divided into two major groups, *dfrA* and *dfrB*. The *dfrA* genes code for proteins of 152 to 189 amino acids, while the *dfrB*-encoded proteins are only 78 amino acids in size. Most of the *dfrA* and *dfrB* genes found in *E. coli* of animal origin are located on class 1 or class 2 integrons. In contrast to *dfrA* genes, *dfrB* genes have rarely been detected in *E. coli* from animals. In previous studies of *E. coli* from food-producing animals, a functionally active *dfrA14* gene was found outside an integron but inserted into a plasmid-borne *strA* gene (Ojo et al., 2002).

Resistance to polymyxins

Colistin (polymyxin E) is a polypeptide antimicrobial agent that targets the LPS in the outer membrane of Gram-negative bacteria. Colistin was a molecule widely used in veterinary medicine for metaphylactic use in different animal species. Recently, due to the increase of AMR phenomenon against this molecule, the possibility of transfer of AMR from animals to humans becomes more risky. For decades, colistin has been the preferred treatment for intestinal infections in pigs, poultry, and cattle (Stefaniuk and Tyski, 2019). Its use has been strongly reduced in human medicine since the 1970s and in veterinary medicine since 2016 due to increasing resistance to the molecule (Min et al., 2018).

Polymyxin resistance in *E. coli* isolates can be developed by genes encoding LPS-modifying enzymes. Normally, the operon *pmrCAB* codes for three proteins, namely, a phosphoethanolamine phosphotransferase PmrC, a response regulator PmrA (also called BasR), and a sensor kinase protein PmrB (also called BasS). Mutations on response regulator PmrA or in sensor kinase protein PmrB were detected as responsible of colistin AMR in *E. coli* isolates

from poultry in Spain (Poirel et al., 2018). Moreover, mutations in the genes *pmrA*, *pmrB*, *mgrB*, *phoP*, and *phoQ* of pigs *E. coli* were found (Delannoy et al., 2017).

Another mechanism conferring colistin resistance is related to plasmid related genes. The first gene discovered was *mcr-1* that encodes for MCR-1 phosphoethanolamine transferase, leading to LPS lipid A modification that induce the resistance mechanism (Poirel et al., 2017).

A few study reported the possible combination of *mcr-1* with ESBL-encoding genes. Eleven variants of the *mcr-1* gene have been found and has been identified from *mcr-1.2* to *mcr-1.12*; variant *mcr-1.3* , *mcr-1.8* and *mcr-1.9* have been found in chickens, poultry and pork respectively. Recently plasmid-mediated colistin-resistance *mcr-2* gene was identified in *E. coli* isolates from piglets in Belgium (Xavier et al., 2016). Then, other genes has been identified with their related variants: from *mcr-3* to *mcr-7*. *mcr-3*, *mcr-4 and mcr-5* has been found in *E. coli* isolated from pigs particularly in Asian and European countries (Poirel et al., 2018).

Resistance to glycylcycline

The glycylcyclines are 3rd generation tetracycline that were synthetized to overcome mechanisms of microbial resistance. They have a broad-spectrum activity on Gram-positive and Gram-negative bacteria, including strains that exabit AMR mechanisms (Rusu and Buta, 2021).

This class of antimicrobials has a bacteriostatic activity by binding to the bacterial 30S ribosomal subunit and by blocking entry of amino-acyl tRNA molecules into the A site of the ribosome. Moreover, amino acid residues incorporation in peptide chains is prevented by those antimicrobials, inhibiting the protein synthesis (Petersen et al., 1999).

Tigecycline is the representative glycylcycline antimicrobial agent and is known to overcome the major causes of tetracycline resistance: active efflux pump and protection of ribosomes. Mutational analysis of 16S rRNA, and structural modeling of tigecycline showed that it is able to

bind site in the 30S ribosomal subunit resulting in a sterile hindrance (Leng et al., 2021).

As mentioned previously, the unique attribute of tigecycline is its stability against these common mechanisms of tetracycline resistance. Tigecycline has demonstrated activity against strains containing *tet* genes, that can code both major forms of tetracycline resistance. It has been hypothesized that tigecycline is not unrecognized by resistant *tet* genes or that the large, bulky side chain of tigecycline prohibits the active expulsion efflux pump and sterically protect ribosome (Yaghoubi et al., 2022).

Nevertheless, the possibility of the development of AMR in new molecules is a significant concern (Pournaras et al., 2016)and few data are available on the molecular basis for resistance to tigecycline. The mechanism of resistance to this molecule is related to acquired mutations of Tet proteins (e.g., Tet(X), Tet(A), Tet(K) and Tet(M)) (Gordon and Wareham, 2009)related to horizontal transfer of mobile genetic elements carrying several resistance genes, particularly the mobile tigecycline-resistance *tet*(X) gene variants found both in humans and animals (Linkevicius et al., 2015). The Tet(X) is a flavin-dependent monooxygenase that was detected in *Enterobacteriaceae* and some *Acinetobacter* spp. isolates (Leski et al., 2013).

In Gram-negative bacteria, the chromosomally encoded, overexpression of resistance-nodulation division (RND) efflux pumps such as AdeABC, AdeFGH, AdeIJK, MexXY, and AcrAB are important molecular mechanisms in the resistance of bacteria to tigecycline (Veleba et al., 2013). Moreover, in Gram negative bacteria, the chromosomally encoded efflux pump, particularly the AcrAB and AcrEF pumps can be a substrate for tigecycline exclusion by bacteria cell (Hirata et al., 2004). The presence of *mar* regulon worsen this phenomenon because of the ability to downregulate the OmpF outer membrane porin stimulating the upregulation of the AcrAB efflux pump (Nishino et al., 2021). Particularly, in high tigecycline MICs *E. coli* strains a frameshift

mutation has been described in *marR* (one of the targets for reduced susceptibility to tigecycline) leading to the overexpression of MarA and AcrAB pumps (Nishino et al., 2021).

A low-level resistance was identified in isolates with mutations of efflux regulatory network genes (*lon*, *acrR*, and *marR*) and related lipopolysaccharide core biosynthesis pathway genes (*lpcA*, *rfaE*, *rfaD*, *rfaC*, and *rfaF*) (Linkevicius et al., 2013).

Escherichia coli as bioindicator of antimicrobial resistance

One of the most investigated microorganisms worldwide is *E. coli*. It is a Gram-negative bacterium belonging to the *Enterobacteriaceae* family. It colonizes gut microbiota of warm-blooded animals and for that reason it constantly influences human and animal life. It is not only a commensal bacterium, but it can cause several infections in humans and animals. Moreover *E. coli* is a ubiquitous bacterium, and it can be found in a wide type of environment. In fact, it can be found on food meaning a direct relation to fecal contamination: the presence of *E. coli* suggests poor hygienic conditions related to contamination or inadequate heat treatments (Reg EU 2073/ 2005).

As a contaminant and a commensal bacterium, *E. coli* come into contact with a variety of other microorganism, and it has the ability to share his mobile genetic elements with the neighbors' bacteria. *E. coli* is the most important reservoir of resistance genes that may be the cause of treatments failure in humans and animals. The number of the resistance genes identified in this microorganism increased in the past ten years and the majority of that are acquired by horizontal gene transfer. *E. coli* can be a recipient of resistance genes, can acquire genetic elements from other bacteria and can also be a donor, particularly with bacteria that share the same living environment (Card et al., 2017).

It must be concerned as a public health issue considering that genomic analysis highlight that

frequently this bacterium shows multi-resistant phenotype with the most frequent AMR to β-lactams and quinolones associated with TEM β-lactamases and gyrA mutation respectively. Moreover, *bla*CMY_2 is frequently harboured by *E. coli*, giving the ability to synthetize AmpC β-lactamases.

As reported by (EFSA, 2012). AMR monitoring from a public health perspective focuses on recommendations on bacterial species, food animal species and/or food products. Routinely, it is important to also evaluate the combination of those elements, focusing firstly on domestic production to compare the AMR found with antimicrobial usages and then on bacterial species, covering both zoonotic agents, as Salmonellae and *Campylobacter* spp., and then indicator organisms of the commensal flora. In fact, indicator organisms of commensal intestinal flora are commonly isolated from animal intestinal content and feces. Commensal *E. coli*, *Enterococcus faecium* and *Enterococcus faecalis* can be used as indicators of the Gram-negative and Gram-positive commensal intestinal flora, respectively. Lots of AMR and MDR profile have been highlighted in those microorganisms but commensal bacteria that contaminate food may be also considered as a potential AMR hazard. In fact, all commensal bacteria can harbour mobile genetic elements leading AMR transmission to host-adapted bacteria or to pathogens in intestine and in food environment too. For those reasons, the diffusion of AMR phenomenon can be more accurately evaluated in indicator bacteria than in food-borne pathogens, because all animals generally carry such indicator bacteria (Poirel et al., 2018).

Chapter 4. Exposure Assessment

Considering the food production chain as a possible transmission route of AMR to consumer, the exposure assessment should describe the pathway of the AMR microorganism in the above mentioned context . The aim is to define the objective considering all relevant pathways and risk factors to generate in the following chapters an estimate of the size of exposure to AMR (Caffrey et al., 2019).

Antimicrobial resistance transmission

Antimicrobial resistance evolution and widespread is mainly characterized by transfer events from ancestral species where resistance genes were shaped. Also, a chromosomal immobile antimicrobial resistance gene (ARGs) can evolve in an acquired resistance genes both for commensal and pathogen bacteria. When associated with insertion sequences or incorporated in integrons, ARGs make the first step of transmissibility followed by the ability to relocate in mobile elements that can be transferred within cells (Razavi et al., 2020). Fecal bacteria are known to often carry such elements and their living conditions frequently favor genes exchange (Flach et al., 2017). Those bacteria must be able to horizontal transferee those elements to a receiver assuming the necessity of "ecological connectivity", high metabolic activity and cell-to-cell contact of the process (Baquero et al., 2019). All the events described must be aligned in order to have a successful transfer.

Three principles are involved:

- DNA transformation. It is the ability of bacteria to internalize part of free DNA from the environment. The transformation must happen in competence physiological state but does not require cell-to-cell contact. This process is different among bacteria, but the uptake of DNA is similar among Gram-positive and Gram-negative bacteria.

Transduction can be generalized by any part of the bacterial chromosome and mobile genetic elements, or specialized involving only DNA adjacent to the original integrated prophages (European Parliament, 2019);

- transduction by bacteriophages. It is a phage mediated process that, after bacterial lysis can incorporate bacteria free DNA instead of phages DNA and transfer it to a new bacterial cell;
- conjugation involving plasmids and integrative conjugative elements. This process is contact dependent and needs the ability to synthetize an donor apparatus to reach the receiver.

Antimicrobials can enhance all those mechanisms through induction of competence for transformations in bacteria, promoting prophage excision and host cell lysis for transduction and increasing the conjugation transfer frequency (Liu et al., 2022).

Use of antimicrobials in swine breeding farms

The use of antibiotics in pig production is a possible route of development of AMR in microorganisms. In a One Health approach, all the three sides of global health (environment, animal and human health) can be affected by this phenomenon originating from swine (Waluszewski et al., 2021). In fact, in different studies resistant bacteria isolated in animals caused hard-to-treat infection in humans (Bonardi et al., 2022; Geenen et al., 2010). Particularly, antimicrobial use as growth promoters have impact on the spread of AMR bacteria in human (Collineau et al., 2018).

The kind of antimicrobials use in pig has been banned since January 2006 by the European Commission (EC) (European Commission, n.d.) and treatments guidelines has been developed to improve the awareness on antimicrobials to turn down the probability to develop AMR.

The therapeutic, metaphylactic, and prophylactic use has been subsequently evaluated to enumerate the risk related to pigs' production in all the different stages of production cycle.

Risks are higher in farms where prophylactic treatments are performed using CIAs (such as 3rd generation cephalosporins or fluoroquinolones), huge farm size, poor biosecurity, and incorrect farm health management (Raasch et al., 2020).

Different studies reported that the stage of production where antimicrobials are most used is weaned piglets and suckling piglets. Moreover, in farms that use lots of antimicrobials during piglets' production stage, the need of more antimicrobial use during finishing stage is even higher (Dewulf et al., 2022).

Despite this, countries all over Europe have different prevalence of antimicrobial usage probably in relation with the diseases that they have to face during pig production and the biosecurity level that they apply in farms (Stygar et al., 2020).

Italy is both an important livestock producer, with 11 million pigs slaughtered per year, and one of the highest consumers of antimicrobials in animals in Europe (Scali et al., 2020). These are the reasons why multi-resistances increased in Italy e.g., from 2002 to 2011 in *E. coli* isolated from pig (Luppi et al., 2015).

To better evaluate the phenomenon, standardized units of measurements were developed by the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project of the European Medicines Agency (EMA) namely, defined daily doses for animals (DDDvet) for cattle, pigs, and poultry (EMA, 2016).

The use of antimicrobials is relatively high in Italy if compared to other European Countries. In fact, the use of Highest Priority CIAs was 16.7% in about the 93% of farms. Macrolides were used in over 60% of farms but EMA recently declared this antimicrobial class as less important than other Highest Priority CIAs because of the minor involvement of pigs in the dissemination

of resistant *Campylobacter* spp. to humans (EMA, CVMP, CHMP, 2019). Colistin is used for the 50% of farms and 3rd and 4th generation cephalosporin were instead used only in 10% probably making nowadays the use of those molecule unnecessary in pigs farms (Jansen et al., 2022).

Pork food chain as potential route of antimicrobial resistance transmission

Commission Implementing Decision 2013/652/EU, monitoring of AMR is mandatory in *Salmonella, Campylobacter jejuni* and indicator commensal *E. coli* in the major domestically produced animal populations and their derived meat (EFSA, 2012). The rotational monitoring is performed on fattening pig and bovine under 1 year of age and their derived meat product. Extended-spectrum-β-lactamases, AmpC and Carbapenemases are specifically monitored in *Salmonella* and commensal indicator *E. coli*. The panel of monitored molecules include CIAs used at concentration ranges following ECOFF and the EUCAST clinical breakpoints (CBPs) to allow the comparison with human data (EFSA, 2012).

Inadequately consumption of food of animal origin is a public health risk. Poorly cooked or raw food, and the cross contamination during raw food manipulation or through the environment can be a source of AMR bacteria spreading from animals to humans. Despite the possibility of direct transmission of resistant bacteria from animals to humans, it seems that the antimicrobial use in food producing animals contribute to AMR phenomenon even if it is not yet understood its full extent (Jans et al., 2018). Monitoring AMR in different animal populations and their derived product instead of a generic categories' evaluation can help to better understand the implications of food production chain. Consumers usually come into contact with meat products derived from a wide variety of animal species and the analysis on those food matrices allows the evaluation of the food-borne AMR bacteria diffusion to consumers by broilers, laying hens,

fattening turkeys, fattening pigs, veal calves (EFSA, 2022). Milk production is frequently related with the pasteurization processes that destroy bacterial flora (O'Callaghan et al., 2018). Vegetables are considered as another important risk factor, but it is known that the origin of microorganisms' contamination is to be attributed to the manure being spread on the soil (Balali et al., 2020; Luna-Guevara et al., 2019). That is the reason why authorities usually limit the observation of their data reports to animal productions and the derived meat trying to monitor and understand the phenomenon (EFSA, 2022).

Different studies reported the detection of AMR bacteria in AMR poultry, swine, goats, cattle, and sheep (Samtiya et al., 2022). Seafoods that grows in aquaculture are more susceptible to genetic elements exchange because of the environmental set and they are one of the major causes of AMR in humans (Reverter et al., 2020). Moreover, different studied showed the presence of AMR genes in animals that not come into direct contact with humans suggesting the involvement of environment and food environment in this phenomenon (Peng et al., 2021). The most reported vehicle is poultry particularly for the transmission of AMR *Campylobacter* spp. (Marotta et al., 2019). Cattle and farm environment are involved in the dissemination of quinolone-resistant *E. coli* (Duse et al., 2016).

Pig farming is the most diffused animal industry worldwide and their derived meat product has high economic impact (Delsart et al., 2020). United Nations Food and Agriculture Organization predicts that pigs farming will have an expected increase of 8.6% by 2030 and 12.7% by 2050. With regulatory differences between countries, the use of antimicrobials in pig farming is impactful on the AMR phenomenon because of the extensive use (Monger et al., 2021).

Pig farms produce a huge amount of pig manure, farm wastewater, that are released in the environment as fertilizer or waste products, can contain antimicrobial residues in relation to the treatments used during breeding farm life (Yang et al., 2020). Streptococcaceae MDR genes are

frequently vehicle by manure, and in water the MDR microorganisms more found are *Moraxellaceae*. In both matrices, Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were the resistant microorganism most found (Zhang et al., 2021). Vegetables grown in manure-amended soils were shown to contain ARGs and antibiotic residues although roots seem to be more contaminated than leaves (Gao et al., 2020). Other studies demonstrated that water waste is equally involved 73 and that air dust in pig farm has an antibiotic concentration that can reach 12.5 mg/kg of dust. The most frequently antimicrobials found were aminoglycosides, tetracycline, and sulphonamides (Hamscher et al., 2003).

Interestingly, studies on pig microbiome and human microbiome highlight that pigs farm and slaughterhouse workers have a different microbiome if compared to the rest of the population with a significant increase in tetracycline, β-lactam and macrolide resistance genes but their resistome is not overlapping with pigs one, suggesting that direct contact with pigs feces is not the main route of ARGs transmission (van Gompel et al., 2020). Moreover, despite the presence of similarities after a prolonged direct contact with farmed animals, it reverts at original situation in humans after the contact stops (Sun et al., 2020). Although *mcr-1* colistin resistance genes were worryingly detected both from pigs and pigs' farmers phylogenetically similar *E. coli* suggesting a common origin (Monger et al., 2021; Viñes et al., 2021).

In addition, humans can be exposed to pigs derived ARGs during food production and consumption process (Deckers, 2016). Pigs are transported to slaughterhouses when the target weight is reached and pre-slaughter management procedures such as fasting to avoid transport related sickness and viscera perforation at evisceration, grouping animals in advance to reset hierarchical position among the newly formed group to be transported, and verification that antibiotic withdrawal period are settled (Driessen et al., 2020). Antimicrobial withdrawal time is important to avoid antimicrobial residues in meat (Monger et al., 2021). All pig tissues are

considered sterile in healthy animals (with the sole exception of lymph nodes for their immune functions) but during carcass dressing and retails it is contaminated by microorganisms derived from their skin, their gut, human workers, and slaughter environment. That's why meat can be a reservoir of AMR bacteria that can derive from farm management if the contamination is originated from pigs' skin or gut and from slaughter process in the other cases (Abass et al., 2020; Rugna et al., 2021). The reduction of antimicrobial usage in livestock can significantly reduce this phenomenon. Moreover, despite the presence of cleaning process in food environments, materials and workers can harbour AMR microorganisms and a possible co-resistance to sanitation products worsens the situation (particularly if caused by the presence of multi drug efflux pump) (Calero et al., 2018). Contamination of raw and ready to eat products can occur in market environment, in fact some AMR bacteria were found in pork ham, pork sausage, salami, and pork luncheon meat sliced at shops (Fijałkowski et al., 2016).

Integrated Program for Antimicrobial Resistance Surveillance monitored *E. coli* resistance prevalence in the different phases of the food producing chain highlighting that more isolates showed resistance profile in abattoirs than in farms and percentages become lower in meat product manipulated in market. It is reported that prevalence of antibiotic resistance is higher in raw meat than in processed meat even if the process can influence both positively and negatively on AMR if not performed correctly (as a stress factor on bacterial cells leading to possibility of bacterial genes mutation) (WHO, 2017).

Chapter 5. Hazard characterization- Materials and methods

The characteristics of the hazard are determine to subsequently evaluate the probability of the exposure to the hazard (Caffrey et al., 2019). The following paragraph describe the way to evaluate the hazard in relation to the aim of the study evaluating the presence of antimicrobial resistant microorganisms in the pork food chain. Results and discussion will be reported in Risk Characterization chapter, as combination of the previous three step of risk analyses.

Sample collection

The study was conducted on pork food chains analyzing samples collected from farm to fork. Samples were collected from 8 different pork food chain (A, B, C, D, E, F, G, H) located in Emilia Romagna region (North Italy) and each one was sampled twice, firstly in 2019/2020 and secondly in 2020/2022.

Fettering pig farms were selected by Azienda Unità Sanitaria Locale (AUSL) of Reggio Emilia and Modena. The selection of farms was based on the slaughterhouses and the meat production companies where animals were destinated; to collect representative samples of Emilia Romagna pork meat production companies both big production facilities (Modena) and small production facilities (Reggio Emilia) were included in the study.

Pigs' feces, pigs' carcasses and pork meat products including a portion of fresh meat, seasoned and fermented meat product were sampled.

Fecal samples were firstly collected using sterile fecal swabs and 15 pigs per farm were selected except for Farm B (17 pigs-2019/2020), Farm E (16 pigs-2019/2020 and 16 pigs 2020/2022) and Farm H (16 pigs-2019/2020). All samples were repeated twice as described before. Pigs were marked with an ear tag and followed along the food production chain. Fecal samples were collected at least 30/40 days before slaughtering and informations on pharmacological treatments

subministrated to pigs in the last 6 months before slaughtering were taken using veterinary electronic prescription system.

The same pigs (almost 15 pigs per farm sampled twice) were followed at slaughterhouses and carcasses' samples using prewetted- sponges were collected following UNI ISO 17604:2015 and Reg. CE 2073/05. Carcasses were sponged in four points: loin, cheek, medial face of the thigh, and belly, each for an area of 100 cm^2. Some carcasses could not be sampled for organizational reasons. At slaughterhouses samples of fresh meat were collected. Not all the pigs considered before were meat sampled: only the ones that gave interesting results at the fecal swab analysis were selected to not interfere excessively with production chain. A portion (at least 25 g) was collected as fresh meat and others were destinated to transformation and were analyzed after proper seasoning (coppa, pancetta) and fermentation (salami). Transformation meat process includes the use of additives, salt and the reduction of the activity water that usually interfere with bacterial growth. However, those kind of meat products were selected to evaluate the exposure to AMR in these ready to eat products.

The total number of samples collected were 245 fecal swabs, 225 carcass sponges, 62 meat samples, 15 seasoned products and 7 fermented products. Fermented products weren't corresponding to one single pig because of necessity of production that gather part of different carcasses of the same pig batch together.

All the samples were sent to the laboratory of Food Hygiene and Inspection of the Veterinary Science Department, University of Parma.

Escherichia coli isolation

E. coli isolation was characterized by samples enrichment phase, an isolation phase and an identification phase following UNI EN ISO 16649-2:2001. The enrichment phase of fecal swabs consisted in put the swab into a sterile tube adding 9 mL of Buffered peptone Water (BPW;

Biolife Italiana, Milan) and incubate at 37°C overnight. The enrichment phase of carcass sponges requires to add the sponge with 225 mL of BPW into a sterile bag and incubate at 37°C overnight. For fresh meat, seasoned and fermented product 25 g of representative cross-section of the samples were portioned in a sterile bag with 225 mL of BPW and homogenized using BagMixer Interscience (Instrument Lab Control, Reggio Emilia, Italy); samples were incubated at 37°C overnight.

The isolation and identification phases were analogous for all the different samples matrices: using a sterile calibrated handle, the broth culture was streaked onto Triptone Bile X-gluc (TBX; Biolife Italiana, Milan, Italy) agar, and incubated at 42°C overnight. This chromogenic medium is selective for the presence of bile salts inhibiting Gram-positive bacteria. Differential action is given by hydrolysis of the chromogenic substrate X-Gluc (5-bromo-4-chloro-3-indoli-b-D-glucuronide) by the enzyme β-glucuronidase. The reaction results in the formation of a blue-green pigment. Five typical colonies were selected and seeded onto basic nutrient medium, Tryptic Soy Agar (TSA, Biolife Italiana, Milan, Italy) and incubated at 37°C. Each colony was than suspended in BPW, incubated again at 37°C overnight and subjected to indole test. This test is used to evaluate the ability of bacteria to decompose the amino acid tryptophane to indole, which is important in the identification of Enterobacteria. This reaction is performed by tryptophanase, a chain of several different intracellular enzymes. Positive reaction was characterized by formation of "cherry-red ring" (pink to red color) in the reagent layer on top of the medium within seconds of adding the Kovaks' reagent (Biolife Italiana, Milan, Italy). One of the indole positive colonies identified for each sample was finally confirmed as *E. coli* with the miniaturized API 20E system (bioMérieux, France). This system is indicated in the identification of *Enterobacteria*; it consists of 20 microtubes containing dehydrated substrates to inoculate with the bacterial suspension created in the dedicated suspension media. Most reactions produced

during the incubation period result in spontaneous color changes or revealed upon addition of reagents. The interpretations of these reactions (positive/negative) are based on manufacturing instruction and recorded as numerical profile that allows identification of the species by comparison with identification software.

β-lactams antimicrobial resistance evaluation- ESBL, AmpC

Phenotypical analysis

All the *E. coli* isolated before were tested for the ability to produce ESBL and AmpC, using the disk diffusion test following the protocol defined by EUCAST, 2019.

Bacterial suspension was prepared at an optical density (OD) of 0.5 McFarland using Mueller Hinton Broth Cation Adjusted (Biolife Italiana, Milan, Italy). Mueller Hinton Agar plates were subsequently swab seeded and filter paper disks impregnated with a standardized concentration of antimicrobial agents were placed on the surface. On those conditions, water is absorbed into the disk from the agar and the antimicrobial begins to diffuse into the surrounding agar. As rapidly as the solubility properties of the drug in MH agar and the molecular weight of the antimicrobial compound. The diffusion rate led to a higher concentration of antimicrobials closest to the disk and a logarithmic reduction in concentration in the plate. Alle factors hesitate in a unique breakpoint zone size indicating resistance or susceptibility to antimicrobial compounds. The diameter of the zone of growth inhibition around the disk is measured using a caliper after overnight incubation at 37°C.

Protocol for the detection of ESBL and AmpC bacteria includes firstly a screening disk test followed by a confirmation disk test.

Screening test was performed using two cephalosporin: cefotaxime 5 μg (CTX05) and ceftazidime 10μg (CAZ10). Bacterial with resistance profile showed a growth inhibition diameter

CTX < 17 mm and CAZ < 19 mm. Intermediate profile had an inhibition diameter 20 ≤ CTX< 17 and 22 ≤ CAZ < 19. Resistant and intermediate *E. coli* were phenotypically confirmed as ESBL and/or AmpC with the combination disk test (CDT) (EUCAST, 2019).

For the detection of ESBL resistance, the test uses one disk of cefotaxime (CTX 30μg) and a disk of cefotaxime (30μg) in combination with clavulanic acid (10μg) (CTX+C). Moreover, a disk of ceftazidime (CAZ 30μg) and one of ceftazidime 30μg + clavulanic acid 10μg (CAZ+C) is used.

The confirmation of AmpC *E. coli* uses a disk of cefotaxime (CTX 30μg) and a disk of cefotaxime 30μg + cloxacillin 10μg (CTX+CX) and a disk of ceftazidime (CAZ 30μg) with a disk of ceftazidime 30μg + cloxacillin 10μg (CAZ+CX).

Clavulanic acid and cloxacillin are two molecules able to inhibit ESBL and AmpC resistance respectively restoring cephalosporin activity.

The inhibition zone around the cephalosporin disk combined with clavulanic acid or cloxacillin is compared with the zone around the disk with the cephalosporin alone.

The combined-disk test was considered positive when the growth inhibition zone around the CTX or the CAZ disks with clavulanate or cloxacillin was 5 mm wider than the diameter around the disks containing CTX or CAZ alone.

Genotypical analysis

DNA from phenotypically confirmed ESBL and AmpC *E. coli* isolates was extracted using a commercial kit (Purelink genomic DNA purification kit, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. In addition, *E. coli* isolates that resulted as being intermediate at the screening with cefotaxime and ceftazidime were also selected and analysed for the presence of ESBL and AmpC genes.

Briefly, an overnight broth culture was prepared starting from five pure colonies of *E. coli* grown on Tryptic Soy Agar (TSA) (Biolife Italiana, Milano, Italia), centrifuged at 15,000 rpm for 5 min,

and the pellet was used for DNA purification using the reagents of the commercial kit mentioned above. Final elution was done in a volume of 50 µL with the elution buffer provided by the kit, and quantity and quality of the extracted DNA were determined with a spectrophotometer (Biospectrometer, Eppendorf, Hamburg, Germany).

A real-time PCR with Sybrgreen (SsoAdvanced SYBR Green Supermix Bio-Rad, Hercules, CA, USA) was applied in order to verify the presence of ESBL-associated genes: *bla*$_{\text{CTX-M1}}$, *bla*$_{\text{CTX-M2}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$, as described by Roschanski et al. (2014). Real-time amplifications were performed in 20 µl reactions containing 10 µl Gotaq qPCR Mix 2× (Promega Italia) at a final concentration of 1×. Forward and reverse primers were added at a final concentration of 0.3 µM. Each gene was tested individually. Supplemental CXR reference Dye was added at 300 nM. A total of 1 µl of sample lysate was added to the reaction mixture and Nuclease Free Water to final volume. Preliminary tests to define the correct annealing temperature for each primer were done, and in each reaction positive (*K. pneumoniae* NCTC 13368 for *bla*$_{\text{SHV}}$, *E. coli* NCTC 13351 for *bla*$_{\text{TEM}}$ and *E. coli* NCTC 13353 for *bla*$_{\text{CTX-M}}$) and negative controls were added. The presence of a nonspecific product was avoided through melting curve analysis. The amplification protocol was characterized by a denaturation step (95 °C for 3 min) and 39 repeated cycles (95 °C for 15 s; 50 °C for 15 s; 72 °C for 20 s). Fluorescence signals were collected in every cycle and each sample was tested four times.

The presence of AmpC genes was verified using the oligonucleotides and the multiplex PCR protocol described by Pérez-Pérez and Hanson (2002), with some modifications. The Multiplex PCR Master Mix was prepared with GoTaq G2 Flexi DNA Polymerase kit (Promega italia S.r.l., Milan, Italy). Master mix was prepared for 50 µl of final volume reaction containing 5×Green GoTaq Flexi Buffer at a final concentration of 1×, 2 mM of $MgCl_2$, 0.2 mM of dNTPs and 1.25U

of GoTaq G2 Flexi DNA Polymerase. *bla*$_{MOX}$, *bla*$_{CIT}$, *bla*$_{DHA}$ primers were added at a final concentration of 0.6 µM, *bla*$_{ACC}$ and *bla*$_{EBC}$ primers at 0.5 µM and *bla*$_{FOX}$ at 0.4µM. The PCR amplification protocol consisted in an initial denaturation of 94°C for 3 min followed by 25 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 64°C for 30 s and extension at 72°C for 1 min. Final extension was performed at 72°C for 7 min. PCR products were evaluated by electrophoresis with 2% of agarose gels stained with SYBR Safe DNA gel stain (Invitrogen, Poland, OR) and visualized by UV light. A 100-bp DNA ladder from Promega was used as marker.

Bacteria belonging to each food production chain were clustered by their AMR pattern considering their sample origin. All the strains that showed the same AMR and AMR genes in feces, carcasses and meat product of the same pig were considered together for performing phylogenetic analysis; all the food chain of the pigs identified were included in the analysis even if not all the strains were phenotypically or genotypically resistant. Moreover, if the resistant strains were found in meat products, the entire food derived chain was analysed. Determination of the *E. coli* isolates' phylogenetic relatedness was performed by using Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) as described by Ventura et al. (2003). DNA extraction was performed suspending five colonies of overnight bacterial culture on Triptic Soy Agar (TSA - Oxoid, Basingstoke, United Kingdom) in 1 mL of sterile distilled water, heating at 95°C for 10 min and removing cellular debris by 15,000 rpm centrifugation for 5 min. The supernatant was used for amplification after proper quantification by Biospectrometer Basic Eppendorf (Eppendorf, Milan, Italy). The amplification was carried out with GoTaq G2 Flexi DNA Polymerase kit (Promega Italia S.r.l., Milan, Italy). Master mix was prepared for 25 µL of final volume reaction containing 5x Green GoTaq Flexi Buffer at a final concentration of 1X, 3 mM of $MgCl_2$, 0,2 mM of dNTPs and 2.5 U of GoTaq G2 Flexi DNA Polymerase. Primers ERIC-1

primer (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5-AAGTAAGTGACTGGGGTGAGCG-3) were added at a final concentration of 1 μM. A total of 3 μL of sample lysate was added to the reaction mixture and Nuclease Free Water to final volume. The PCR protocol consists in an initial denaturation of 94°C for 3 min followed by 35 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 48°C for 30 s and extension at 72°C for 4 min. Final extension was performed at 72°C for 6 min. PCR products were evaluated by electrophoresis with 2% of agarose gels stained with 0.5 μg/mL of SYBR Safe DNA gel stain (Invitrogen, Poland, OR) at a voltage of 7 V/cm for 40 mins and finally visualized by UV light. A 100-bp DNA ladder from Promega (Milan, Italy) was used as marker.

Comparative Genomic Analysis - Average Nucleotide Identity

Bacterial strains, after biochemical identification up to 99% with API 20E, were delivered to Department of Chemistry, Life Sciences and Environmental Sustainability of the University of Parma where comparative genomic analyses were performed following Alessandri et al. (2022). After an overnight culture of cells, 10 mL were centrifugated at 6000 rpm for 8 min and the pellet was used for DNA extraction using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, Darmstadt, Germany) following the manufacturer's guidelines.

The bacterial chromosomal DNA was decoded through a MiSeq platform (Illumina, San Diego, CA, USA) according to the manufacturer's protocol by using the Nextera XT DNA Library Prep kit (Illumina). The library samples obtained were then pooled into a Flow Cell V3 600 cycle (Illumina). Subsequently, the .fastq files of paired-end reads generated from each genome sequences were employed as input for the genome assembly by using the MEGAannotator pipeline (Lugli et al., 2016). Then, to predict protein-coding open reading frame (ORFs), MEGAnnotator employed contigs longer than 1,000 bp, through Prodigal (Hyatt et al., 2010).

A value of average nucleotide identity (ANI) was calculated through the program fastANI, using each genome pair as the input (Alessandri et al., 2022; Lugli et al., 2020) Subsequently the generated ANI matrix was reordered by using a Hierarchical Clustering Analysis (HCL) performed through OriginPro 2021.

The whole-genome ANI has emerged in the last years as a fundamental method to assess species boundaries in the estimation of genetic relatedness. Microorganisms belonging to the same species typically shows ≥95% ANI to each other (Donovan, 2017).

FastANI enables accurate estimation of pairwise ANI values for large cohorts of genomes or evaluation of the novelty of a query draft genome by comparing it against the full collection of available prokaryotic genomes (Lugli et al., 2022).

Multi drug resistances evaluation- MDR

Phenotypical analysis

All *E. coli* were tested for susceptibility to a set of molecules on Sensititre plates™ (Thermofisher Scientific, Italy) defining the Minimum Inhibitory Concentration (MIC) following manufacturer's instructions. It is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). The bacterial suspension inoculated was 5 x 10^5 CFU/mL and plates were incubated at 35 $\pm$ 1°C for 18$\pm$2 h as defined by EUCAST, 2019.

Each plate was customized with

- β-lactams: meropenem (MERO: Sensible (S)≤0,25 – Resistant (R)>8), piperacillin/tazobactam (P/T4: S≤8 - R>16), amoxicillin/clavulanic (AUGC: S≤8 - R>8), ceftolozane/tazobactam (C/T4: S≤1 – R>1), cefotaxime (FOT: S≤1 – R>2), ceftazidime

(TAZ: S≤1 – R>4), ceftazidime/tazobactam (CZA: S≤8 – R>8), imipenem (IMI: S≤ 2 - R>8), ertapenem (ETP: S≤ 0,5 - R>1); aztreonam (AZT: S≤1 - R>4);

- aminoglycosides: amikacin (AMI: S≤8 - R>16), gentamicin (GEN: S≤2-R>4), tobramycin (TOB: S≤2 - R>4);
- quinolones: ciprofloxacin (CIP: S≤0,25 - R>0,5);
- polymixin: colistin (COL: S≤2 – R>2);
- glycylcyclines: tigecycline (TGC: S≤1 - R>2);
- sulphonamides: sulphamethoxazole/trimethoprim (SXT: S≤2 -R>4).

Optical density of bacterial growth was recorded at 620 nm by Multiskan FC Version 1.00.75 by Thermofisher Scientific.

Genotypical analysis

Bacteria belonging to each food production chain were clustered by their AMR patter considering their sample origin. All the strains that showed the same AMR and AMR genes in feces, carcasses and meat product of the same pig were considered together for performing phylogenetic analysis; all the food chain of the pigs identified were included in the analysis even if not all the strains were phenotypically or genotypically resistant. Moreover, if the resistant strains were found in meat products, the entire food derived chain was analysed.

Determination of the *E. coli* isolates' phylogenetic relatedness was performed by using Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) as described by Ventura et al. (2003). DNA extraction was performed suspending five colonies of overnight bacterial culture on Triptic Soy Agar (TSA - Oxoid, Basingstoke, United Kingdom) in 1 mL of sterile distilled water, heating at 95°C for 10 min and removing cellular debris by 15,000 rpm centrifugation for

5 min. The supernatant was used for amplification after proper quantification by Biospectrometer Basic Eppendorf (Eppendorf, Milan, Italy).

The amplification was carried out with GoTaq G2 Flexi DNA Polymerase kit (Promega italia S.r.l., Milan, Italy). Master mix was prepared for 25 µL of final volume reaction containing 5x Green GoTaq Flexi Buffer at a final concentration of 1X, 3 mM of $MgCl_2$, 0,2 mM of dNTPs and 2.5 U of GoTaq G2 Flexi DNA Polymerase. Primers ERIC-1 primer (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5-AAGTAAGTGACTGGGGTGAGCG-3) were added at a final concentration of 1 µM. A total of 3 µL of sample lysate was added to the reaction mixture and Nuclease Free Water to final volume. The PCR protocol consists in an initial denaturation of 94°C for 3 min followed by 35 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 48°C for 30 s and extension at 72°C for 4 min. Final extension was performed at 72°C for 6 min. PCR products were evaluated by electrophoresis with 2% of agarose gels stained with 0.5 µ/mL of SYBR Safe DNA gel stain (Invitrogen, Poland, OR) at a voltage of 7 V/cm for 40 mins and finally visualized by UV light. A 100-bp DNA ladder from Promega (Milan, Italy) was used as marker.

Chapter 6. Risk characterization

Risk characterization is based on the findings obtained from the hazard identification, exposure assessment and hazard characterization steps. The analysis is performed to obtain a Risk Estimate that is described through the result obtained (Caffrey et al., 2019).

Results- ESBL, AmpC

Escherichia coli isolation

Collected samples were processed for *E. coli* isolation. *E. coli* were isolated from all carcasses, fresh meat, and fermented meat product samples: 225 strains from carcasses, 62 strains from fresh meat, and 7 strains from fermented meat products. In fecal and seasoned meat product samples, 243/245 and 8/15 *E. coli* were isolated, respectively.

Antimicrobial Resistance Evaluation- ESBL and AmpC

All the isolates were tested for their ability to produce ESBL and AmpC β-lactamases through disk diffusion method. Data showed that in the first sampling period (2019/2020) the 4.1% (CI 95% = 2.3-5.9) of the isolates from fecal swabs harboured ESBL enzyme. This percentage in 2020/2022 reached the 9.9% (CI 95% = 4.8-15) as shown in table 1. Carcasses *E. coli* resistance prevalence showed the same trend; in particular, ESBL prevalence was 6.8% (CI 95% = 1.6-12) in the first sampling period and was 13.1% (CI 95% = 11.3-14.9) in the second. High ESBL percentages were shown in pork meat product of the 2019/2020 sampling period: fresh meat resistant isolates were 15.8% (CI 95% = 0-34.1); seasoned meat product resistant isolates were 28.6% (CI 95% = 11-46.2), fermented meat product resistant isolates were 20% (CI 95% = 0-55). Data changed in the 2020/2022 to 9.3% (CI 95% = 4.2-14.4) in fresh meat an 0% in processed meat product.

AmpC resistance prevalence were 3.3% (CI 95% = 0.3-6.3) in the first sampling period (2019/2020) and 5.8% (CI 95% = 1-10.6) in 2020/2022 of fecal *E. coli*, 2.9% (2019/2020) (CI 95% 0-6.1) and 5.7% (2020/2022) (CI 95% = 3.2-10.2) of carcass *E. coli*, 0% (2019/2020) and 4.8% (CI 95% = 0-13) of fresh meat *E. coli* while no AmpC isolates were detected in processed meat product isolates.

Intermediate resistance profiles were found in fecal, carcass and fresh meat product ranged approximately from 7 to 16% of isolates in both sampling period. In seasoned and fermented meat product *E. coli* of the first sampling period, percentages reached the 28.6% (CI 95% = 0-46.2) and 60% (CI 95% = 0-100) respectively and were absent in 2020/2022.

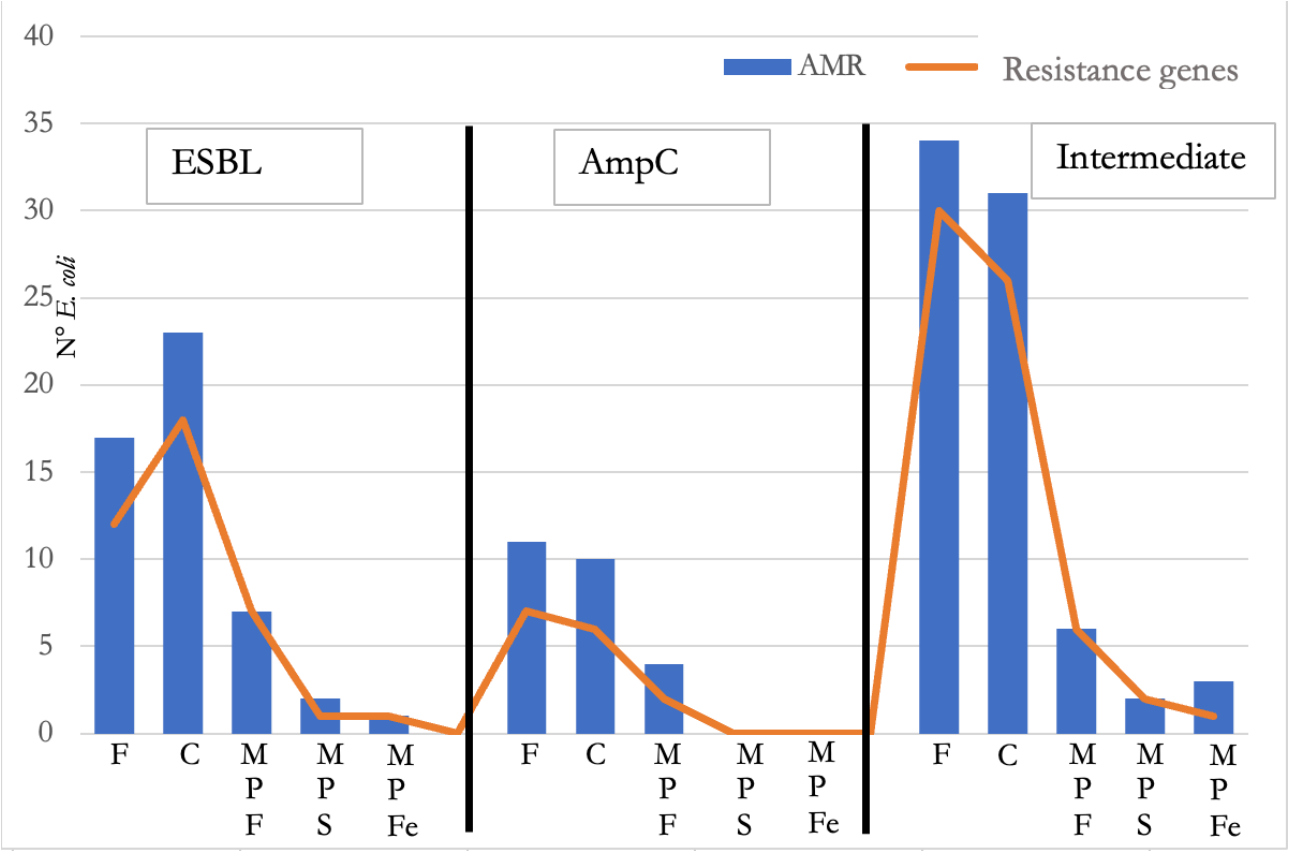
Table 1. Prevalence of ESBL, AmpC and Intermediate *E. coli* isolated in the entire pork food chain. (MP= meat product).

	2019/2020			2020/2022		
ESBL	**AMR *E. coli***	***E. coli* isolates**	**%**	**AMR *E. coli***	***E. coli* isolates**	**%**
Feces	5	122	**4,10**	12	121	**9,92**
Carcasses	7	103	**6,80**	16	122	**13,11**
Fresh MP	3	19	**15,79**	4	43	**9,30**
Seasoned MP	2	7	**28,57**	0	0	**-**
Fermented MP	1	5	**20,00**	0	0	**-**
AmpC						
Feces	4	122	**3,28**	7	121	**5,79**
Carcasses	3	103	**2,91**	7	122	**5,74**
Fresh MP	0	19	**0,00**	2	42	**4,76**
Seasoned MP	0	7	**0,00**	0	0	**-**
Fermented MP	0	5	**0,00**	0	0	**-**
Intermediate						
Feces	19	122	**15,57**	15	121	**12,40**
Carcasses	13	103	**12,62**	18	122	**14,75**
Fresh MP	3	19	**15,79**	3	43	**6,98**
Seasoned MP	2	7	**28,57**	0	0	**-**
Fermented MP	3	5	**60,00**	0	0	**-**

All resistant strains were tested for the presence of the most common plasmidic resistance genes. Data showed that not all the strains that were phenotypically ESBL or AmpC harboured resistance genes. In fact, in 2019/2020, only the 40% (2/5) of fecal ESBL *E. coli* harboured resistance genes, the 57.1% (4/7) of carcass ESBL *E. coli* were genotypically confirmed by the presence of resistant genes, and the 100% (3/3) of fresh meat ESBL *E. coli*, the 50% (1/2) of ESBL seasoned meat product isolates and the 100% (1/1) of fermented meat product ESBL *E. coli* were genotypically confirmed. In AmpC isolates the 25% (1/4) of fecal *E. coli*, 33.3% (1/3) carcasses *E. coli*, ESBL resistance genes were detected. Moreover, no AmpC resistance genes were found. Despite their no full-blown resistant profile, intermediate *E. coli* were genotypically tested to evaluate the possible relation with genetic elements. The 84.2% (16/19) of intermediate fecal *E. coli*, the 69.2% (9/13) of intermediate carcasses isolates and the 100% of meat product isolates, with the sole exception of fermented meat products (33.3% - 1/3) harboured ESBL resistance genes (data not shown).

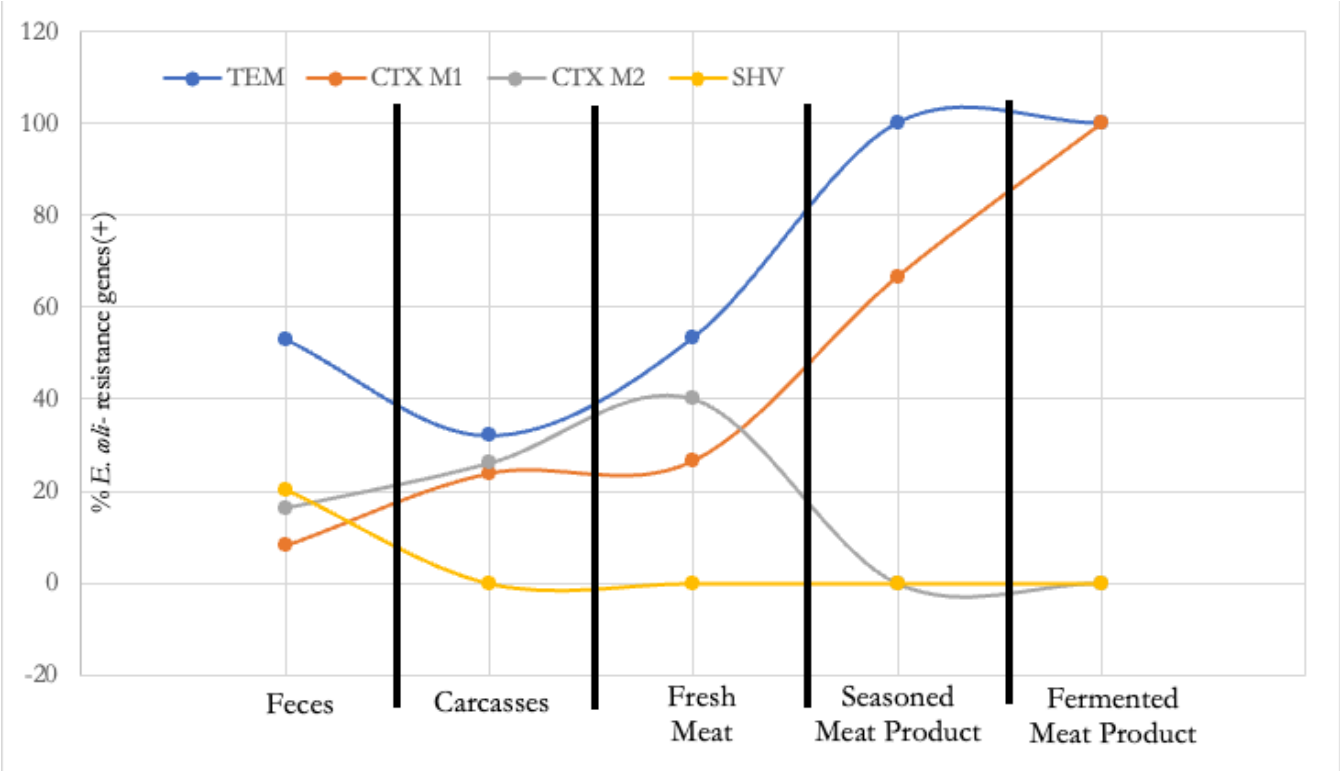
In 2020/2022, the 83.3% (10/12) of fecal ESBL *E. coli*, the 87.5% (14/16) of carcass ESBL isolates and the 100% (4/4) of fresh meat ESBL isolates harboured ESBL resistant genes. AmpC resistant isolates harboured ESBL mobile genetic elements in the most of resistant isolates: 85.7% (6/7) of fecal AmpC *E. coli*, 71.4% (5/7) of carcass AmpC *E. coli* and the 100% (2/2) of fresh meat AmpC *E. coli*; no AmpC related resistance genes were found. Isolates with an intermediate profile harboured ESBL mobile genetic elements in most of the cases: 93.3% (14/15) of fecal intermediate *E. coli*, 94.4% (17/18) of carcass intermediate *E. coli* and the 100% (3/3) of fresh meat intermediate isolates. In Figure 4, a comparison between phenotypic and genotypic resistance prevalence was reported considering overall data.

Figure 5. ESBL, AmpC and Intermediate *E. coli* prevalence and the plasmidic related genes in 2019/2022. (F = feces, C = carcasses, MPF = fresh meat product, MPFe= fermented meat product, MFS= seasoned meat product).



As shown in Figure 5, the most frequently found mobile genetic element conferring resistance was *bla*$_{TEM}$ followed by *bla*$_{CTX-M1}$. They were frequently found in association in all the phases of the pork food chain.

Figure 6. ESBL related genes trend along the different stages of the pork food chain.



Bla$_{CTX-M2}$ was frequently found in carcass and fresh meat resistant isolates and *bla*$_{SHV}$ was found only in fecal resistant isolates. Considering both sampling periods, the resistant and intermediate *E. coli* harboured *bla*$_{CTX-M1}$ in 8.2%, 24.4% and 33.3% of the fecal, carcass and fresh meat resistant *E. coli* respectively. *Bla*$_{CTX-M2}$ was harboured by the 16.3%, 26% and 50% of fecal, carcass and fresh meat isolates respectively. *Bla*$_{TEM}$ was harboured by the 53%, 28%, 66.7% of fecal, carcass and fresh meat isolates respectively and *bla*$_{SHV}$ was found only in fecal isolates with the prevalence of 20.4%. Resistant or intermediate strains isolated from processed (seasoned and fermented) meat product were found only in 2019/2020 and the plasmid genes found were *bla*$_{TEM}$ in the 100% of isolates (3/3 *E. coli* for seasoned meat product and 2/2 *E. coli* for fermented meat

product), and *bla*$_{\text{CTX-M1}}$ in the 33.3% of seasoned meat product and in the 100% of fermented meat product, in combination with *bla*$_{\text{TEM}}$.

As described before, phylogenetical analysis was performed on pork food chain isolates chosen using the obtained data. Nine pork food chains were identified in the first year of sampling and 19 pork food chains were chosen in the second year. The bacteria isolated from Pig 3 of Farm A, in the first sampling period showed an intermediate profile both in fecal isolates and fermented meat product isolates: all the isolates along the food chain were included in the study. At the same time, in Pig 9 from Farm A (2019/2020) all the strains isolated from feces to seasoned meat product showed an intermediate profile and they were chosen for further analysis. In Pig 34 from Farm B (2020/2022) only the isolates from meat product showed a resistant profile but all the isolates of the same food chain were included for the further investigations. The same criteria were applied to all the isolates of the other pork food chains.

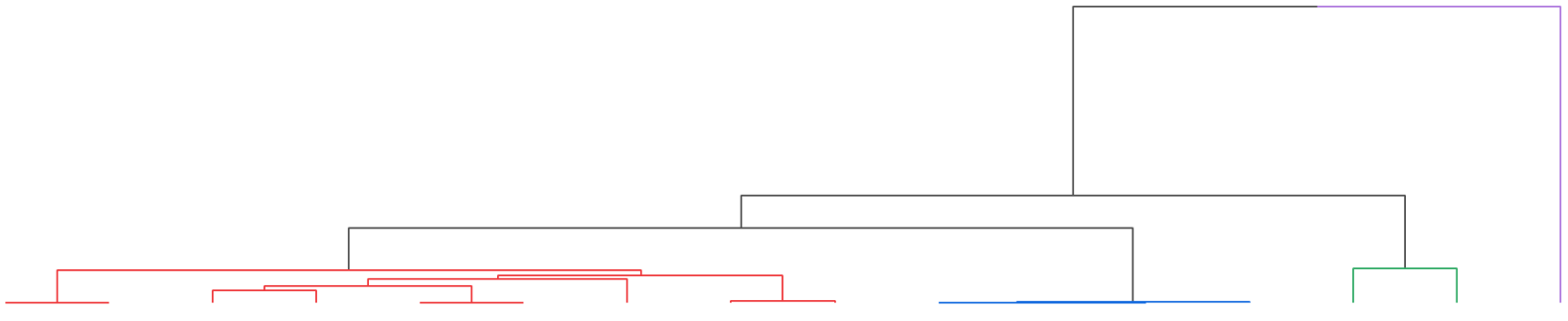
Phylogenetic analysis showed that some similarities were found in Pig 3 (Farm A) including feces, carcasses and fresh meat isolates. The same results were found in Pig 24 belonging to farm F, that showed similar phenotypic and genotypic profiles too. Similarities between carcasses *E. coli* and fresh meat isolates were shown in Pig 14 (Farm A), Pig 57 (Farm A) and Pig 9 (Farm H) even if phenotypical and genotypical analysis didn't highlight the same resistance profile. Similarities were detected in *E. coli* from carcasses and fermented meat product isolates in Pig 34 from Farm F and between isolates from fresh meat and seasoned fermented product in Pig 40 from Farm C. All data are reported in Table 2.

Table 2. Pig food chain selected for ERIC analysis. Phylogenetic similarities are highlighted in the table (bold and underlined). The enumeration of "Pig" is independent and related to each farm. (Int=Intermediate AMR profile)

Sampling period	Farm	Feces	AMR	AMR Genes	Carcasses	AMR	AMR Genes	Fresh Meat	AMR	AMR Genes	Seasoned MP	AMR	AMR Genes	Fermented MP	AMR	AMR Genes
2019/2020	A	**Pig 3**	**Int**		**Pig 3**			**Pig 3**			Pig 3			Pig 3	Int	
		Pig 8	ESBL		Pig 8			Pig 8			Pig 8	Int	*bla* CTXM1 *bla* TEM			
		Pig 9	Int	*bla* TEM	Pig 9	Int	*bla* CTXM1 *bla* CTXM2 *bla* TEM	Pig 9	Int	*bla* CTXM1 *bla* CTXM2 *bla* TEM	Pig 9	Int	*bla* CTXM1 *bla* TEM			
		Pig 14	Int	*bla* TEM	**Pig 14**			**Pig 14**	**ESBL**	***bla* CTXM1 *bla* TEM**						
	C	Pig 36	AmpC		Pig 36			Pig 36						Pig 36	Int	*bla* CTXM1 *bla* TEM
		Pig 40	Int		Pig 40	Int		**Pig 40**			**Pig 40**	**ESBL**				
		Pig 46	Int	*bla* CTXM2 *bla* SHV	Pig 46	Int		Pig 46			Pig 46	ESBL	*bla* TEM			
	F	**Pig 24**	**ESBL**	***bla* TEM**	**Pig 24**	**ESBL**	***bla* TEM**	**Pig 24**	**ESBL**	***bla* TEM**				Pig 24	ESBL	*bla* TEM
	G	Pig 34			Pig 34			Pig 34	Int	*bla* CTXM2				Pig 34	Int	
2020/2022	A	Pig 47	Int	*bla* CTXM1 *bla* TEM	Pig 47	ESBL+AmpC	*bla* CTXM1 *bla* CTXM2 *bla* TEM *bla* SHV	Pig 47								
		Pig 57	AmpC	*bla* TEM	**Pig 57**			**Pig 57**	**ESBL**	***bla* CTXM1 *bla* CTXM2 *bla* TEM**						
		Pig 58	ESBL+AmpC	*bla* CTXM1 *bla* TEM	Pig 58	ESBL	*bla* TEM	Pig 58								
		Pig 59	Int	*bla* CTXM1 *bla* CTXM2 *bla* TEM	Pig 59			Pig 59	Int	*bla* CTXM1 *bla* CTXM2 *bla* TEM						
	B	Pig 34			Pig 34			Pig 34	ESBL	*bla* CTXM1 *bla* CTXM2 *bla* TEM						
		Pig 35	ESBL+AmpC	*bla* CTXM1 *bla* CTXM2 *bla* TEM	Pig 35	ESBL+AmpC	*bla* CTXM1 *bla* CTXM2 *bla* TEM *bla* SHV	Pig 35								
	C	Pig 17	ESBL	*bla* CTXM2	Pig 17	Int	*bla* CTXM1 *bla* TEM	Pig 17								
		Pig 21	Int	*bla* CTXM2 *bla* TEM	Pig 21	Int	*bla* CTXM1 *bla* CTXM2 *bla* TEM	Pig 21								
		Pig 27	ESBL		Pig 27	Int	*bla* CTXM1 *bla* CTXM2 *bla* TEM	Pig 27	AmpC	*bla* CTXM1 *bla* CTXM2 *bla* TEM						
		Pig 29	Int	*bla* CTXM2 *bla* TEM	Pig 29	Int	*bla* CTXM1 *bla* CTXM2 *bla* TEM	Pig 29								
	D	Pig 1	ESBL	*bla* TEM	Pig 1	Int	*bla* TEM	Pig 1	AmpC	*bla* CTXM2 *bla* TEM						
		Pig 3			Pig 3	ESBL		Pig 3	Int	*bla* TEM						
		Pig 13	Int	*bla* CTXM2	Pig 13	AmpC	*bla* TEM	Pig 13								
	E	Pig 30	Int	*bla* CTXM1 *bla* CTXM2 *bla* TEM	Pig 30	ESBL	*bla* TEM	Pig 30	Int	*bla* TEM				Pig 30		
	F	Pig 34	Int	*bla* CTXM2 *bla* TEM	**Pig 34**	**ESBL**	***bla* CTXM1 *bla* CTXM2 *bla* TEM**	Pig 34						**Pig 34**		
		Pig 38	ESBL+AmpC	*bla* CTXM2 *bla* TEM	Pig 38			Pig 38	ESBL	*bla* CTXM1 *bla* CTXM2 *bla* TEM						
	G	Pig 48	Int		Pig 48			Pig 48			Pig 48			Pig 48		
	H	Pig 9			**Pig 9**	**Int**		**Pig 9**			Pig 9					
		Pig 13	Int	*bla* CTXM1 *bla* CTXM2 *bla* TEM	Pig 13	Int	*bla* CTXM1 *bla* TEM	Pig 13								

All the strains that showed phylogenetic similarities were subjected to ANI sequencing technique. Phylogenetic relations found in Pig 3 were confirmed by sequencing analysis only for feces and fresh meat isolates with 99.1% of similarities. The relations of isolates obtained from Pig 24 were confirmed by ANI with 99.1% of similarities between all the strains isolates (feces, carcasses and fresh meat *E. coli*). Similarities between carcasses and fresh meat *E. coli* were strongly confirmed with 99.9% in Pig 14 and Pig 57 while in Pig 9 no similarities were found. The 99.9% of similarities were highlighted by sequencing analysis in *E. coli* isolated from carcass and fermented meat product of Pig 34 (99.9%) and in fresh meat and seasoned meat product of Pig 40 (99.8%).

Figure 7. ANI results. The isolates subjected to the analysis are the ones phylogenetically related using ERIC. Colors reflect the abundance of the identified genes, starting from zero (black) to 100% (red). Hierarchical clustering is reported. (F = feces, C = carcasses, MPF = fresh meat product, MPFe= fermented meat product, MFS= seasoned meat product).



			HCA_order	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
			Cluster	1	1	1	1	1	1	1	1	1	2	2	2	2	3	3	4
			HCA_input	1	2	5	7	15	16	8	6	10	3	11	12	4	9	14	13
HCA_order	**Cluster**	**HCA_input**		**Pig 14 C**	**Pig 14 MPF**	**Pig 3 F**	**Pig 24 C**	**Pig 34 C**	**Pig 34 MPFe**	**Pig 24 MPF**	**Pig 24 F**	**Pig 3 MPF**	**Pig 40 MPF**	**Pig 57 C**	**Pig 57 MPF**	**Pig 40 MPS**	**Pig 3 C**	**Pig 9 MPF**	**Pig 9 C**
1	1	1	**Pig 14 C**	100,000	99,986	99,174	99,171	99,146	99,131	98,979	99,170	99,118	98,912	98,882	98,872	98,929	98,486	98,309	96,659
2	1	2	**Pig 14 MPF**	99,986	100,000	99,170	99,157	99,141	99,130	98,934	99,151	99,111	98,874	98,851	98,836	98,872	98,461	98,266	96,638
3	1	5	**Pig 3 F**	99,174	99,170	100,000	99,268	99,286	99,276	99,071	99,224	99,153	98,829	98,842	98,829	98,889	98,490	98,315	96,710
4	1	7	**Pig 24 C**	99,171	99,157	99,268	100,000	99,257	99,251	99,135	99,124	99,144	98,878	98,886	98,883	98,936	98,518	98,422	96,719
5	1	15	**Pig 34 C**	99,146	99,141	99,286	99,257	100,000	99,997	99,115	99,144	99,145	98,898	98,902	98,895	98,910	98,551	98,434	96,738
6	1	16	**Pig 34 MPFe**	99,131	99,130	99,276	99,251	99,997	100,000	99,116	99,161	99,132	98,898	98,914	98,900	98,905	98,557	98,414	96,764
7	1	8	**Pig 24 MPF**	98,979	98,934	99,071	99,135	99,115	99,116	100,000	99,102	99,010	98,720	98,745	98,721	98,736	98,405	98,213	96,800
8	1	6	**Pig 24 F**	99,170	99,151	99,224	99,124	99,144	99,161	99,102	100,000	99,765	98,895	98,907	98,879	98,850	98,462	98,361	96,680
9	1	10	**Pig 3 MPF**	99,118	99,111	99,153	99,144	99,145	99,132	99,010	99,765	100,000	98,905	98,883	98,873	98,900	98,464	98,436	96,714
10	2	3	**Pig 40 MPF**	98,912	98,874	98,829	98,878	98,898	98,898	98,720	98,895	98,905	100,000	99,997	99,992	99,854	98,462	98,367	96,801
11	2	11	**Pig 57 C**	98,882	98,851	98,842	98,886	98,902	98,914	98,745	98,907	98,883	99,997	100,000	99,995	99,865	98,455	98,352	96,781
12	2	12	**Pig 57 MPF**	98,872	98,836	98,829	98,883	98,895	98,900	98,721	98,879	98,873	99,992	99,995	100,000	99,847	98,470	98,315	96,794
13	2	4	**Pig 40 MPS**	98,929	98,872	98,889	98,936	98,910	98,905	98,736	98,850	98,900	99,854	99,865	99,847	100,000	98,493	98,339	96,750
14	3	9	**Pig 3 C**	98,486	98,461	98,490	98,518	98,551	98,557	98,405	98,462	98,464	98,462	98,455	98,470	98,493	100,000	98,889	96,803
15	3	14	**Pig 9 MPF**	98,309	98,266	98,315	98,422	98,434	98,414	98,213	98,361	98,436	98,367	98,352	98,315	98,339	98,889	100,000	96,592
16	4	13	**Pig 9 C**	96,659	96,638	96,710	96,719	96,738	96,764	96,800	96,680	96,714	96,801	96,781	96,794	96,750	96,803	96,592	100,000

Discussion - ESBL, AmpC

In Europe, monitoring ESBL, AmpC and Carbapenemases producing *Salmonella* and indicator *E. coli* is mandatory since 2013 (According to Commission Implementing Decision 2013/652/EU) in the major food producing animals' populations including pigs. In commensal *E. coli* isolated all over Europe the percentage of ESBL and AmpC isolates is reported at low levels. In fact, only the 0.6% of those bacteria were phenotypically resistant to 3rd and 4th generation cephalosporin. Despite those comforting data, the food producing animals' populations vary among European countries and can influence resistance at all levels of the food producing chain.

The most common genes encoding ESBL in animals are *bla*$_{\text{CTX-M1}}$ and *bla*$_{\text{CTX-M14}}$, followed by *bla*$_{\text{TEM-52}}$ and *bla*$_{\text{SHV-12}}$ while the gene mainly associated with resistance in AmpC-type β-lactamases is *bla*$_{\text{CMY-2}}$ (EFSA, 2012). In enteric bacteria of domestic animals and large game animals in Europe, *bla*$_{\text{CTX-M1}}$ is one of the most prevalent ESBL genes (Aguirre et al., 2020; Ewers et al., 2012; Guenther et al., 2011) and is also frequently found among *Enterobacteriaceae* recovered from meat of farm animals (Kola et al., 2012; Zarfel et al., 2014).

Data highlighted in this study showed that resistant phenotypical profiles were more frequent that the median data reported by EFSA, (2020). In fact, in fecal *E. coli* the percentage of ESBL strains was approximately 7% considering the two-sampling periods. The prevalence of phenotypically resistant bacteria increase in the pork food chain with the 10% of ESBL *E. coli* isolated from carcasses and the 12.5% of isolates from fresh meat products. A great difference in prevalence is shown in seasoned and fermented meat products where percentages are higher in the first sampling period than in the second one they settled at 0%. This can be due to a non-complete transformation process that brought to a higher bacteria population in meat product and consequently an increasing possibility of AMR prevalence. In the second sampling period, samples were collected after the proper transformation process giving more accurate results.

AmpC *E. coli* were found only in feces, carcasses, and fresh meat *E. coli* in low percentages. Those are even higher than the ones reported by other European countries (EFSA, 2020).

In the present study, *E. coli* isolated from feces, carcasses, and pork meat products frequently harboured *bla*$_{\text{TEM}}$ followed by *bla*$_{\text{CTX-M1}}$ and *bla*$_{\text{CTX-M2}}$. No bacteria strains were genotypically identified as AmpC in our study.

The Farm to Fork Strategy developed by European Green Deal aims to ensure food systems fair, health and being environmentally friendly.

This strategy is focused on ensuring food safety and public health making sure that everyone has access to sufficient, safe, nutritious, sustainable food (European Commission, 2017). Moreover, the pork food chain can be a source of resistant microorganisms for consumers and food manipulation and preparations can be a transmission route for resistant bacteria.

The present study focuses on the pork food chain analysing ESBL and AmpC profiles in fecal, carcasses and meat products of the same pigs evaluating the role of the food chain in the AMR dissemination. The major strength of this study was the traceability of samples that allowed to analyse the farm to fork system and to better understand the spread of resistant bacteria in the entire food chain. Results showed that on 243 pork food chain fully or partially analysed from feces, through carcasses, fresh meat to fermented and seasoned meat product, only in one case AMR bacteria were genetically similar from farm to fork (isolates in feces, carcasses and fresh meat). Frequent similarities were shown in AMR *E. coli* isolates from carcasses and fresh meat or fermented product (three pork food chain) and in one case, bacteria isolated from fresh meat and fermented product were genotypically similar.

Results highlighted that the transmission directly from farm to fork is possible but not frequent. Food processing techniques are used to extend the shelf life of food products, but some bacteria can survive or be only sub-lethally damaged. At the same time, cell death and lysis can release

DNA and resistant genes into the environment, allowing other bacteria to receive or incorporate and express parts of free DNA. The increasing demand of raw or minimally processed food can also contribute to the spread of AMR due to the bacterial replication.

Moreover, the food producing environment and cross contamination can play an important role in the dissemination of AMR.

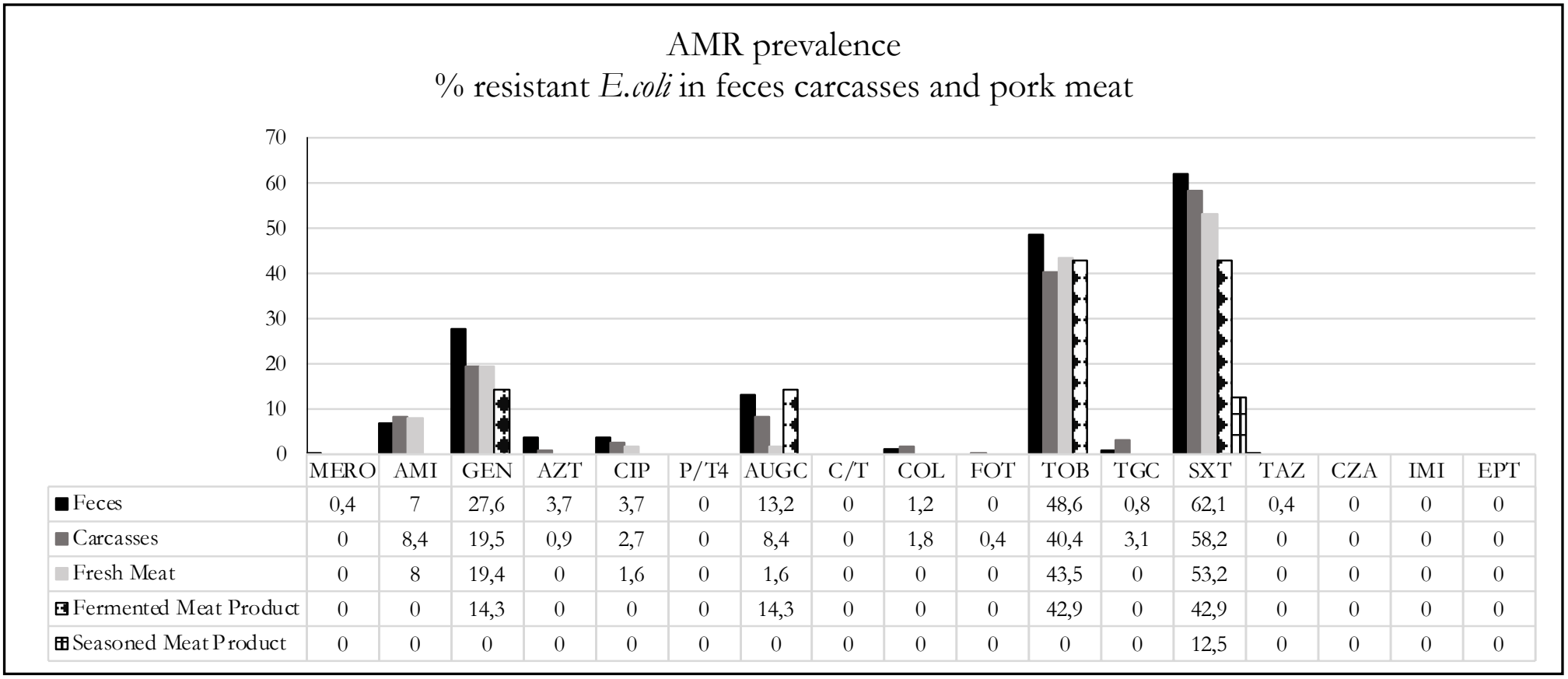
Results- MDR

Reference: *Rega, M., Andriani, L., Poeta, A., Bonardi, S., Conter, M., Bacci, C. (2023). The Pork Food Chain as a Route of Transmission of Antimicrobial Resistant Escherichia coli: A Farm-to-Fork Perspective. Antibiotics 12(2), 376. https://doi.org/10.3390/antibiotics12020376.*

Multi Drug Resistance Evaluation- MDR

All strains were tested for their susceptibility to 17 antimicrobials. The antimicrobial resistances most detected were against SXT, TOB and GEN as reported in figure 1.

Figure 8. Prevalence of antimicrobial resistant *E. coli* isolated from feces, carcasses, and meat products.



	MERO	AMI	GEN	AZT	CIP	P/T4	AUGC	C/T	COL	FOT	TOB	TGC	SXT	TAZ	CZA	IMI	EPT
Feces	0,4	7	27,6	3,7	3,7	0	13,2	0	1,2	0	48,6	0,8	62,1	0,4	0	0	0
Carcasses	0	8,4	19,5	0,9	2,7	0	8,4	0	1,8	0,4	40,4	3,1	58,2	0	0	0	0
Fresh Meat	0	8	19,4	0	1,6	0	1,6	0	0	0	43,5	0	53,2	0	0	0	0
Fermented Meat Product	0	0	14,3	0	0	0	14,3	0	0	0	42,9	0	42,9	0	0	0	0
Seasoned Meat Product	0	0	0	0	0	0	0	0	0	0	0	0	12,5	0	0	0	0

The 62.1% (CI 95% = 56-68.2) of *E. coli* isolated from feces, 58.2% (CI 95% = 51.8-64.6) from carcasses and 48% (CI 95% = 36.8-59.2) from pork meat products were resistant to SXT. TOB resistance was observed in 48.6% (CI 95% = 42.3-54.9), 40.4% (CI 95% = 34-46.8) and 39% (CI 95% = 28-50) of *E. coli* isolated from feces, carcasses e pork meat products, respectively. GEN resistance was detected in 27.6% (CI 95% =22-33.2), 19.5% (CI 95% = 14.3-24.7) and 16.9% (CI 95% = 8.5-25.3) of *E. coli* isolated along the food chain (feces, carcasses, and meat products). From feces, only one strain (0.4%) was non-susceptible to MERO and one (0.4%) was resistant to TAZ. Moreover, only one strain isolated from carcasses (0.4%) was resistant to FOT. Despite the presence of resistant isolates from feces and carcasses, strains isolated from pork meat products were not resistant to AZT, TGC or COL (figure 1). All strains detected along the food chain were susceptible to C/T, P/T4, CZA, IMI, EPT.

MDR patterns were evaluated in a farm-to-fork perspective. Particularly, MDR profiles were frequently found in isolates from feces, in a few from carcasses and never in pork meat products. The MDR patterns were: i) AUGC-TOB-SXT (10/243 fecal *E. coli* and 4/225 carcasses *E. coli*); ii) GEN-AUGC-TOB-SXT (7/243 fecal *E. coli* and 1/225 carcasses *E. coli*); iii) AMI-AUGC-TOB-SXT (1/243 fecal *E. coli*); iv) GEN-CIP-AUGC-TOB-SXT (2/243 fecal *E. coli* and 1/225 carcasses *E. coli*); v) AMI-GEN-AUGC-TOB-SXT (1/243 fecal *E. coli* and 2/225 carcasses *E. coli*); vi) AZT-CIP-COL-TOB-TGC-SXT-TAZ (1/243 fecal *E. coli*); vii) GEN-CIP-TOB-SXT (2/243 fecal *E. coli*) viii) GEN-CIP-AUGC-TOB-SXT (1/225 carcasses *E. coli*); ix) CIP-TOB-SXT (1/240 fecal *E. coli*); x) GEN-AUGC-SXT (5/243 fecal *E. coli*); xi) MERO-AMI-AZT (1/243 fecal *E. coli*); xii) CIP-AUGC-SXT (1/243 fecal *E. coli*); xiii) CIP-TGC-SXT (1/225 carcasses *E. coli*).

Moreover, other antimicrobial patterns were evaluated along the food chain (feces, carcasses, meat products). SXT, TOB, CIP, AUGC, SXT-TOB, GEN-TOB, GEN-TOB-SXT, AMI-

GEN-TOB-SXT were considered along the same food chains (table 1) or in groups of animals that showed the same resistance profile along the food chains evaluated (table 2). Considering the 12 pork food chains reported in table 1, phylogenetic analysis was performed on all the *E. coli*.

Table 3. *E. coli* AMR pattern isolated along the food chain (feces, carcasses and pork meat products of the same pig). The enumeration of "Pig" is independent and related to each farm.

Farm	Resistance Pattern	Feces	Carcasses	Fresh Meat	Fermented MP	Seasoned MP
A	SXT	Pig 3		Pig 3		Pig 3
		Pig 8	Pig 8	Pig 8		
B	SXT-TOB		Pig 34	Pig 34		
C	SXT		Pig 44	Pig 44		
	SXT-TOB	Pig 27		Pig 27		
			Pig 21	Pig 21		
F	AMI-GEN TOB-SXT	Pig 46		Pig 46		
G	SXT		Pig 57	Pig 57		
	SXT-TOB	Pig 51		Pig 51		
		Pig 52			Pig 52	
		Pig 55	Pig 55	Pig 55		
H	SXT-TOB		Pig 9	Pig 9		

Table 4. *E. coli* AMR pattern detected in groups of pigs along the different food chains. The enumeration of "Pig" is independent and related to each farm.

Farm	Resistance Pattern	Group	Feces	Carcasses	Fresh Meat	Fermented MP	Seasoned MP
A	SXT-TOB	3	Pig 6, 12, 13, 48, 49	Pig 53, 55, 56, 58	Pig 54, 57		
	GEN-TOB-SXT	4	Pig 11, 53, 60	Pig 1, 3, 51, 57	Pig 47, 58		
B	SXT	1	Pig 36, 37	Pig 32, 33, 38, 39, 41, 45	Pig 35, 42		
	GEN-TOB-SXT	2	Pig 18, 40, 46	Pig 36	Pig 44		
C	GEN-TOB-SXT	2	Pig 41	Pig 33, 18, 20	Pig 17, 23		
	GEN-TOB	3	Pig 35	Pig 23, 24	Pig 26		
D	SXT-TOB	1	Pig 29	Pig 6, 10	Pig 12	Pig 49	
	GEN-TOB-SXT	2	Pig 48, 49, 53, 54, 57, 58, 60, 62	Pig 4, 5, 13	Pig 15		
	AUGC	3		Pig 48, 58	Pig 1		
	CIP	4		Pig 49, 50	Pig 3		
E	SXT-TOB	1	Pig 9, 11, 13, 15	Pig 16, 18, 19, 23, 26, 27, 29, 32	Pig 28, 30		
G	AMI-GEN-TOB-SXT	4	Pig 59	Pig 40, 41, 44	Pig 42		
	GEN-TOB-SXT	5		Pig 47, 63		Pig 33	
H	GEN-TOB-SXT	1	Pig 50		Pig 8		
	TOB	2	Pig 52, 53	Pig 1	Pig 13		

In farm A, resistance to SXT was detected in feces, fresh meat, and seasoned meat product isolates derived from Pig 3. Moreover, in the same farm, SXT resistance was detected in fecal, carcasses, and fresh meat *E. coli* of Pig 8. In farms C and G, *E. coli* resistant to SXT were found in each farm in carcass samples and their related fresh meat samples (Pig 44 and Pig 57 respectively).

The SXT-TOB resistance pattern was the most frequently found. In fact, in Farm B and H resistant *E. coli* strains were found in the carcasses and fresh meat samples of Pig 34 and Pig 9 respectively (as shown in table 1). In Farm C, one *E. coli* isolated from feces (Pig 27) and one from carcasses (Pig 21) have the same SXT-TOB patter as their fresh meat isolates (Pig 27 and 21 respectively). In Farm G, 2 fecal *E. coli* strains (Pig 51 and Pig 52) showed the same AMR patter of their meat products (fresh meat and fermented product respectively) and in Pig 55 SXT-TOB resistant strains were found along all the food chain.

The AMI-GEN-TOB-SXT pattern was found in *E. coli* isolated from feces and fresh meat belonging to Pig 46 in farm F (table 1).

As reported in table 2, 15 groups of pigs were selected for their identical antimicrobial resistant profile along the food chain.

SXT resistant strains belonging to the same group were identified in farm B. Resistance against TOB was detected only in one group of isolates from farm H (table 2).

CIP and AUGC resistances were in carcasses and fresh meat *E. coli* only in Farm D group 3 and 4. Three different groups were considered in Farm A (group 3), D (group 1) and E (group 1) for their SXT-TOB resistance along the food chain (table 2).

The GEN-TOB pattern was found only in one group of samples belonging to Farm C while GEN-TOB-SXT pattern was detected in *E. coli* of farm A, B, C, D, G, H (table 2).

AMI-GEN-TOB-SXT pattern was found in one group of samples in Farm G.

Other AMR patterns were detected in *E. coli* isolates but were not considered for phylogenetic analysis because they did not harbour the same resistances along the food chain and/or food chain groups.

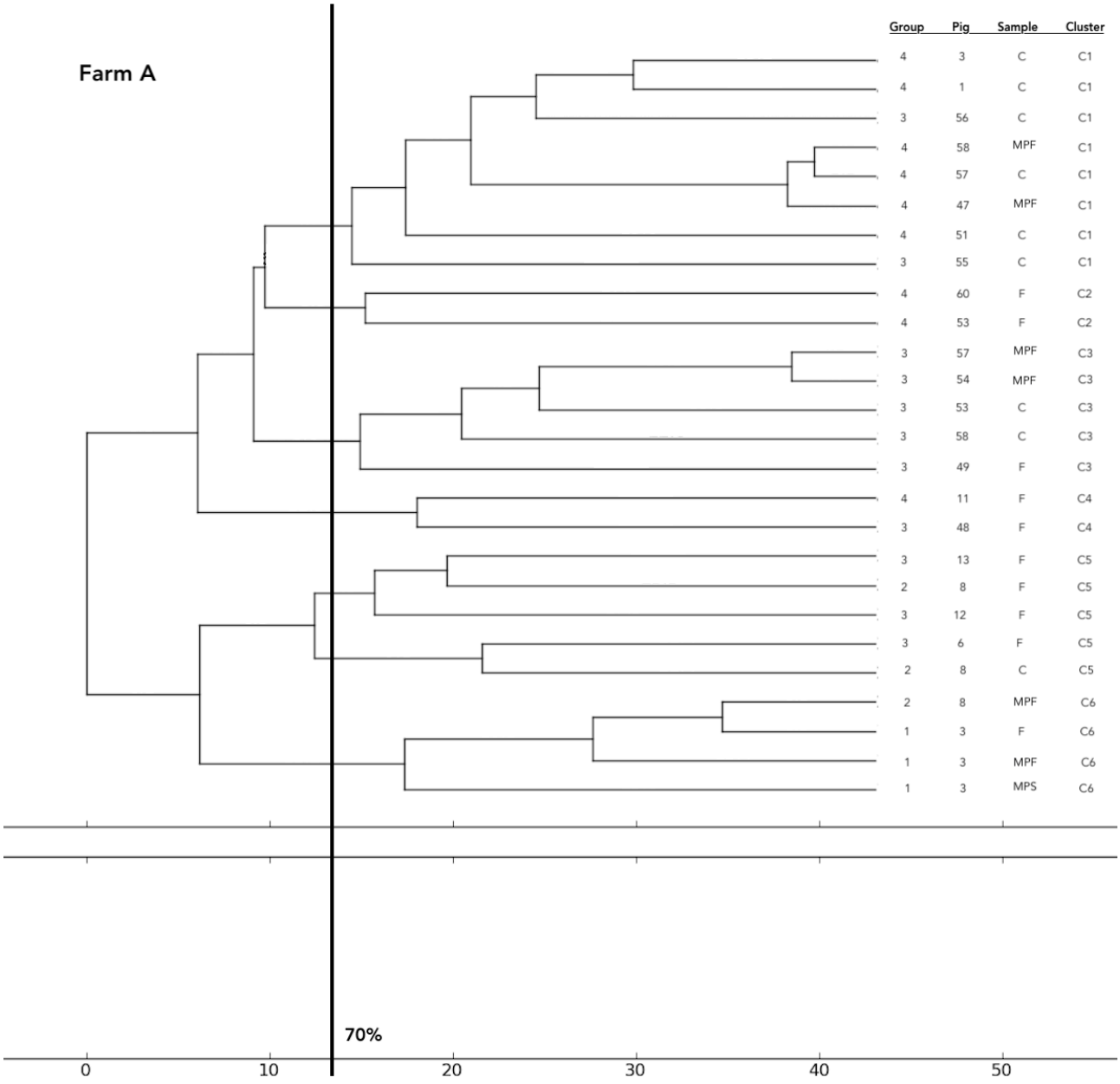
Overall, 26 *E. coli* were tested in farm A, 17 isolates in farm B, 16 in farm C, 23 in farm D, 14 in farm E, 2 in farm F, 17 in farm G and 8 in farm H. Phylogenetic analysis performed by ERIC-PCR revealed the presence of 36 clusters.

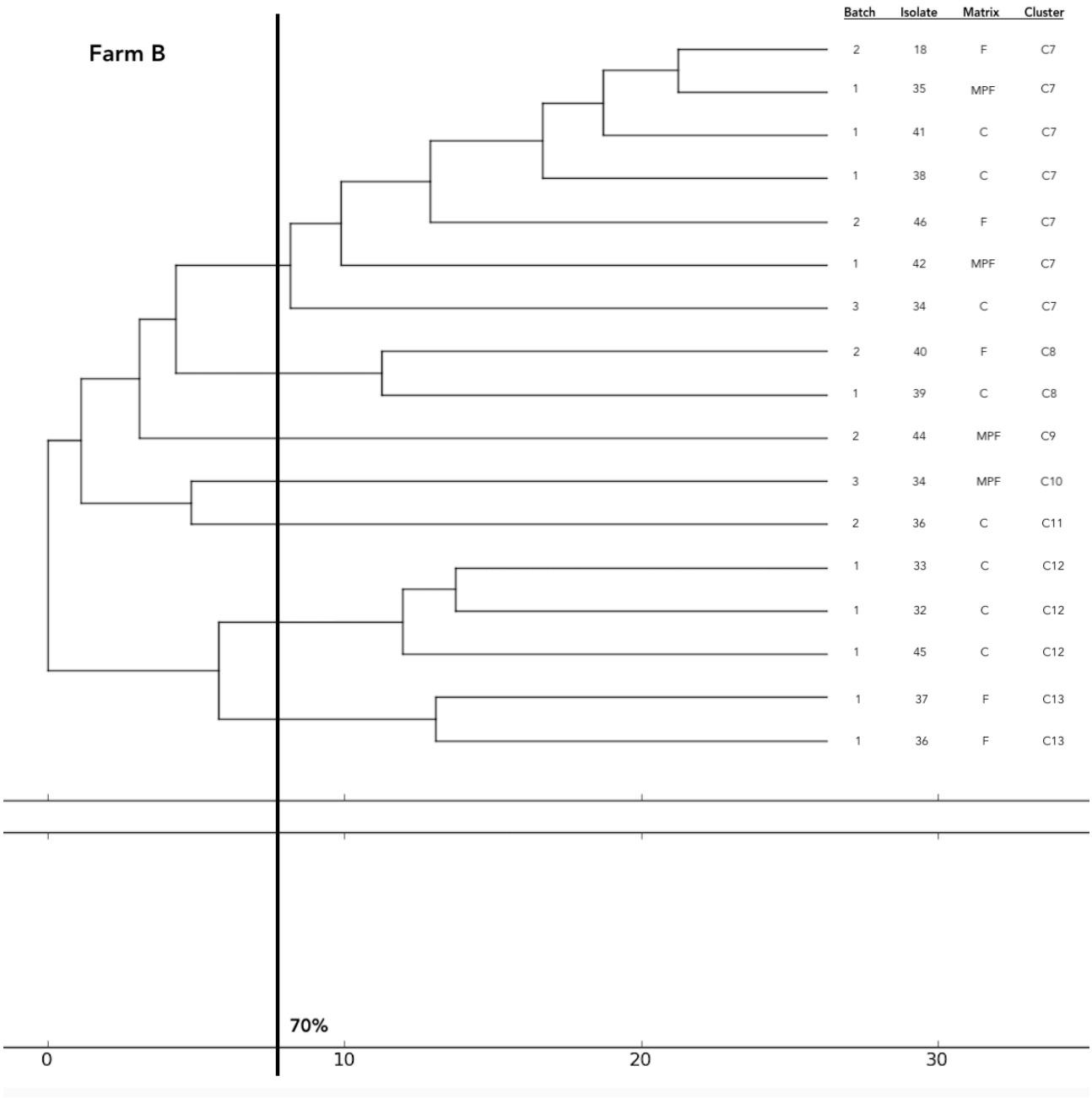
Analysis of the 12 food chains highlighted the relationship between *E. coli* isolated in Pig 3 (Farm A) from feces, fresh meat product and seasoned meat product in Cluster 6 (C6); in C5 and C32, only feces and carcass *E. coli* (Pig 8 and Pig 55 respectively) were phylogenetically related despite their related fresh meat *E. coli* isolates belonged to another cluster. Feces and fresh meat isolates of the same food chain were related in C14 (Pig 27) and C31 (Pig 51) where they are related to fermented product too (Pig 52). Carcasses and fresh meat isolates were related in C15 (Pig 44) and C29 (Pig 57). The other 4 pork food chains were not phylogenetically related (Pig 9, 21, 34). Pig 46 (Farm F) phylogenetically relations were not calculated by the software and the used method UPGMA.

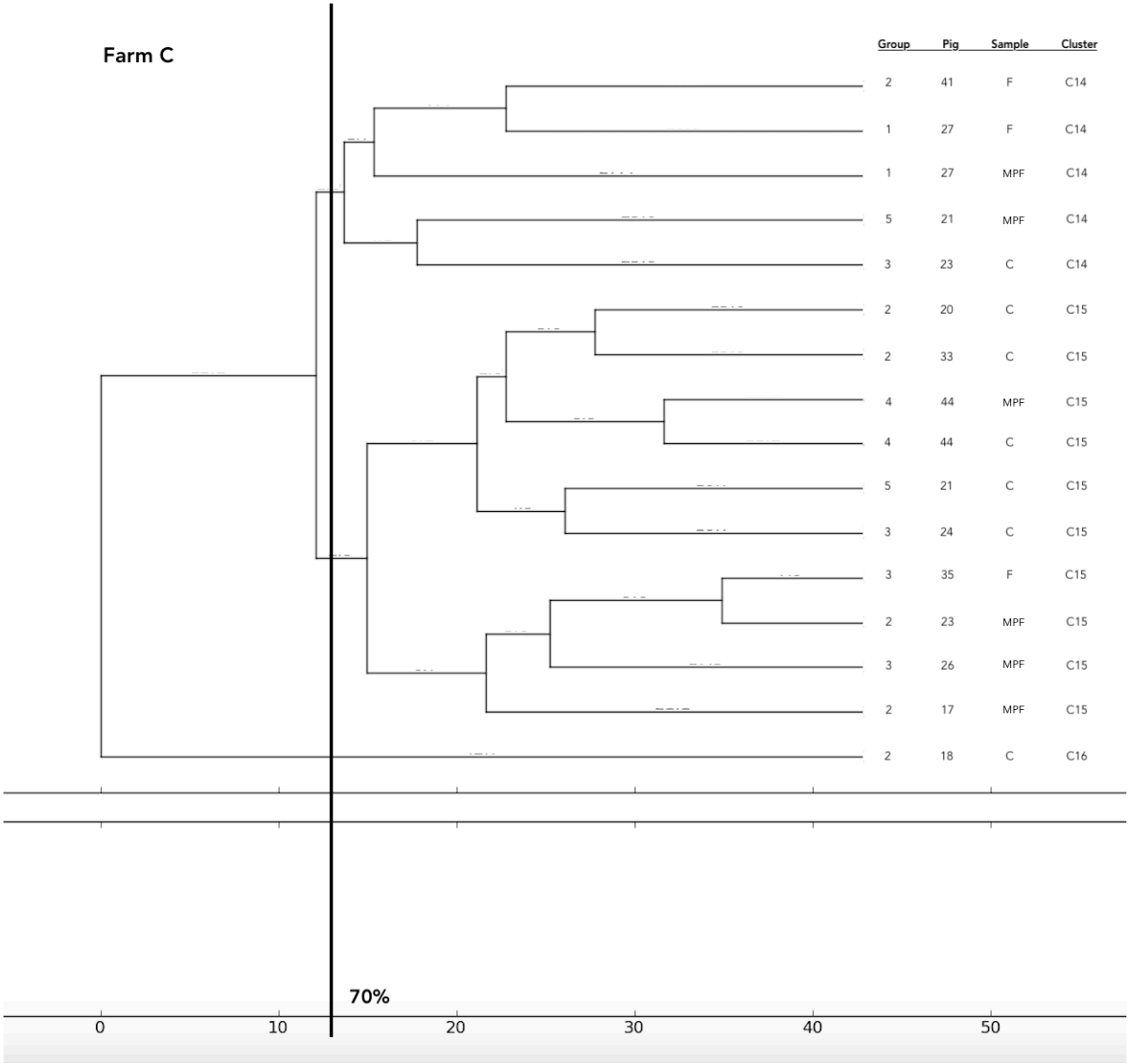
Considering the phylogenetic analysis of 15 groups of pigs along the different food chains, *E. coli* isolated from feces belonging to the same groups harboured phylogenetic similarities in 6/36 clusters (C 2, 5, 7, 13, 22 and 26). Moreover, isolates from carcasses of the same groups belonged to the same cluster in 6/36 cases (C1, 12, 19, 23, 24, 25) and *E. coli* of meat products from the same groups showed genetic similarities in only one group (C 27). Frequently, phylogenetic similarities were found in *E. coli* from carcasses and meat products of the same groups (C1, 7, 15, 19, 21, 29) and, in 3/36 cluster (C 3, 15, 30), *E. coli* isolated from feces, carcasses and meat products of the same groups were similar. In 2/36 cluster similarities were found in fecal and

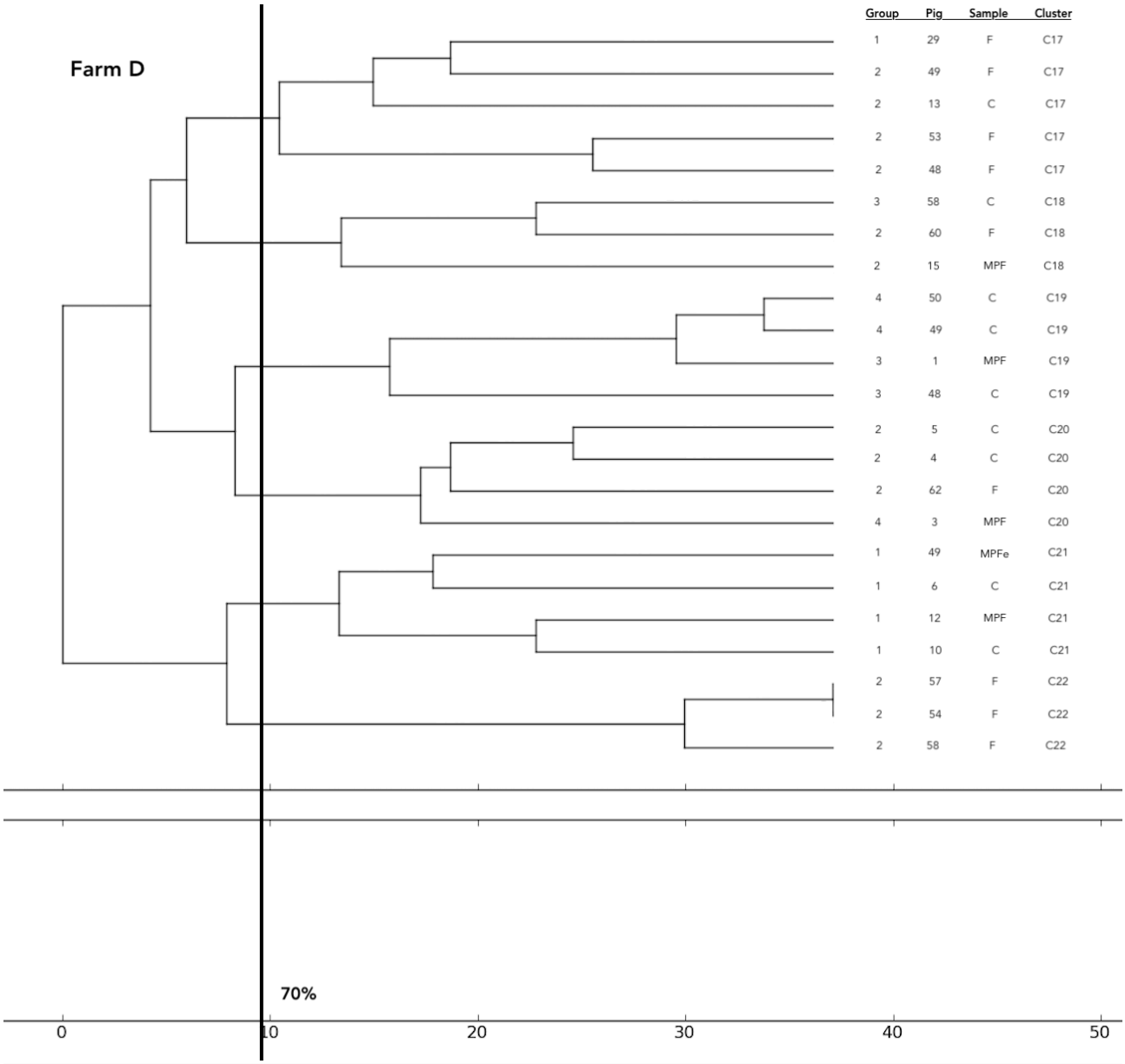
carcasses *E. coli* of the same groups (C 17, 20) and in 3/36 in fecal and fresh meat products isolates (C 18, C 33, C 36). All data are reported in Figure 5.

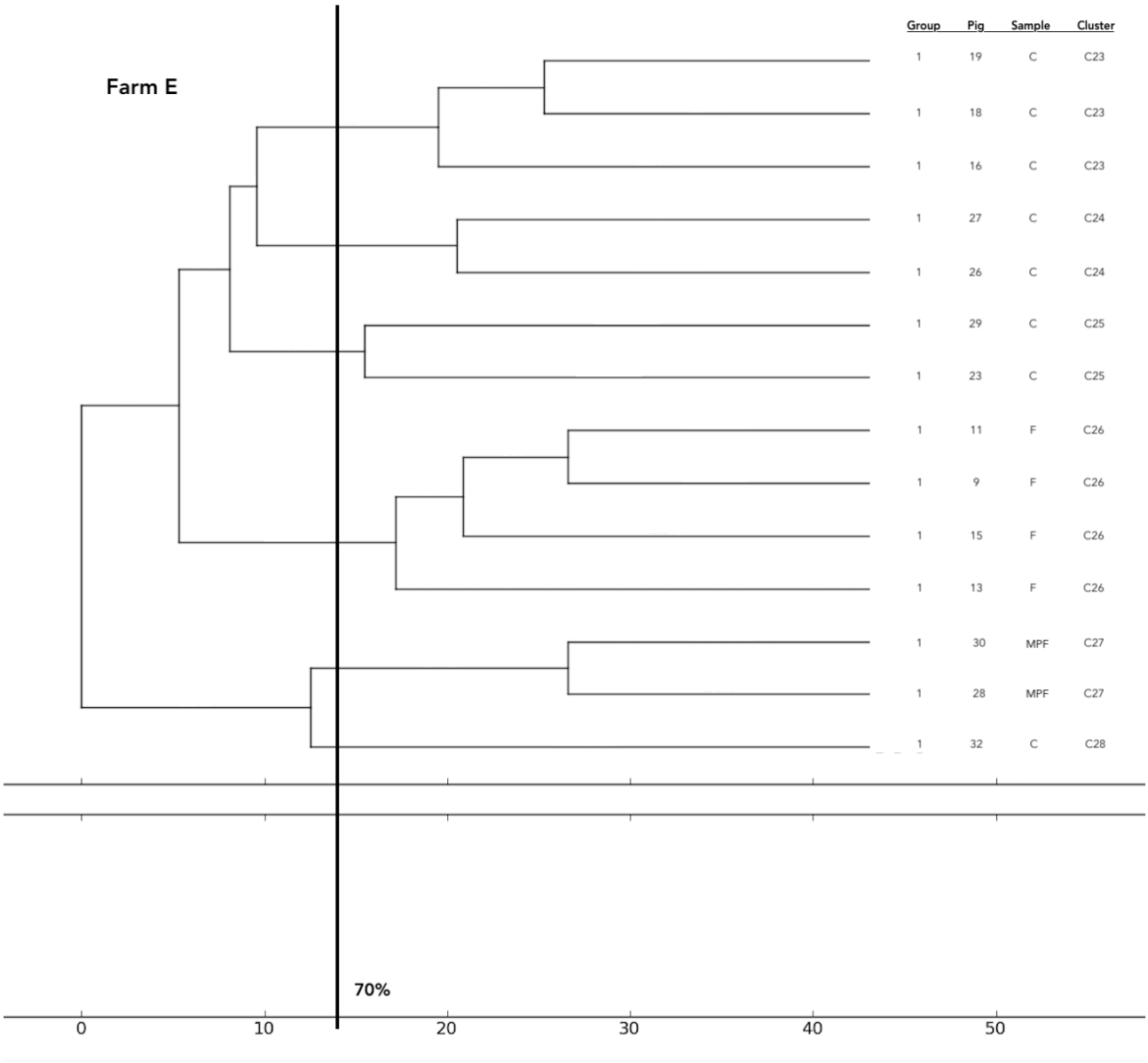
Figure 9. Phylogenetic relations of resistant *E. coli* highlighted in 11 pork food chain and 15 group food chain analysis (F = feces, C = carcasses, MPF = fresh meat product, MPFe= fermented meat product, MFS= seasoned meat product).

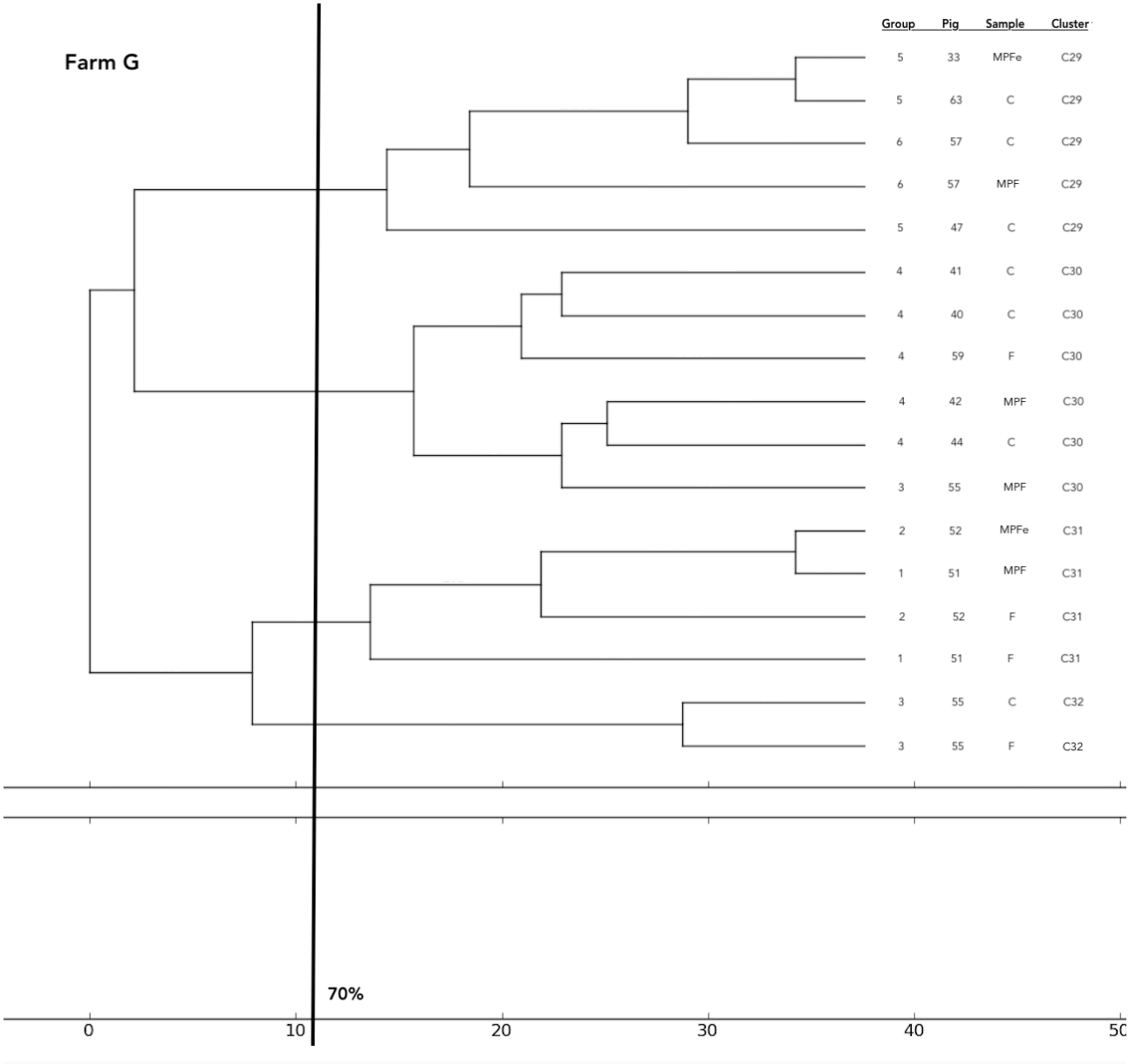


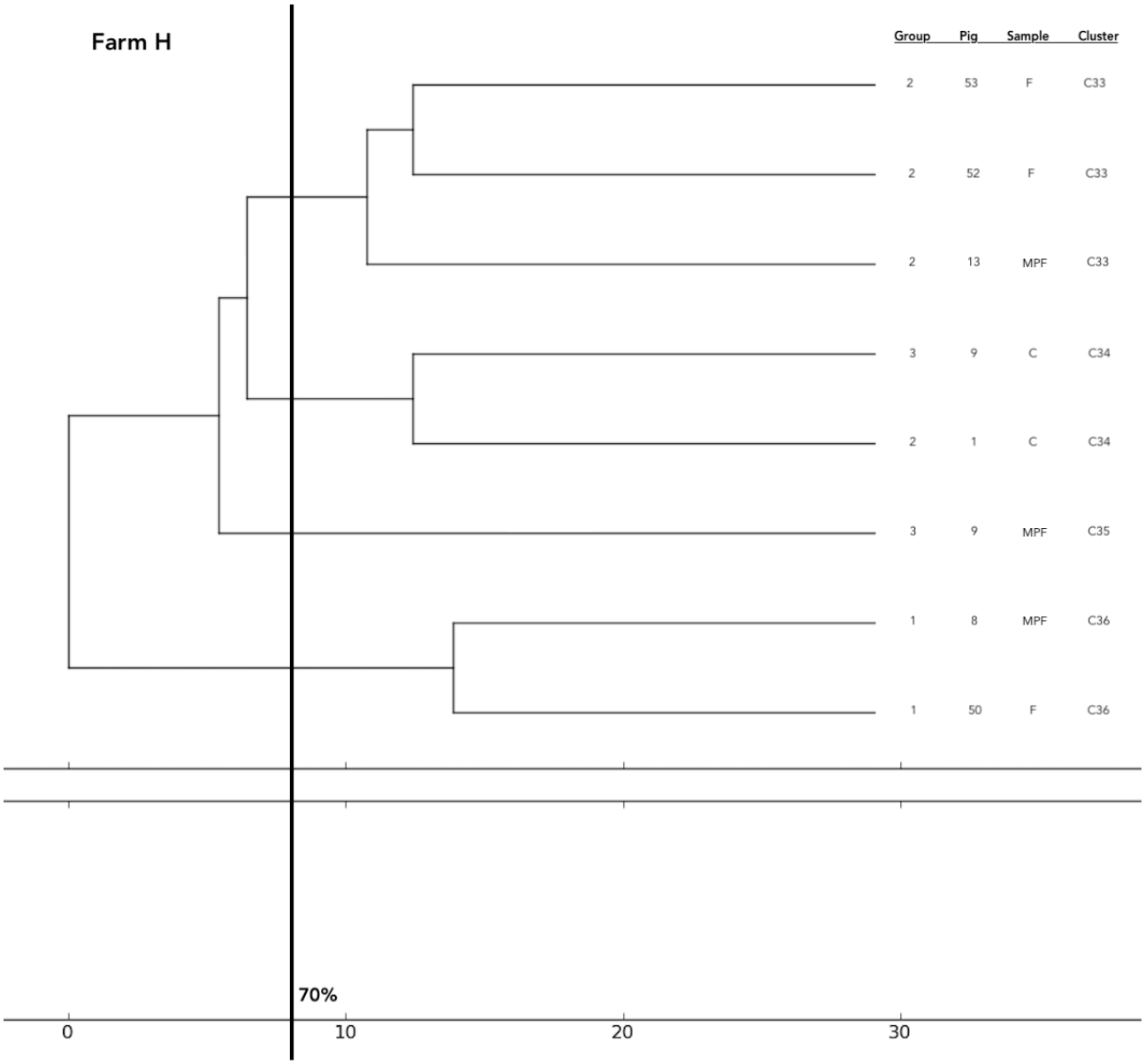

Farm B
Batch Isolate Matrix Cluster
2 18 F C7
1 35 MPF C7
1 41 C C7
1 38 C C7
2 46 F C7
1 42 MPF C7
3 34 C C7
2 40 F C8
1 39 C C8
2 44 MPF C9
3 34 MPF C10
2 36 C C11
1 33 C C12
1 32 C C12
1 45 C C12
1 37 F C13
1 36 F C13
70%
0
10
20
30


Farm C
Group Pig Sample Cluster
2 41 F C14
1 27 F C14
1 27 MPF C14
5 21 MPF C14
3 23 C C14
2 20 C C15
2 33 C C15
4 44 MPF C15
4 44 C C15
5 21 C C15
3 24 C C15
3 35 F C15
2 23 MPF C15
3 26 MPF C15
2 17 MPF C15
2 18 C C16
70%
0
10
20
30
40
50


Farm D
Group Pig Sample Cluster
1 29 F C17
2 49 F C17
2 13 C C17
2 53 F C17
2 48 F C17
3 58 C C18
2 60 F C18
2 15 MPF C18
4 50 C C19
4 49 C C19
3 1 MPF C19
3 48 C C19
2 5 C C20
2 4 C C20
2 62 F C20
4 3 MPF C20
1 49 MPFe C21
1 6 C C21
1 12 MPF C21
1 10 C C21
2 57 F C22
2 54 F C22
2 58 F C22
70%
0
10
20
30
40
50


Farm E
Group Pig Sample Cluster
1 19 C C23
1 18 C C23
1 16 C C23
1 27 C C24
1 26 C C24
1 29 C C25
1 23 C C25
1 11 F C26
1 9 F C26
1 15 F C26
1 13 F C26
1 30 MPF C27
1 28 MPF C27
1 32 C C28
70%
0
10
20
30
40
50


Farm G
Group Pig Sample Cluster
5 33 MPFe C29
5 63 C C29
6 57 C C29
6 57 MPF C29
5 47 C C29
4 41 C C30
4 40 C C30
4 59 F C30
4 42 MPF C30
4 44 C C30
3 55 MPF C30
2 52 MPFe C31
1 51 MPF C31
2 52 F C31
1 51 F C31
3 55 C C32
3 55 F C32
70%
0
10
20
30
40
50


Farm H
Group Pig Sample Cluster
2 53 F C33
2 52 F C33
2 13 MPF C33
3 9 C C34
2 1 C C34
3 9 MPF C35
1 8 MPF C36
1 50 F C36
70%
0
10
20
30

Discussion-MDR

Reference: *Rega, M., Andriani, L., Poeta, A., Bonardi, S., Conter, M., Bacci, C. (2023). The Pork Food Chain as a Route of Transmission of Antimicrobial Resistant Escherichia coli: A Farm-to-Fork Perspective. Antibiotics 12(2), 376. https://doi.org/10.3390/antibiotics12020376.*

Commensal bacteria in animals are currently recognized as a reservoir of AMR and, at the same time, as a source of AMR transmission. However, the role of longitudinal transmission of those bacteria directly from livestock to human by means of meat products is still poorly understood (Lugsomya et al., 2018). This study monitored the AMR pattern and phylogenetic analysis in *E. coli* isolated from feces, carcasses and meat products following the pigs along the entire food producing chain, evaluating the risk of AMR transmission to consumers.

According to Commission Implementing Decision (2013/652/EU), AMR monitoring in indicator commensal *E. coli* is mandatory in the major domestic producing animal populations and their derived meat. A specific monitoring of extended-spectrum-β-lactamase (ESBL)-,AmpC- and carbapenemase-producing indicator commensal *E. coli* is also required.

According to the EFSA 2022b, resistance to ampicillin, sulfamethoxazole, trimethoprim and tetracycline is at high levels in all animal categories in Europe, frequently causing the development of MDR bacterial profile. Results highlighted in our study confirm those data particularly for sulphamethoxazole resistant *E. coli,* although high levels of resistance to aminoglycosides were also found (GEN and TOB). In fact, in previous studies, aminoglycoside resistant *E. coli* were frequently detected in pork at slaughter associated with penicillin and tetracycline resistances (Bacci et al., 2020; Li et al., 2016). As reported in other European countries, resistance to colistin, azithromycin, cefotaxime and ceftazidime is less common, particularly to meropenem. Notably, in our study, only one meropenem resistant strain isolated from feces was found while no strain was detected all over Europe (EFSA, 2022b).

The MDR *E. coli* pattern is frequently characterized by tetracycline, ampicillin, sulphamethoxazole and trimethoprim across Europe (EFSA, 2022b). In our study the most frequently highlighted pattern included AUGC-GEN-TOB-SXT and MDR strains were detected in feces and carcasses. Fortunately, no MDR strains were isolated from pork meat products.

Bacterial isolation along the food production chain in the present study was essential to evaluate the farm-to-fork involvement in the dissemination of antimicrobial resistant bacteria. Phylogenetic analysis allowed to divide strains in 36 cluster and to understand their phylogenetic similarities along the food chain.

Data showed that the 50% of phenotypical AMR observed along the pork food were related phylogenetically. Our results showed that the contamination of fresh meat, in half of the cases, is not directly related to contamination from feces or carcasses. Despite this, some similarities were found from feces and carcasses.

The analysis of groups along the different food chains showed that most frequent relation was found between *E. coli* isolated from carcasses and meat products of the same groups (16.7%). The frequent relations found between *E. coli* isolated from feces (16.7%) and *E. coli* from carcasses of the same groups (16.7%) highlight the possible cross contamination during farming and processing at slaughter, respectively. In group analysis, phylogenetic similarities from farm to fork were detected in 3/36 cluster (8.3%).

Chapter 7. Risk management

The risk management approach bases on the search of control measures to manage a risk assessment. Risk Management has a structured approach that includes preliminary Risk Management activities, identification and selection of Risk Management options, implementation of Risk Management activities (Caffrey et al., 2019).

Test of new synthesis metal based compounds to inhibit β-lactams resistance

Reference: *Scaccaglia, M., Rega, M., Vescovi, M., Pinelli, S., Tegoni, M., Bacci, C., Pelosi, G., Bisceglie, F. (2022). Gallium(III)-pyridoxal thiosemicarbazone derivatives as nontoxic agents against Gram-negative bacteria. Metallomics, 14(10), mfac070.* https://doi.org/10.1093/mtomcs/mfac070.

Materials and methods

The synthesis of molecules has been entirely provided by Department of Chemistry, Life Science and Environmental Sustainability of the University of Parma.

The desired thiosemicarbazones were synthetized from adapted known synthetic procedures. (Belicchi-Ferrari et al., 2005; Ferrari et al., 2002; Ferrari et al., 2004)

L1: Pyridoxal-5-phosphate (0.8 mmol, 201 mg) was completely dissolved in water (20 mL) under reflux. Thiosemicarbazide (0.8 mmol, 73.5 mg) was added. A bright yellow precipitate started to form. The reaction was stirred at room temperature for 8 more hours. The solid was filtered on filter paper, washed with cold ethanol, diethyl ether and dried under vacuum. Yellow powder. Yield: 80%. CHNS analysis $C_9H_{13}N_4O_5PS$. Calc: C 33.75, H 4.09, N 17.49, S 10.01. Found: C 33.90, H 4.22, N 17.15, S 9.65. ^{1}H NMR (400 MHz, D_2O, pH 13) δ 8.36 (s, 1H, NH-N=), 7.66(s,

1H, CH arom.), 4.86 (m, 2H, CH_2), 2.29 (s, 3H, CH_3). ^{13}C NMR (400 MHz, D_2O, pH 13) δ 176.30 (C=S), 160.38 (C-OH), 146.40 (C arom.), 143.37 (C=N), 133.59 (C arom.), 127.07(C arom.), 126.65(C arom.), 62.32(-CH_2-), 16.26(-CH_3). ^{31}P NMR (400 MHz, D_2O, pH 13) δ 3.76. ESI-MS (+, m/z, MeOH): calc. 343.0, found 343.2 $[M+Na]^+$. IR (ATR, cm^{-1}): 3354 cm^{-1}, 3283 cm^{-1}, 3142 cm^{-1} ν NH and ν OH; 1642 cm^{-1} ν C=N; 1585 cm^{-1} ν C=C, 1040 cm^{-1} ν N-C=S; 852 cm^{-1} ν C=S.

L2: Pyridoxal-5-phosphate (0.25 mmol, 624 mg) was dissolved in hot water (60 mL) with 1 mL of acetic acid. The mixture was kept under stirring and reflux until complete dissolution of the powder. Then 4,4'-dimethyl-3-thiosemicarbazide (301 mg, 0.25 mmol) was added. The formation of a yellow to orange precipitate started shortly after the addition of 4,4'-dimethyl-3-thiosemicarbazide. The reaction mixture was stirred at room temperature for 8 h. The solid was filtered on filter paper, washed with cold ethanol, diethyl ether and dried under vacuum. Yellow powder. Yield: 82 %. CHNS analysis $C_{11}H_{17}N_4O_5PS$. Calc: C 37.93, H 4.92, N 16.09, S 9.20. Found: C 37.66, H 4.90, N 15.82, S 9.36.^1H NMR (400 MHz, D_2O, pH 13) δ 8.51(s, 1H, NH-N=), 7.64 (s, 1H, CH arom.), 4.80 (m, 2H, CH_2), 3.16 (s, 6H, N-CH_3), 2.31(s, 3H, CH_3).^{13}C NMR (400 MHz, D_2O, pH 13) δ 181.46 (C=S), 168.36 (C-OH), 150.69 (C arom.), 146.16 (C=N), 134.47(C arom.), 131.99 (C arom.), 129.52 (C arom.), 61.77 (-CH_2-), 40.04 (N-CH_3), 18.20 (-CH_3).^{31}P NMR (400 MHz, D_2O, pH 13) δ 3.68. ESI-MS (+, m/z, MeOH): calc. 349.07, found 349.21 $[M+H]^+$; calc. 371.05, found 371.17 $[M+Na]^+$. IR (ATR, cm-1): 3167 cm^{-1}, 2987 cm^{-1} ν NH and ν OH; 1625 cm^{-1} ν C=N,1555 cm^{-1} ν C=C, 1098 cm^{-1} ν N-C=S, 841 cm^{-1} ν C=S.

L3: Pyridoxal-5-phosphate (1.43 mmol, 353 mg) were dissolved in hot water (13 mL) under reflux for 30 minutes. Semicarbazide hydrochloride (1.43 mmol, 160 mg) were dissolved in water (2 mL) and then slowly added to the reaction solution. A precipitate starts to form shortly after. The reaction was stirred at room temperature for 4 hours. The solid was filtered on filter paper, washed with cold ethanol, diethyl ether and dried under vacuum. White precipitate. Yield: 88%.

CHNS analysis $C_9H_{13}N_4O_6P$. Calc: C 35.54, H 4.31, N 18.42; Found: C 35.28, H 4.44, N 18.39. ^1H NMR (400 MHz, D_2O, pH 13) δ 8.20 (s, 1H, NH-N=), 7.69 (s, 1H, CH arom.), 4.79 (m, 2H, CH_2), 2.25 (s, 3H, CH_3). ^{13}C NMR (400 MHz, D_2O, pH 13) δ 196.01 (C=O), 160.22 (C-OH), 158.12 (C arom.), 151.70 (C arom.), 146.25 (C arom.), 141.16 (C=N), 61.95 (-CH_2-), 16.44 (-CH_3). ^{31}P NMR (400 MHz, D_2O, pH 13) δ 3.32.ESI-MS (+, m/z, MeOH): calc. 305.0, found 305.2 $[M+H]^+$. IR (ATR, cm^{-1}): 3376 cm^{-1}, 3160 cm^{-1} ν NH and ν OH; 1742 cm^{-1} ν CO; 1560 cm^{-1} ν C=C.

L4: Pyridoxal hydrochloride (0.9 mmol, 200 mg) and KOH (0.9 mmol, 59 mg) were dissolved in 20 mL of water at room temperature. Thiosemicarbazide (0.9 mmol, 82 mg) was then added. A precipitate starts to form shortly after. The reaction was stirred at room temperature for 4 hours and then filtered on paper filter. The solid was filtered on filter paper, washed with cold ethanol, diethyl ether and dried under vacuum. Yellow powder. Yield: 40%. CHNS analysis for $C_9H_{12}N_4O_2S$: C 44.99, H 5.03, N 23.32, S 13.34. Found: C 44.79, H 5.05, N 23.16, S 13.02.^1H NMR (400 MHz, DMSO-d_6): δ 11.61 ppm (s broad, 1H, NH=N), 9.67 ppm (s broad, 1H, phenolic OH), 8.58 ppm (s, 1H, CH arom.), 8.33 ppm (s broad, 1H, NH_2), 8.10 ppm (s broad, 1H, NH_2), 8.0 ppm (s, 1H, CH=N), 5.26 ppm (s, 1H, aliphatic OH), 4.59 ppm (s, 2H, CH_2), 2.41 ppm (s, 3H, CH_3).^{13}C NMR (400 MHz, DMSO-d_6): δ 149.56, 147.46, 142.50, 139.44, 133.35, 122.09, 59.40, 31.17, 19.60. ESI-MS (+, m/z, MeOH): calc. 263.3, found 263.1 $[M+Na]^+$. IR (ATR, cm^{-1}): 3440 cm^{-1}, 3374 cm^{-1}, 3260 cm^{-1}, 3160 cm^{-1}, 3090 cm^{-1} ν NH and ν OH, 1620 cm^{-1} ν C=N, 1528 cm^{-1} ν C=C, 1030 cm^{-1} ν N-C=S, 820 cm^{-1} ν C=S.

L5: Pyridoxal hydrochloride (3.3 mmol, 682 mg) and KOH (3.3 mmol, 157 mmol) were dissolved in 10 mL of hot MeOH. 4,4'-3-thiosemicarbazide (3.3 mmol, 398 mg) was then added. A precipitate starts to form shortly after. The reaction was stirred at room temperature for 8 hours. The solid was filtered on filter paper, washed with cold ethanol, diethyl ether and dried under

vacuum. Yellow powder. Yield: 86 %. CHNS analysis for $C_{11}H_{16}N_4O_2S$: C 49.24, H 6.01, N 20.88, S 11.95. Found: C 49.11, H 6.16, N 20.83, S 12.16; ^{1}H NMR (400 MHz, DMSO-d_6): 12.38 ppm (s, 1 H, NH), 11.67 ppm (s, 1H, phenolic OH), 8.90 ppm (s, 1 H, CH), 7.94 ppm (s, 1H, CH=N), 5.38 ppm (s, 1H, aliphatic OH), 4.60 ppm (s, 2H, CH_2), 2.42 ppm (s, 3H, CH_3), methyl peaks rely under water peak. ^{13}C NMR (400 MHz, DMSO-d_6): δ 181.64, 164.24, 150.36, 145.52, 132.46, 128.90, 127.53, 61.59, 38.84, 20.72. ESI-MS (+, m/z, MeOH): calc. 269.1, found 269.2 $[M+H]^+$; calc. 291.1, found 291.2 $[M+Na]^+$. IR (ATR, cm^{-1}): 3451 cm^{-1}, 3232 cm^{-1} ν NH and ν OH, 1678 cm^{-1} ν C=N, 1514 cm^{-1} ν C=C, 1046 cm^{-1} ν N-C=S, 806 cm^{-1} ν C=S.

L6: Pyridoxal hydrochloride (0.96 mmol, 196 mg) and KOH (0.96 mmol, 46 mg) were dissolved in 5 mL of water at room temperature. Semicarbazide hydrochloride (0.96 mmol,106 mg) were dissolved apart in water (2 mL) and then slowly added to the pyridoxal solution A precipitate starts to form shortly after. The reaction was stirred at room temperature for 4 hours. The solid was filtered on filter paper, washed with cold ethanol, diethyl ether and dried under vacuum. White powder. Yield: 95%. CHNS analysis for $C_9H_{12}N_4O_p$: C 48.21, H 5.39, N 24.99; Found: C 48.31, H 5.46, N 25.14.^{1}H NMR (400 MHz, DMSO-d_6): 11.08 (s, 1H, NH), 8.40 (s, 1H, CH arom.), 8.09 (s, 1H, CH=N), 6.62 (s, 2H, NH_2), 5.62 (s broad, 1H, OH aliphatic), 4.69 (s, 2H, CH_2), 2.53 (s, 3H, CH_3). ^{13}C NMR (400 MHz, DMSO-d_6): δ 155.28 (C=O), 151.48 (C-OH), 144.15 (C arom.),137.36 (C=N), 135.28 (C arom.), 132.98 (C arom.), 127.05 (C arom.), 59.98 (-CH_2-), 16.58 (-CH_3); ESI-MS (+, m/z, MeOH): calc. 247.1, found 247.2 $[M+Na]^+$. IR (ATR, cm^{-1}): 3461 cm^{-1}, 3350 cm^{-1}, 3278 cm^{-1} , 3134 cm^{-1} ν NH and ν OH, 1699 cm^{-1} ν C=O, 1579 cm^{-1} ν C=C.

Preparation of the complexes

Stock solution of 3 mM Ga^{3+} complexes were freshly prepared in situ by mixing equimolar solution of $Ga(NO_3)_3$ dissolved in HCl 0.1M and the ligands dissolved in NaOH 0.1M. The pH

of the resulting stock solution was neutral. Ga(III) complex solution were immediately used for antibacterial and cytotoxicity screening. The stock solutions were diluted 1: 200 with a mixture H_2O/MeOH 1:1 for HR-MS. Full HR-MS spectra are reported in Fig. SX-SX.

GaL1: [GaL1/$C_9H11O_5N_4GaPS]^+$ calculated: 386.94436; found: 386.94381.

GaL2: [GaL2/$C_{11}H_{15}O_5N_4GaPS]^+$ calculated: 414.97566; found: 414.97511.

GaL3: [GaL3/$C_9H_{11}GaN_4O_6P]^+$ calculated: 370.96720; found: 370.96692: [GaL3-HCOOH/$C_{10}H_{13}GaN_4O_8P]^+$ calculated: 416.97268; found: 416.97213; [GaL3-HCOOH-HCOONa-$C_{11}H_{14}GaN_4NaO_{10}P]^+$ calculated: 484.96011; found: 484.95966; found: 416.97213; [GaL3-2HCOONa/$C_{11}H_{13}GaN_4Na_2O_{10}P]^+$ calculated: 506.94205; found: 506.94154. GaL3 has a high affinity for formic acid, and it ionizes with the track of the solvent in the source of ESI.

GaL4: [GaL4-H_2O / $C_9H_{12}GaN_4O_3S]^+$ calculated: 324.98859; found: 324.98804; [GaL4-CH_3OH/$C_{10}H_{14}GaN_4O_3S]^+$ calculated: 339.00424; found: 339.00369; [GaL4-Cl / $C_9H_{11}ClGaN_4O_2S]^+$ calculated: 342.95471; found: 342.95461; [GaL4-HCOOH / $C_{10}H_{12}GaN_4O_4S]^+$ calculated: 352.98351; found: 352.98324. GaL4 has a high affinity for formic acid, and it ionizes with the track of the solvent in the source of ESI.

GaL5: [GaL5-H_2O / $C_{11}H_{16}GaN_4O_3S]^+$ calculated: 353.01989; found: 353.01934; [GaL5-CH_3OH / $C_{12}H_{18}GaN_4O_3S]^+$ calculated: 367.03554; found: 367.03595; [GaL5-Cl / $C_{11}H_{15}ClGaN_4O_2S]^+$ calculated: 370.98601; found: 370.98659; [GaL5-HCOOH / $C_{12}H_{16}GaN_4O_4S]^+$ calculated: 381.01481; found: 381.01426. GaL5 has a high affinity for formic acid, and it ionizes with the track of the solvent in the source of ESI.

GaL6: [GaL6-H_2O / $C_9H_{12}GaN_4O_4]^+$ calculated: 309.01144; found: 309.01124; [GaL6-CH_3OH / $C_{10}H_{14}GaN_4O_4]^+$ calculated: 323.02709; found: 323.02679; [GaL6-Cl / $C_9H_{11}ClGaN_4O_3]$+ calculated: 326.97755; found: 326.97732.

Microdilution MIC and MBC assay.

Klebsiella pneumoniae NDM-1 (NTCT14331); *Klebsiella pneumoniae ESBL* (NCTC13368), *Pseudomonas aeruginosa* clinical isolate (NCTC13713) were purchased from Star Ecotronics s.r.l. *E. coli* ESBL, *E. coli* biofilm, *Enterococco spp. VRE* were isolated in the laboratory of Food Inspection of Parma University. The isolates were tested for their AMR profiles both phenotypically, through Kirby Bauer test, and genotypically by end-point PCR and Real time PCR technique. PCR allows the detection of the most common resistance genes in ESBL, AmpC, CRE and VRE bacteria (Doyle et al., 2012; Pérez-Pérez & Hanson, 2002; Roschanski et al., 2014; Tatsing Foka et al., 2019).

MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) values were determined by standard broth micro-dilution method following ISO 20776-1 (2019). Briefly, frozen stock bacteria were put into 5 mL of Buffered Peptone Water (BPW) and incubated for 2 h at 37 °C or until 0.5 McFarland. The bacterial density was adjusted to reach a final concentration of 5×10^5 $CFUmL^{-1}$. Tested antimicrobials were added in triplicate into 96-microwells plates, performing 2-fold serial dilution, followed by the addition of water (for antibacterial screening) or antibiotic (for combination therapy screening) and the prepared bacterial inocula. The plates were incubated at 37 °C for 18-24h. The MIC values were determined by OD reading at 620 nm using a spectrophotometer plate reader. MIC is the lowest concentration of a drug/broth dilution of antimicrobials that results in inhibition of the growth of the tested bacteria (Andrews, 2001). The MBC values were determined by transferring 100 μL of broth microdilution to a Triptic Soy Agar plate, a solid nonselective medium, and incubated at 37 °C for 18-24h. The MBC values were chosen as the lowest concentration of a drug/broth dilution of antimicrobials that results in killing 99.9% of the tested bacteria(Andrews, 2001). The FICI values were determined as follows: $FICI=FIC_A+FIC_B=C_A/MIC_A+C_B/MIC_B$, where MIC_A and MIC_B are the MIC values of compounds A and B alone, and C_A and C_B are the effective

concentration of A and B when administrated. A FICI index of 0.5 or less indicates synergistic effect; between 0.5 and 4 the effect is additive. FICI index greater than 4 denotes antagonism(Huang et al., 2019; Cecchini et al., 2015). The compounds were tested starting from the 1 mM. Meropenem and cefotaxime concentration were chosen following EUCAST (2019) guidelines and breakpoints table (meropenem resistance MIC>8 μg/mL and cefotaxime resistance MIC>2 μg/mL).

UV-vis spectroscopy

UV-vis spectra were collected in a range of 250-450 nm using 1 cm quartz cuvette at room temperature. Spectrophotometric titrations were performed on ligand samples alone or in combination with Ga(III) with 1:1 stoichiometry over a pH range between 3 and 11 at an ionic strength of 0.1M KCl in water at 25 °C. The pH of the samples was corrected using a standard HCl 0.1M solution. The UV titration curve were fitted and protonation and stability constants were calculated with the program Hypspec2014(Gans et al., 1996). Speciation diagram were obtained from Hyss2009 (Gans et al., 1996).

Cytotoxicity

Human dermal fibroblasts HuDe were purchased from the Istituto Zooproflattico Sperimentale della Lombardia e dell'Emila (IZSLER-Brescia, Italy). Cells were grown inRPMI 1640 medium contain- ing 10% heat inactivated foetal bovine serum, 10 U m1-l peni- cillin, and 100 mg ml-1 streptomycin a t 37°C w%i5th CO2. Ex- periments were conducted with cells ni the log phase growth. Cells (100 000 ml-1) were seeded into 96-well plates for 24 h and then exposed to compounds at concentrations between 0.1 and 100/M. After incubation at 37°C for 24 h, cells were stained with Trypan Blue and counted with hemacytometer. At least three in- dependent experiments were performed for each sample. Cell viability was assessed by the MTT (3-(4,5-

dimethyIthiazol-2-yl)-2,5-diphenyItetrazolium bromide) test, and samples were read yb spectrophotometer (Varioskan LUX Multimode Microplate Reader, Thermo Fisher Scientific).

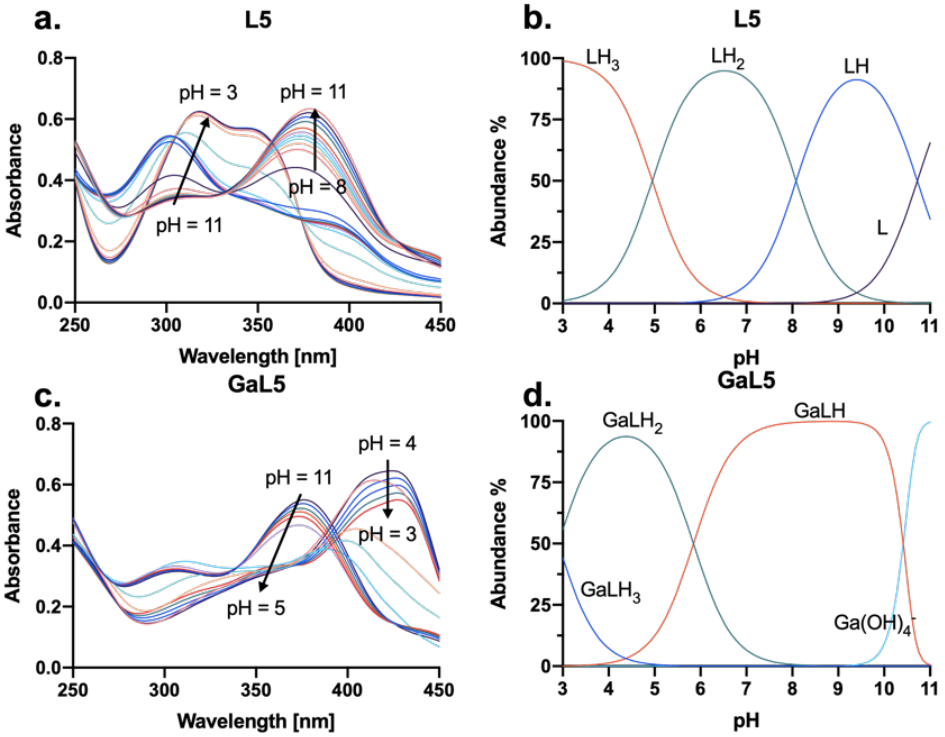
Results

The ligands were synthetized according to adapted general procedures previously reported. (Belicchi-Ferrari et al., 2005; Ferrari et al., 2002; Ferrari et al., 2004). Briefly, the reaction is a condensation between the corresponding aldehyde and the various thiosemicarbazides or semicarbazide. The reaction was conducted in methanol or water, according to the better solubility of the reactants, with acetic acid as catalyst. The compounds were characterised by ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, ESI-MS, IR spectroscopy and elemental analysis. The corresponding Ga(III) complexes **GaL1–GaL6** were prepared in situ and characterized by UV/VIS spectroscopy and HR-MS.

The biological activity of the metal complexes strongly dependβs on the prevalent species at the physiological pH of 7.4. The distribution of the species is in turn related to their stability at different pH. To determine the speciation of the gallium(III)/ligand systems, a series of pH-metric UV/VIS titrations were performed on solutions of the ligands and of the ligands in the presence of 1 eq. of Ga^{3+} nitrate.

Alkaline solutions of the ligands and of the Ga(I)/L systems Hp(.ac 1w1er)e added with increasing amounts of HCI 0.1 M down ot pH ca.3. The UV/Vis spectra were collected after each titrant addition. Representative spectra for the titration of L5 and Ga(III)/L5 are reported in Figure 10A and C.

Figure 10. a. UV-Vis absorption spectra of L5 at different pH (CL5 = 50μM). b. Representative species distribution diagram of L5 (CL5 = 100μM). c. UV-Vis absorption spectra of the Ga3+/L5 system at different pH (CL5 = 50μM; Ga/L5 = 1:1). d. Representative species



Treatment of the spectra dataset for the titration of the ligands allowed to determine the proton dissociation constants (K_a), which are reported in Table 5 in the form of pK_a. In Table 5, the logarithm of the global formation constants of the Ga(III) complexes (logβ) is also reported along with the calculated conditional formation constants of the 1:1 GaL1-GaL6 adducts at pH 7.4 (log K_{app}).

Table 5. Proton dissociation (pKa) of the H+ of the ligands L1-L6 with overall stability (log β) and conditional stability (log Kcond) constants of Ga(III) complexes formed with the ligands L1-L6.

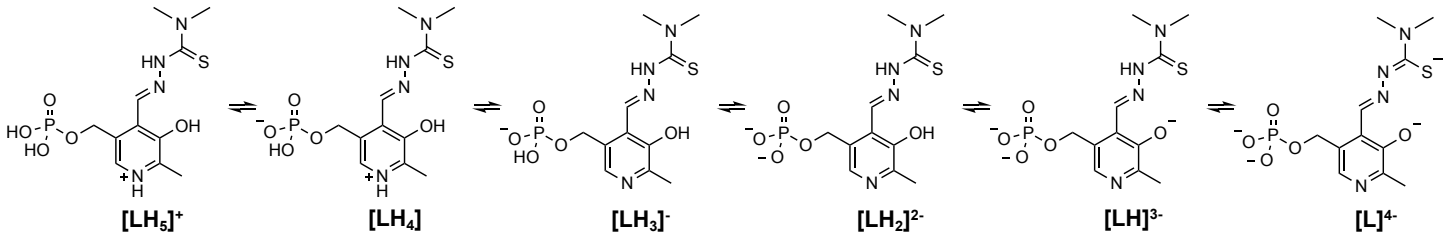
Species/pK_a	Functional gr.	L1	L2	L3	Functional gr.	L4	L5	L6
pK_a								
$[LH_5]^+$; pK_{a1}	$OP=O(OH)_2$	<2.5	<2.5	<2.5				
$[LH_4]$; pK_{a2}	Pyridyl NH^+	4.104(3)	4.82(2)	4.457(4)				
$[LH_3]^-$; pK_{a3}	$OP=O(O^-)(OH)$	6.098(7)	5.93(9)	5.97(2)	Pyridyl NH^+	4.066(2)	4.949(2)	4.551(2)
$[LH_2]^{2-}$; pK_{a4}	Phenolic OH	8.06(1)	7.39(7)	9.52(4)	Phenolic OH	8.048(4)	8.034(3)	8.603(8)
$[LH]^{3-}$; pK_{a5}	(C = S)NH	9.21(2)	8.71(1)	–	(C = S)NH	10.914(5)	10.720(8)	–
logβ								
$[GaLH_4]^{3+}$		45.1(3)	44.7(3)	–		–	–	–
$[GaLH_3]^{2+}$		41.1(3)	42.1(2)	44.61(3)		43.85(6)	43.64(9)	42.4(6)
$[GaLH_2]^+$		35.5(3)	36.9(2)	41.37(3)		39.72(6)	40.74(3)	38.1(6)
[GaLH]		–	30.9(3)	35.43(3)		34.67(5)	34.90(3)	30.8(6)
log K_{cond}(pH = 7.4)		–	8.2(3)	12.27(3)		9.38(5)	9.78(3)	8.8(6)
pH range		3.2–6.3	3.0–7.4	2.8–9.3		3.5–8.5	3.3–8.9	3.3–7.7

[a] For the generic equilibrium $pGa + qL + rH = [Ga_pL_qH_r]$, $\beta = [Ga_pL_qH_r]/([Ga]^p \cdot [L]^q \cdot [H]^r)$.
[b] K_{cond} = [Ga(III) bound to L]/([Ga(III) not bound to L]·[L not bound to Ga(III)]).

In their fully protonated forms, the pyridoxal phosphate derivatives **L1-L3** present four functional groups which can undergo proton dissociation in aqueous solution: the phosphate moiety (for two protons), the protonated pyridyl nitrogen, the phenolic OH and the N^2H of the (thio)semicarbazone moiety. Out of the corresponding five pK_a values for **L1** and **L2**, four of them could be determined by spectrophotometric data. The first pK_a of the phosphate group expectedly resulted too low to observe the proton dissociation equilibria in the experimental pH range. Evaluation of the data suggested that for all three L1-L3 the pK_{a1} is lower than 2.5. Conversely, in the semicarbazone derivates L3 the NH(C=o) group does not appreciably dissociate in water, and indeed consistently only three pK_a were determined in the experimental pH range of the titrations. On the other hand, the L4-L6 ligands do not bear the phosphate group, and therefore only three pK_a values were expected and indeed found for L4 and L5. The determined pK_a values are in good agreement with those reported for similar compounds

(Jakusch et al., 2018). The deprotonation equilibria are represented, for L2, in Figure 12, and a representative distribution diagram is reported in Figure 10B.

Figure 11. Representative proton dissociation equilibria for L2.



As stated, in these ligands the first proton dissociation in the pH range investigated. The second proton dissociation (pK_{a2} = 4.1-4.9) likely occurs at the protonated pyridy] nitrogen, since pyridines functionalized with electron withdrawing groups often present pK_a, values lower than 5. The third proton dissociation corresponds to the second dissociation ta the phosphate group, which occurs close ot the neutral pH (pK_a ca. 6), as expected. As for the last two dissociation steps, literature data suggest the phenol deprotonation occurs at lower pH than the thiosemicarbazide NH (pH 8 vs. 11.5). In the current series the ligands L4, L5, and L6 have the values of pK_{a4} and pK_{a5} fully consistent with the literature value. The pK_a values of L1, L2 and L3 for the dissociation of the phenolic OH site are spread over quite large range of values, from 8.6 to 10.9 and their pK_{a5} values are much lower than 11. The spectra dataset for the pH titration of the ligands in the presence of Ga(III) is different from that with the ligand alone at acidic and neutral pH. At alkaline pH (e.g. pH 11) the spectra are very similar, in agreement with the probable presence of uncomplexed ligand Ga(III) in the form of hydroxo-species . The spectra dataset were used to determine the stability constants of the gallium(III) complexes with the

ligands in their different protonation forms as fixed parameters. With the purpose of accurate data treatment, the formation constants of the hyroxo-complexes of Ga(III) were also included in the calculation as fixed parameters. The logβ values of the Ga(III)/ligand adducts are reported in Table 5.The pH range reported in Table 5 refers to the pH range where the fitting of the spectral data could be obtained using the molar spectra of the different protonated forms of the ligand as fixed parameters, together with the related pK_a values. Within

these pH ranges, five out of the six ligands form the $[GaLH_3]^{2+}$, $[GaLH_2]^{+}$, and [GaLH] species, the latter prevalent species at pH 7.4 (Figure 3). For the L1 ligands, the [GaLH] species was not detected, likely because a good fitting of spectral data could be obtained only below pH 6.3. Finally, with the L2 which forms also a $[GaLH_4]^{3+}$ specie. The $[GaLH_2]^{+}$, species for L6 is relatively more stable (compared to [GaLH]) and therefore more abundant at pH 7.4 compared to the observed values for L4 and L5. The hypothesis generated is that the ligand in solution coordinates to Ga(III) through the chelating moiety O^N^S or O^N^O, with formation of a 5- and 6- membered chelation system. This coordination mode of the ligands is supported on the basis of the computational calculations.

As for the presence of the C= S or C= O coordination sites, the logK_{cond} values suggest that the presence of either group does not play a major role in determining the stability of the adducts. Actually, while the logK_{cond} are higher for GaL3 in the GaL1-GaL3 series (C = O in place of C = S), the opposite was observed for the GaL4 GaL6 series. The logK_{cond} obtained for our ligands are in good agreements with those reported for other O^N^S or O^N^O ligands and Ga(III) (Enyedy et al., 2014).

For our ligands, proton dissociation equilibria in the pH range examined (see Table 5) are associated with relevant changes in the absorption spectra, and therefore likely associated to protonation-deprotonation of the coordinated ligand. Deprotonation of coordinated water

molecules and subsequent formation of hydroxo-species may however occur at higher pH and may be associated to limited spectral changes, which actually were observed for our liand in alkaline conditions. It seems evident from the speciation data (Table 1) that almost all Ga(III) is present in the form of Ga(III) complexes and not of Ga(III) hydroxo-species (with the exception of L1 for which no precise information at this pH can be obtained from species distribution). This evidence is indispensable for the further evaluation of their biological activity, in this case investigating their antimicrobial activities against Multidrug resistant bacteria and exploring their cytotoxicity.

The minimal Inhibitory concentration (MIC) was evaluated in different Gram negative bacteria: *Klebsiella pneumoniae* (NTCT14331) that carries the bla_{NDM-1} metallo-carapenemase gene and was first isolated from human blood infection; *Klebsiella pneumoniae* (NCTC13368) that has the ability to produce Extended-Spectrum-Beta-Lactamases (ESBL) encoded by bla_{SHV-18} gene; *Pseudomonas aeruginosa* clinical isolate (NCTC13713) with an intrinsic upregulated AmpC beta-lactamase activity. We tested the compounds also against a series strain isolated in the laboratory of Food Inspection Unit of Parma University which include two Gram negative *E. coli* ESBL (genes bla_{CTXM1} bla_{CTXM2} bla_{TEM} bla_{SHV}) and a non-pathogenic *E. coli* (phylogenetic group A), characterized by the ability to produce biofilm, and one Gram positive *Enterococco spp* for which a resistant at vancomycin (VRE) was carried by VanA gene.

The lowest MIC values were recorded for **GaL2** and **GaL5**, which were active against all Gram-negative bacteria tested. The corresponding ligand **L2** and **L5** were also active, but with a higher MIC value. The compounds **GaL2** and **L2** which presented the $-OPO_3^{2-}$ moiety were slightly less active in comparison to the compounds which presented the hydroxyl in the same position, indication that compounds with higher charges may encounter more difficulties getting inside the cells. The active class of compounds **GaL2**, **L2**, **GaL5** and **L5** are thiosemicarbazones which

present the N^4 double methylated. The corresponding compounds with no substitution at N^4 were active only at high concentration, with no enhanced activity for gallium(III) compounds. Comparing MIC values, it is shown that ligands L2 and L5 and their complex GaL2 and GaL5 activities are more effective against the *E. coli* than the *K. pneumoniae* tested. This phenomenon is more evident in *E. coli* able to produce biofilm suggesting a major efficacy in bacteria susceptible to the antibiotics considered. All the compounds were not active again the Gram-positive bacteria tested, indicating that this group of compounds may be suitable for the developing of selective for Gram negative antibacterial agents. We also noted that all the semicarbazone derivatives **L3**, **GaL3**, **L6**, **GaL5** did not show any biological activity. Minimum Bactericidal Concentration (MBC) was also investigated but all the compounds showed only an inhibition of the growth with MBC values higher than the tested concentrations. Notably that the pure $Ga(NO_3)_3$ was not active, indicating once again the metal complexes have properties that are not the sum of the one of the respective metal and ligand.

Table 6. Antibacterial activity displayed as minimum inhibitory concentration (MIC; μM) against a panel of resistant bacteria strains.

Bacteria	*Klebsiella pneumoniae* NDM-1	*Klebsiella pneumoniae* ESβL NDM-1	*Escherichia coli* ESβL	*Escherichia coli* biofilm	*Pseudomonas aeruginosa* AmpC NDM-1	*Enterococcus* spp. VRE	HuDe[a]
L1	1000	500	>1000	500	500	>1000	>100
GaL1	>1000	>1000	>1000	250	>1000	>1000	>100
L2	500	250	500	31.25	125	>1000	>100
GaL2	62.5	62.5–31.25	15.63	15.63	31.25	>1000	>100
L3	>1000	>1000	>1000	>1000	>1000	>1000	>100
GaL3	>1000	>1000	>1000	>1000	>1000	>1000	>100
L4	1000	500	1000	500	500	>1000	>100
GaL4	>1000	500	500	500	500	>1000	>100
L5	250	125	250	31.25	125	>1000	>100
GaL5	31.25	31.25	15.63	15.63	62.5	>1000	>100
L6	>1000	>1000	>1000	>1000	>1000	>1000	>100
GaL6	>1000	>1000	>1000	>1000	>1000	>1000	>100
Meropenem	256*	–	–	–	–	–	–
Cefataxime	–	8*	16*	2*	32*	–	–
$Ga(NO_3)_3$	>1000	>1000	>1000	>1000	>1000	>1000	–

[a]Cytotoxicity against HuDe cells as IC_{50} value. * $\mu g\ ml^{-1}$.

Klebsiella pneumoniae NDM-1 and *Klebsiella pneumoniae* ESBL were reference type culture (NCTC) and were chosen to test a combination therapy with our compounds and the antibiotics (meropenem and cefotaxime respectively). The MIC of the antibiotics were firstly investigated and secondly were evaluated with a sub inhibitory concentration of the compounds synthetized (MIC/4). The *Klebsiella pneumoniae NDM-1* produces a metallo beta-lactamase able to hydrolyse the beta-lactam of carbapenems and show a meropenem MIC of 256 μg/mL. We noted that the pure ligands **L1-L6**, and the Ga(III) semicarbazone derivatives **GaL3**, **GaL6** did not show any activity. In the presence of the **GaL1**, **GaL2**, **GaL4** and **GaL5** the meropenem MIC lowered down to 64-32 μg/mL, with a FICI index of 0.5 indicating a synergism between meropenem and the gallium complexes of the thiosemicarbazone ligands. GaL2 and GaL5 have the best synergic effect because the concentration needed is lower if compared with the other compounds. However, the synergism was not enough to restore carbapenem sensibility. Interestingly, comparing the ligands and complexes activity, only Ga(III)-complexes showed synergism with the MBL-producing strain. Further studies may be needed to know the molecular target in the biological system and their possible inhibitory activity. *Klebsiella pneumoniae* ESBL which produces serine-beta-lactamase (SHV-18) with cefotaxime MIC of 8 μg/mL did not show any relevant biological activity for the combination therapy of all the compounds.

Table 7. Minimum inhibitory concentration of the antibiotic* against resistant strains of *K. pneumoniae* in combination therapy with MIC/4 of the compounds.

Bacteria	*Klebsiella pneumoniae* NDM-1		*Klebsiella pneumoniae* EsβL	
Compounds	Effective concentration (MIC/4) (μM)	MIC meropenem (μg ml^{-1})	Effective concentration (MIC/4) (μM)	MIC cefotaxime (μg ml^{-1})
L1	250	>64	125	>4
GaL1	250	64	250	>4
L2	125	>64	62.5	>4
GaL2	15.63	64	15.63	>4
L3	250	>64	250	>4
GaL3	250	>64	250	>4
L4	250	>64	125	>4
GaL4	250	64	125	>4
L5	62.5	>64	31.25	>4
GaL5	7.81	64-32	7.81	>4
L6	250	>64	250	>4
GaL6	250	>64	250	>4
$Ga(NO_3)_3$	250	>64	250	>4
Antibiotic[a]	–	256	–	8

[a]Meropenem for *K. pneumoniae* NDM-1, cefotaxime for *K. pneumoniae* ESβL.

The selectivity of our compounds against bacteria strains was evaluated studying the cytotoxicity effect against human epithelial HuDe cells that we used as model of eukaryotic cells. All the ligands and all the complexes exhibited no cytotoxicity after 24 hours from the treatment with IC_{50}>100 μM. The morphology of the cell was also checked, and no significative difference was detected in comparison with the control lines. For this reason, the compounds have an high selectivity towards bacterial cells, showing no activity against the tested healthy human cells.

Discussion

The development of new antimicrobial molecules is a strategy to ensure effective treatments and European Medicines Agency (EMA) develops regulatory approaches and guidelines. They set up preclinical trials to establish antimicrobial spectrum of activity, the ability of molecules for select resistances *in vitro* and *in vivo* considering intrinsically resistant microorganisms. It is also necessary to establish the correct dose, dosing intervals and duration of treatments by pharmacokinetic and pharmacodynamic evaluations. The molecules need to be tested on the target bacterium to

evaluate again the spectrum of activity, MIC and MBC, time or concentration dependent activity or co-activity and finally the ability to reach and be affective at the site of infection. Then the evaluation of bio-availability in accordance with the route of administration, the concentration, the metabolism, and the excretion routes of the molecules are needed (OIE, 2020). Finally clinical trials in targeted animal species can confirm the validity of the proof taken following regulatory aspects established by authorities. Assessment of the potential of new antimicrobial agents able to select for resistance is needed. (ECDC, 2022).

A series of ligands bearing a thiosemicarbazone or semicarbazone moiety, derived from pyridoxal and their respective 5'-phosphates, have been synthesized and characterized in the present study. The compounds were tested against different strains of *K. pneumoniae*, *E. coli*, and *P. aeruginosa* which have developed different type for the production of β-lactamase (NDM-1, ESBL or AmpC) or biofilm production. GaL2 and GaL5 showed an antibacterial activity against drug-resistant strains of Gram-negative bacteria. GaL2 and GaL5 are gallium(III) complexes of thiosemicarbazone ligands which present a dimethylated N-terminal and present MIC values in the range 62.5-15.63 μM. The corresponding ligands L2 and L5 showed also antibacterial activity but with higher MIC values. A any growth inhibition was detected in compounds derived from semicarbazide moieties L3, GaL3, L6, and GaL6. The compounds were not active against the only Gram-positive *Enterococcus* spp. tested. The combination therapy studies with NDM-1 producing *K. pneumonia* showed synergism between meropenem and GaL1, GaL2, GaL4 , and GaL5. Since this group of compounds did not show any cytotoxicity against eukaryotic cells, they maybe suitable for the developing o selective antibacterial agents for Gram-negative bacteria.

Prevention is even recognized as the crucial way to counteract the spread of infectious diseases and reduce the prevalence of AMR phenomenon. For instance, research must be continuously supported in the development of new effective vaccines both for humans and animals or

eventually focusing on always new rapid and reliable diagnostic techniques to avoid the incorrect therapeutical approach.

Chapter 8. General discussion and conclusion

Limiting the AMR spread is based on surveillance and monitoring strategies of resistant bacteria in animals, food, environment, and humans. Focusing on products of animal origin intended for human consumption, the surveillance is fundamental at every step of the food chain considering processing, packing and retailing (OIE, 2020). In fact, globally the largest amount of antimicrobial is used in food producing animals (Collignon et al., 2016).

As defined by OIE, 2020:

"National antimicrobial resistance monitoring and surveillance programmes should be scientifically based and may include the following components:

1. *statistically based surveys;*
2. *sampling and testing of food-producing animals on the farm, at live animal markets or at slaughter;*
3. *organised sentinel programme, for example targeted sampling of food producing animals, herds, flocks and vectors (e.g. birds, rodents);*
4. *analysis of veterinary practice and diagnostic laboratory records;*
5. *sampling and testing of products of animal origin intended for human consumption;*
6. *sampling and testing of feed ingredients or feed".*

Moreover, it is encouraged the collaboration between Member States to determine the trends and sources of new antimicrobials, evaluate the emergence of new AMR mechanisms, recommend a prudent antimicrobial use and prescription, and organize common action plan to combat AMR. This phenomenon has no boundaries and needs international action plan (ECDC, 2022). In 2021, 25 EU countries that participated to an action plan to trace AMR across Europe that include the food safety and environmental sector; the WHO simultaneously supported a

Global Action Plan (WHO-GAP) focusing on economic investment for research, development and raise awareness on the prudent antimicrobial use.

This global action is necessary to avoid AMR development that can impact in the dissemination of AMR in a ONE Health perspective. A prudent antimicrobial use is important to ensure animal health, optimize efficacy and safety of antimicrobial molecule and respect residues of antimicrobial agents in food of animal origin (Magnusson, 2020). All Member States of the EU should avoid manufacture, importation, unlicensed use of antimicrobials and the Competent Authority guarantees and provide correct authorizations and appropriate use information on antibiotics. Moreover, more studies are needed to evaluate the ability of antimicrobials to select for resistance considering the concentration of either active antimicrobial agents or metabolites in the gut of the animal, the human antimicrobial exposure, and every intrinsic AMR mechanism in microorganisms (OIE, 2020).

Since 2011, several initiatives brought to a reduction of antimicrobial use and sales, in most of European countries, although in 2020 the consumption of broad-spectrum antibiotics was still 3.5 times higher than consumption of narrow-spectrum antibiotics. Since 2020, the trend of antimicrobial use was increasing in nine EU countries including Italy. WHO introduced in 2017 the 'Access, Watch, Reserve' (aWaRe) (WHO, 2022b) classification suggesting that at least the 60% of the total antimicrobial national consumption should be based on molecules (listed as "Access group") that offers the best therapeutic value minimizing the potential for AMR. In Italy the use of those molecules reached the 47% but in the majority of EU countries the pre-established rates have been successfully reached (ECDC, 2022).

Despite this, in the EU the percentages of health care associate infection of *K. pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter* resistant to 3rd and 4th generation cephalosporin and carbapenems is still high (approximately 30%) (WHO, 2021).

Food-producing animals (such as pig) receive more than 70% of the antimicrobials produced worldwide. Antimicrobials are used for treating, controlling and preventing diseases, but they have also been used in the past for their effect in improving growth performances (Marshall and Levy, 2011).

In 2021, veterinary medicine sales reached the encouraging lowest data reported ever, declining for the 46.5% from 2011(from 161.2 mg/PCU to 86.2 mg/PCU) and the last 5.5% decrease was registered between 2020 and 2021. Particularly, the sales of 3rd and 4th generation cephalosporins decreased of 37.8% (from 0.24 mg/PCU to 0.15 mg/PCU) from 2011 to 2021, the fluoroquinolones and quinolones sales of 14.2% (from 2.5 mg/PCU to 2.2 mg/PCU) and 83.1% (from 1.1 mg/PCU to 0.18 mg/PCU) respectively and polymyxins decreased by 79.5% (from 11.0 mg/PCU to 2.2 mg/PCU) (EMA, 2022).

Pig can be a reservoir of antimicrobial resistant bacteria that can be transferred intra ed inter species (Heidemann Olsen et al., 2016). This study showed that in the eight selected farms, antimicrobial resistant bacteria were easily found along the entire food chain, from farm, through slaughterhouse, to meat products. The analysis showed that close phylogenetic similarities have been found between strains isolated from pig carcasses and pork meat product, rarely involving fecal *E. coli*. Despite this, in one case, sequencing analysis highlighted relations between the strains isolates in all the step of the food chain. It suggests that even the use of antimicrobials can cause the development of AMR in animal fecal microbiota. Possible fecal contamination of meat product during slaughter can consequently affect the safety of food which consumers came into contact every day. Moreover, it has been demonstrated that good farming conditions is clearly correlated with a lower frequency of animal disease and a lower rate of bacterial pathogens at gut level (i.e *Salmonella* and *Campylobacter*) (Alpigiani et al., 2017).

It is necessary to highlight that processed meat products were not frequently found as antimicrobial resistant *E. coli* carriers. This suggests that proper seasoning of meat products is still a good method to reduce bacterial load. This study, however, was not able to evaluate any potential cross contamination during handling and processing of food at consumer level, which can be an added risk factor (Rega et al., 2022; Sacher-Pirklbauer et al., 2021). In fact, it is well known that food can be a source of transmission for pathogens and the high demand of ready-to-eat foods as well as raw or inadequately cooked meal amplifies this phenomenon (Rega et al., 2021). At the same time, the contribution of food in transmission of live bacterial strains (both commensal and pathogenic) or resistance genes to humans is still poorly established and underestimated worldwide (Antunes et al., 2020; Jian et al., 2021). AMR surveillance in the pig production chain has provided evidence of genetic fingerprint similarities for nosocomial human infection using multi-locus sequence typing (MLST) and whole-genome sequencing (Bonardi et al., 2022; Sirichokchatchawan et al., 2021). Transmission from livestock and/or retail meat to humans of ESBL and AmpC β-lactamases plasmid related genes harboured by *E. coli* strains have been reported by several studies (Dorado-García et al., 2018). Linkages between poultry meat, pork and humans were detected in the USA and clones of gentamicin and vancomycin resistant genes by *Enterococcus* spp. were detected in feces of healthy humans in Europe and USA (Manoharan et al., 2022; Novais et al., 2006) .

Improve research, development and innovation is the unique way to provide new solutions to prevent AMR diffusion. The first step is to improve knowledge on detection, control, and surveillance of infectious diseases to understand AMR related challenges; combine data of human hospital, agri-food sectors and society can make the process of identify the correct care solutions earlier (European Commission, 2017).

New antimicrobial molecule development is an effective strategy to ensure treatment efficacy. In the present study, a series of ligands bearing a thiosemicarbazone or semicarbazone moiety, derived from pyridoxal and their respective 5'-phosphates, have been synthesized and characterized. The compounds were tested against β-lactamase (NDM-1, ESBL or AmpC) or biofilm producers Gram-negative bacteria. Two compounds (GaL2 and GaL5) showed an antibacterial activity with MIC values in the range 62.5-15.63 μM.

Since this group of compounds did not show any cytotoxicity against eukaryotic cells, they maybe suitable for the developing of selective antibacterial agents for Gram-negative bacteria.

Despite this, to manage the foodborne AMR risk is necessary to primary follow good agricultural practices, good veterinary practices (GVP), good hygienic practices (GHP), and good manufacturing practices (GMP) along the entire food production chain, considering:

- pre-harvest options that include minimizing the presence of antimicrobials and AMR microorganisms in feed, the proper use of veterinary antimicrobials (restricting extra and off label use, limiting marketing status) and the development of national or regional treatment guidelines, targeting a specific AMR food safety issue. Moreover, promoting the availability of diagnostic microbiological tests and controlling the waste management to prevent AMR diffusion is fundamental;
- post-harvest options that consist in preventing foods contamination by AMR microorganisms able to reach the consumer through products identification before marketing or withdrawal for reprocessing or destruction (WHO, 2021).

Moreover, food safety systems such as Hazard Analysis and Critical Control Points (HACCP) that are conceptualize on risk, should guarantee hazard control in the different step of the food production chain, and must be implemented to include foodborne AMR in management measure (Pérez-Rodríguez and Mercanoglu Taban, 2019).

Nevertheless, further studies are need in order to guarantee a correct risk communication to consumers and give access to clear and reliable information and health concerns on food labels. Consumers' awareness on antimicrobial resistance phenomenon and its possible spread from animals to humans is low. Less than half of consumer population in Europe identified consumption of meat products as a route of transfer of antibiotic resistant bacteria from animals to humans (BfR, 2015; Etienne et al., 2017). The percentages are even higher than the number of consumers that evaluate the risks posed by contacts with live animals. Despite this, consumers are reported as having seldom changed behaviors because of AMR risks (Etienne et al., 2017). Scientific professionals and authorities are considered as trusted sources of information on this matter compared to the media and food industry (Etienne et al., 2017).

Conclusion

In conclusion, results showed that AMR bacteria can be easily found along the different stages of the pork food chain. Proper meat transformation processes (fermentation and seasoning) highly reduce the bacterial load in meat product and the possibility of transmission of AMR bacteria to consumers.

From farm-to-fork AMR bacteria diffusion is not frequent but in one case it has be evidenced that genomically related bacteria were found in feces, carcasses and fresh meat derived from the same pig. Other phylogenetic relations between bacteria showed frequent similarities between strains isolated from pig carcasses and pork meat product, rarely involving fecal *E. coli*.

The use of antimicrobials during farm life can impact on the AMR development and that AMR bacteria can persist in the different stages of meat production. Moreover, the food producing environment and cross contamination have an important role in the dissemination of this phenomenon in the food chain. The development of new antimicrobials able to inhibit AMR can be an option even in the food production world to face this public health risk. In the One Health context, it is a duty to clearly and effectively communicate AMR risks to consumers to reduce its diffusion and to protect human, animal and environmental health.

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