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Occurrence of Extended spectrum β-lactamases-producing *Escherichia coli* and Methicillin-resistant *Staphylococcus* spp. in swine farms and association with sanitary and productive parameters

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Preface - Antimicrobial resistance (AMR)

Antimicrobial resistance (AMR) is a global threat for humans, animals and environment. It is estimated that more than 60% of the total antibiotic consumption is employed for livestock treatment. Recently, WHO has listed pathogenic antibiotic-resistant bacteria based on the urgent need of new drugs to treat related infections. In this list, two bacterial groups of human and animal interest, namely Extended spectrum β-lactamase (ESBL) - producing Enterobacterales and methicillin-resistant *Staphylococcus aureus* (MRSA), are at critical and high level of priority respectively. Considering this, surveillance on animal farms and assessment of farmers' exposure to antibiotic-resistant bacteria is paramount to uncover the real burden of AMR at farm level. In this thesis, two main antibiotic-resistant bacterial groups will be considered. More precisely, the first bacterial group is ESBL-producing *Escherichia coli (E. coli*). The presence of this resistant microorganism will be reviewed in livestock, swine and poultry farming, taking into account ESBL-producing *Escherichia coli* related infections in exposed humans. Moreover, pathotypes and virulence determinants will be described in depth **[Chapter 1].** The second bacterial group is composed by MRSA and methicillin-resistant coagulase-negative staphylococci (MRCoNS). These two bacterial subgroups will be considered in swine and poultry productive chains, taking into account also farm working associated human infections **[Chapter 2].** Next section will be on the review of factors that can be implemented to counteract AMR, like farm biosecurity and animal welfare. Reduction of antibiotic usage (AMU) will be considered too. All these subjects will be discussed in the light of the new Italian system called "ClassyFarm", born to classify farms based on the risk level **[Chapter 3].** The following chapters will investigate MRSA, MRCoNS and ESBL-producing *E. coli* in field studies. **Chapter 4** describes the investigations of MRCoNS and MRSA from pigs and farm environment of Northwestern Italy through phenotypic and genotypic analysis. Farm management is also taken into account. **Chapter 5** illustrates the level of exposure of workers to methicillin-resistant staphylococci (MRS) and ESBL- producing *Escherichia coli* in an intensive broiler farm located in Northwestern Italy. In this chapter AMU will be also examined. A similar study is described in **Chapter 6,** focusing on the occurrence of MRCoNS, MRSA and ESBL-producing *Escherichia coli* in one swine farm from Northern Italy, to understand the exposure of farm workers to these resistant bacteria. **Chapter 7** focuses on the WGS analysis of ESBL-producing *E. coli*, sampled from pigs and farm environment, to describe AMR and virulence determinants in depth. In the last chapter of the thesis **[Chapter 8]**, the concept of antimicrobial stewardship in swine farming is introduced. AMU (frequency and routes of administration) and antibiotic susceptibility in MRS and ESBL- producing *E. coli* will be jointly evaluated, in order to find potential association between antibiotic usage and bacterial resistance.

Part I - Review of the literature

Chapter 1 - Review of Extended-spectrum β-lactamase (ESBL)- producing *Escherichia coli*

1.1 General characteristics of Extended-spectrum β-lactamase (ESBL)- producing *Escherichia coli*

In the last decades, antimicrobial resistance has become a pandemic worldwide; in this context antibiotics, particularly the categories belonging to the class of β-lactams, are necessary to tackle pathogens in human and veterinary medicine. β-lactams are hugely prescribed at the hospital settings and they are mainly from four classes: penicillins, cephalosporins, carbapenems, and monobactams. However, resistance to these antibiotic categories is growing rapidly at a global scale, indicating that monitoring of this phenomenon is paramount [1]. Extended- spectrum β-lactameses (ESBLs) are enzymes able to hydrolyse and inactivate last generation cephalosporins, monobactams and penicillins. Some of these enzymes can inactivate even carbapenems. Important health agencies, such as the Center for Disease Control and Prevention (CDC) and the World Health Organization (WHO), have indicated β-lactamase-producing Gram-negative bacteria as critical pathogens, against which there is urgent need to formulate novel therapies [2,3].
Despite the fact that in scientific literature have been reported around 3000 different β-lactamases from different epidemiological settings [4], few are relevant from a clinical perspective [5]. In 1980, Ambler suggested the classification of β-lactamases, based on the four β-lactamase amino acid sequences that were discovered till that period: class A enzymes, characterised by a serine in the active site, and class B metalloenzymes (MBLs) [6]. After this classification, other two classes, C and D serine-based β-lactamases, were described, considering the molecular structure and specific homologous motifs [7,8]. β-lactamases are able to hydrolyse the β-lactam ring through a covalent acyl-enzyme intermediate compound originated between the β-lactam molecule and the active site serine, or due to a reaction catalysed by one or two zinc ions that are present in the MBL active site [4]. For their aminoacidic similar composition, class A, C, and D serine β-lactamases can be considered structurally related, while class B β-lactamases, that are metallo-β-lactamases, can be considered separately (Figure 1). Bacterial strains expressing MBLs generally are resistant to penicillins, cephalosporins, carbapenems, and the commercially available β-lactamase inhibitors; for these reasons, they are considered the most threatening from a clinical perspective [9]. The most frequently identified MBLs comprise IMP, VIM, and NDM type [10]. Even in class D β-lactamases, some type of enzymes, namely OXA, can have carbapenemase activity, these variants are mainly represented by OXA-23, OXA-40, and the more frequently identified OXA-48 [1].

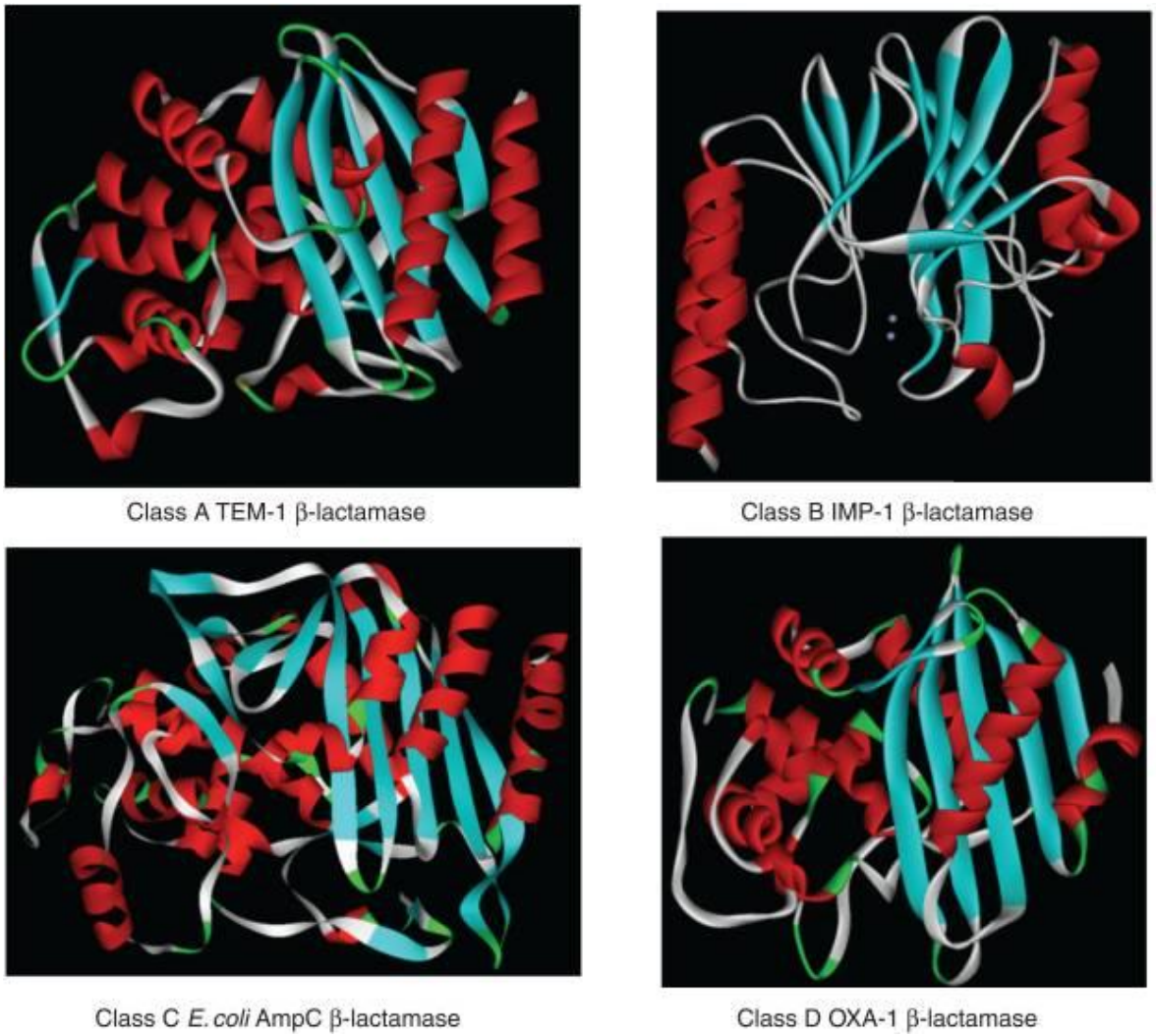

Figure 1: structural resemblance of class A, C, B and D serine β-lactamases (modified from Bonomo, 2017 [9]).

In the Enterobacterales bacterial family, SHV- and TEM-variant enzymes (class A) are 240 each [11], and they are mostly plasmidic. All these different enzymes came from point mutations that gave way to amino acid substitutions in the ancestral gene. In the 2000s, a new extended spectrum β-lactamase (ESBL) -class A enzymes (CTX-M type) became prominent, causing the so-called CTX-M pandemic; nowadays, this is the most frequently detected ESBL-type in Enterobacterales around the world. Contrary to the ESBLs enzymes TEM and SHV, that are related to plasmid-borne penicillinases, the CTX-M variants are of chromosomal β-lactamase origin, derived from species of the environmental bacteria *Kluyvera* [11,12,13,14]. *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are the most often identified ESBL-producing bacteria and they can be responsible of urinary tract infections (UTI), pneumonia or, in the worst scenario, sepsis [15].
All these enzymes are able to inactivate antimicrobial molecules such as cephalosporins of 3rd and 4th generations (e.g. cefotaxime, ceftriaxone, ceftazidime, and cefepime) and some monobactams (e.g. aztreonam). The presence of antibiotic- resistance genes on mobile genetic elements, such as plasmids and transposons, allows their rapid transmission and the dissemination among the bacterial population between invasive and commensal strains [16,17,18]. AmpC genes can be also plasmid-linked, or in *E. coli,* chromosomal, with a mutation in the promoter region, that leads to an overexpression of the enzyme [19]. AmpC group enzymes are considered β-lactamases, but they have not the "extended spectrum" action; they confer resistance to aminopenicillins and early-generation cephalosporins (e.g. cephalothin, cefuroxime and cefoxitin). They can have spontaneous

mutations in the AmpC regulatory genes, that cause AmpC overproduction, conferring the resistance to expanded- spectrum cephalosporins, like cefotaxime, ceftriaxone, and ceftazidime. In human medicine, CMY-2 can be responsible for the resistance to 4th generation cephalosporins, due to a mutation, caused by the therapeutic use of cefepime. In this case, the CMY-2 enzyme switches to CMY-33 variant [20]. These enzymes are inhibited by cloxacillin, oxacillin, and aztreonam [21]. CMY-2 is the enzyme most frequently found in livestock in Europe among the AmpC group-related enzymes [16,22,23]. CMY-2 belongs to the specific enzymatic group CIT [24], and it is almost the only detected in *E. coli* isolated in poultry sector. Belonging to the AmpC group, CMY-2 is less inhibited by clavulanic acid, given false-negative results, when coproduced with other β-lactamases during the ESBL-screening with CLSI tests [25]. The same enzyme can confer resistance to carbapenems, as was reported in *E. coli*, due to a decreased membrane permeability. This β-lactamase can be generally recovered on mobile genetic elements (MGE) like IncI1-Iβγ, IncA/C, IncF and IncK plasmids [26].
In human medicine, the epidemiological dissemination of ESBLs in the last two decades was linked to the global diffusion of the CTX-M-type enzymes. This enzymatic class comprehends more than 220 variants grouped in 5 subfamilies depending on the amino acids composition that consider the CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 subfamilies. The variants arising from subfamilies CTXM-1 and CTX-M-9 are globally spread and usually identified worldwide [27,28]. CTX-M-15 is the most important variant in many Regions of Europe [29,30], Asia [31], Africa [32], and in the USA [33]. In Southeast Asia, another variant, CTX-M-14, is the prevalent ESBL identified in *E. coli*, in particular in South Korea and Japan, while CTX-M-2 variant is frequently found in South America [13].

1.2 Extended-spectrum β-lactamase (ESBL)- producing *Escherichia coli* in livestock

During the last decades ESBL-producing bacteria, particularly *E. coli*, have often occurred not only in human clinical cases, but also, in companion animals and more frequently in livestock. For this reason, a considerable amount of surveillance studies targeting specifically livestock have been published [34,35]. ESBL-producing *E. coli* strains are not rare, and they can be isolated in a growing number of livestock species [36,37,38]. From these increasing number of reports, it has emerged the possibility that animals, with particular relevance livestock, can be reservoirs of these AMR bacteria enhancing the risk of human infection [39]. In farm animals and environment, the most frequently recovered *bla* genes, encoding for "real" extended-spectrum β-lactamases enzymes, are *bla*$_{TEM}$, *bla*$_{SHV}$ *e bla*$_{CTX-M.}$ The gene *bla*$_{CTX-M}$ is the most frequently found in livestock, especially the type *bla*$_{CTX-M-1}$ [37,38]. They can be associated with other genes that confer resistance to non- β-lactams antibiotics, like aminoglycosides (*aadA*), fluoroquinolones (*qnrB1, aac(6')-Ib-cr*), fosfomycin (*fosA*), phenicols (*floR*), sulfonamides (*sul*), tetracyclines (*tet*) and trimethoprim (*dfrA*) [40,41,42,43,44,45]. AmpC group enzymes are less frequently detected, but genes encoding for

these β-lactamases are commonly found in the poultry productive chain [24]. Colonised animals do not show clinical symptoms, because these antibiotic resistant strains are often less virulent in animals, so it is impossible to identify positive subjects without phenotypic and genotypic microbiological tests. In livestock, ESBL- producing *E. coli* can be often identified among cattle, swine and broiler samples. Indeed, in the study of Dahms et al., high percentages of phenotypic positivity, were recovered in cattle (54.5%), pigs (88.2%) and broilers (75%) [46]. Prevalence higher than 90% were detected in dairy calves in Germany [42].
In the next paragraphs, the occurrence, epidemiology and molecular characterizations of ESBL-producing *E. coli* will be accounted specifically in the swine and poultry productive chains, with a particular attention to the possible transmission to farm workers. Virulence genes present in ESBL-producing *E. coli* will be summarized in a final paragraph.

1.3. Extended-spectrum β-lactamase (ESBL)- producing *Escherichia coli* in swine farming

Among farm animals, pigs are recognized ESBL-producing *E. coli* reservoir [38]. They are routinely sampled in European countries, to monitor these antibiotic resistant bacteria at animal and meat level; through mandatory surveillance (Commission Implementing Decision (EU) 2020/1729), high percentages of phenotypic positive animals were found in certain countries, particularly Spain (80.3%), Italy (69.2), Belgium (60.7) and Austria (58.8%) [47,48]. Moreover, transmission of the genes coding for ESBL enzymes from pigs to farm workers was hypothesized in some European countries like Denmark, Germany and the Netherlands. ESBL-associated genes can be transmitted from livestock bacteria to human-associated commensal and pathogen bacteria. Humans working on pig farms are at major risk to be carriers of bacteria with these types of genes, with respect to the general population. The main reason is the direct and continuous contact with carrier animals during daily farm work [16,19,49]. Furthermore, the farm environment seems to play a major role in bacterial transmission. In fact, ESBL-producing Enterobacterales (ESBL-producing E) have been detected even in the swine barn air of seven German pig farms with 9.5% of positive samples [49].

Several studies assessed the prevalence of ESBL-producing E in animals, pig farmers and farm environment. In Swiss pig farms, the isolated ESBL-producing E were just *E. coli*, and the occurrence in pigs was 13%, and 12% in workers. None of the farmers showed nasal carriage, while in some cases pigs had nasal contamination, due probably to the natural habit of snuffle for food. The Authors also found a statistically significant association between the use of antibiotics in the pig housing and the presence of ESBL- producing E. The most frequently detected β-lactamase enzyme was from CTX-M group 1 [50].

As said before, the airborne transmission can be a way to acquire ESBL- positive bacteria [49]. In the study of García-Cobos et al. (2015), dust and manure from 47 pig farms in Germany were analysed to detect ESBL-E [51]. ESBL- producing *E. coli* were present in the 27.3% of manure samples and 10.3% of dust samples.

In a study of Dohmen et al., dust samples from 32 non-organic Dutch pigs' farms were screened for ESBL ($bla_{\text{CTX-M}}$ group 1). Pig farmers, their family components and other farms workers were included in the survey, which also comprised faecal samples from 60 pigs per farm. A double sampling was done with 12 months distance. The results indicated that in pig farmers, ESBL carriage was linked to exposure to contaminated dust and pigs with the same ESBL-associated gene. The comprehensive

human faecal prevalence of the specific ESBL-associated gene was 3.6%, while the prevalence of *bla*$_{CTX-M}$ group 1 in the dust was 9% during the first sampling and 3% in the second. Furthermore, the ESBL detection decreased in pigs too, from the first sampling (18%) to the second (12%). These data confirm air as a possible transmission source for humans and suggest the need to implement hygiene standards to reduce the possibility to be exposed to contaminated air in the pigsty environment [52].

A very low occurrence of ESBL-producing *E. coli* was found in Finland, as reported in the article of Päivärinta et al. [53]. The study analyzed 531 pigs at the slaughterhouse, some of which previously treated with antibiotics (294 animals). ESBL/AmpC positive *E. coli* were found in 1.5% (8 samples) of pigs. All pigs with AmpC positive *E. coli* samples had previously been treated with antibiotics, and the difference in comparison with non-treated animals was significant. No positive sample was due to plasmidic *bla*$_{ESBL/AmpC}$ genes. AmpC-producers *E. coli* had mutations in the chromosomal AmpC promoter area. This low occurrence can be explained with the low usage of antibiotics for farm animals in Finland, with respect to the rest of Europe, and probably can be linked to the fact that pigs are treated singularly and not in-group. Also, Finland imports a very low number of pigs, and this can positively influence the ESBL/AmpC prevalence in swine productive chain, because there are less chances to import ESBL/AmpC positive pigs [53].

In the study of Dohmen et al., the evaluation of ESBL-producing *E. coli* prevalence was carried out between farms with high use of third- or fourth- generations' cephalosporins and farms where these antibiotics were not used [54]. Prevalence in the highly treated farms was significantly higher than the other farms (79% versus 20%). *bla*$_{CTX-M-1}$ was the gene most frequently found; also, *bla*$_{CTX-M-14}$, *bla*$_{SHV-12}$ and *bla*$_{CTX-M-97}$ were detected. Humans that resulted positive to ESBL-*E. coli* had more often direct contact with pigs positive for ESBL-producing *E. coli* (20%), compared to people with direct contact with negative pigs (5%) [54].

Another study of Dohmen et al. recovered a prevalence of 6% (12/142) of ESBL-producing *E. coli* colonised farmers working on positive farms in the Netherlands. Again, *bla*$_{CTX-M-1}$ was the most often recovered gene; moreover *bla*$_{TEM-52}$ and *bla*$_{CTX-M-14}$ were identified from farm personnel. All these genes resembled the genetic pool found in the animals. The Authors observed that ESBL-producing *E. coli* human carriage was associated to the mean of hours working at the pigsty per week; contact with positive animals was also a risk factor considered important; indeed, colonized farmers, said to have daily contact with animals [55].

In the survey of Hansen et al., it was highlighted that the stage with the highest colonization by ESBL-producing *E. coli* during the swine productive life was the end of farrowing period; two types of *bla* genes (*bla*$_{CTX-M-14}$ and *bla*$_{TEM-1}$) were recovered in positive bacterial strains during the first weeks of life [56].

In the study of Dorado-García et al., different potential ESBL/AmpC- producing bacteria reservoirs (general population, farmers and livestock animals) were analysed in the Netherlands. Using the rarefaction analysis, it emerged that the farming groups in direct contact with pigs, as well as poultry, has the lowest different genetic pool (~4 diverse genes) regarding ESBL/AmpC genes [57]. Through the proportional similarity index (PSI) it was detected a high level of similarity between cephalosporinase genes from livestock farmers and their own animals (PSI=0.8 for swine farming). It was highlighted that in pigs and people linked to these animals, the most prevalent gene was *bla*$_{CTX-M-1}$. This finding allowed to separate the farming community and their animals from a group composed by general population, water sources and human clinical population, in which the occurrence of *bla*$_{CTX-M-15}$ was elevated [57]. These data support the theory that an intense and direct contact with livestock animals can be the source for farmers, or vice versa for animals, to

acquire ESBL/AmpC-associated genes.

In the article of Fischer et al. on 51 German farms, ESBL- producing E was detected in 61% of the sampled farms, while 6% of farmers (5/84) were ESBL-producing E positive [58]. Using a specific cgMLST (core genomeMLST), the Authors found that only one farmer sample was associated with the environmental isolate from the corresponding farm. The other human samples were poorly related to the ones from their farm. The human isolate which was similar to the corresponding environmental one, belonged to the lineage ST10 with the ESBL-associated gene *bla*$_{\text{CTXM-X-1}}$, frequently found in livestock environment [58]. The prevalence in this German farmers' community (6%) is really low compared to the elevate occurrence of ESBL carriers among farmers in the Netherlands (27%) [55], and is in line with carrier status among German general population (~ 6%) [59,60]. In another study from German intensive and organic farms, results from farmers highlighted that 2 out of 32 tested humans were ESBL-producing *E. coli* carriers (all from ESBL- positive farms); 15 out of 17 pig farms were found ESBL- positive (88.2%), including organic farms. *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ genes were identified from farmers, while swine samples were positive for *bla*$_{\text{CTX-M}}$, or for both *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M}}$. Although farmers had the same enzymes found in ESBL- positive pigs (CTX-M-1/-61; TEM-104/-206), the ST profile was different. These results do not prove a clonal transmission of a resistant bacterial species between animal and human. However, these occurrences can be associated to the genetic mechanism of horizontal resistance gene transfer, not necessarily directly from the animal carrier, but maybe indirectly, from a common source (e.g. contaminated air, soil, water or even rodents) present on farm [46].
De Been et al. state that transmission of a mobile genetic element, namely a plasmid, is the easiest way to acquire cephalosporinase transcription genes. To corroborate this hypothesis, the Authors used a high generation molecular technique, WGS (Whole Genome Sequencing), to analyse antibiotic-resistant *E. coli* strains potentially transmitted between two reservoirs, farmers and pigs. Running this complex analysis, they discovered that the same plasmid, IncI1, carrying the antibiotic resistance gene *bla*$_{\text{CTX-M-1}}$, could be found in different phylogenetic strains of *E. coli* in distinct reservoirs (human and pigs) [17]. Furthermore, in the IncI1 plasmid, there were other antibiotic-resistant genes, *sul*, *dfrA*, *aadA* or *tet*, which are often found in livestock related *E. coli* strains [17]. A limitation to this analysis was the restricted number of samples, 8 in total, 4 from farmers and 4 from pigs; furthermore, the study included poultry samples, which were less closely related to human *E. coli* strains, but they carried the same resistance gene *bla*$_{\text{CMY-2}}$, supporting a conjugative transmission of the plasmid between bacteria [17].

Although CTX-M-1 is the most frequently ESBL enzyme detected in pigs, Wang et al. identified in 2016 seven samples, six from animals and one from the pigsty environment, that carried other ESBL-associated genes, not typical of the livestock group: *bla*$_{\text{CTX-M-14}}$ was detected in 3 weaner pigs, *bla*$_{\text{CTX-M-15}}$ was identified in 2 piglets and *bla*$_{\textit{TEM-20}}$ was found in an environmental sample and in piglets' faeces [61]. This study highlights the presence of ESBL-associated genes, normally detected in human clinical setting, in pigs. In line with these results, a Swedish survey recovered the presence of the most frequent human ESBL-associated gene, *bla*$_{\text{CTX-M-15}}$, in isolates from pigs and calves [62]. These results are relevant because they are from a country that employs a lower amount of antibiotics in veterinary and in human medicine with respect to the rest of Europe.

In the study of Munk et al., the overall highest level of antibiotic resistance among nine European countries was registered in Italian pigs; β-lactam resistance genes were infrequently isolated from the faecal samples. The analysis was performed through shotgun metagenomics to find the acquired antibiotic-resistant genes, called resistome, in pigs and poultry, before the slaughter [63]. Data from the 2015 EFSA official monitoring program from fattening pigs, reported in Italy an overall prevalence

of 64% of *E. coli* showing an ESBL-phenotype; the prevalence of AmpC mediated resistance was much lower (5.9%). In Italy, 309 caecal samples were tested and molecular analysis reported CTX-M-1 as the most prevalent enzyme, while CMY-2 was the one recovered more frequently among AmpC types [64]. In the article of Stefani et al., analysis on swine farms in the Italian province of Modena revealed a phenotypic prevalence of 30%. *bla*$_{\text{CTX-M-1}}$ was the most frequently recovered gene, followed by *bla*$_{\text{TEM-52}}$. In some samples, *bla*$_{\text{CTX-M-1}}$ was present with *bla*$_{\textit{TEM-201}}$ (2/15) or *bla*$_{\text{TEM-1}}$ (2/15). *bla*$_{\text{CTX-M-15}}$ positive samples were identified [65].

In the review by Valentin et al., the Authors made comparisons between animal and human ESBL-associated data from the German National interdisciplinary research project (RESET). CTX-M-1 was the enzyme most often found among all livestock animals (63%), while CTX-M-15 was the one most often detected among humans (48%) [59].

In the article of Schmithausen et al., the prevalence of ESBL-producing E among pigs was 32%, mainly *E. coli.* The study highlighted a different carriage status depending on the age of the animal; in fact, finishing farms had a lower prevalence of ESBL-producing E isolates (26%), compared to farrowing and nursery farms, that together accounted for 36% [66]. Looking at the different areas of the farms, in the young farrowing/newly weaned sectors, a prevalence of 83.3% was detected, while the farrowing/nursery areas had a 66.7% of positivity. Furthermore, this study considered the ESBL-producing E contamination at the slaughterhouse for ESBL- negative pigs; these data evidenced a prevalence of 29.4% of newly colonised animals. ESBL-producing E were recovered in 17.1% of air sampled from the farm environment, while a higher prevalence was found in the slaughterhouses air, with 50% of positive isolates. This elevate amount of ESBL-producing E in the abattoirs air is probably due to the high humidity present inside these premises, while at pig farms air is drier. Molecular tests (PCR) highlighted that the majority of ESBL *E. coli* - positive samples belonged to the *bla*$_{\text{CTX-M}}$ group of genes (95.7%) [66].

A study of Dohmen et al. assessing ESBL- positive strains of *E. coli* after reducing cephalosporins usage in the Dutch swine farms, evidenced the reduction of the total prevalence of positive animals. The research found an association between use of the cephalosporins in pig farms, before and in the course of the study, and the detection of ESBL-producing *E. coli* in animals. The enrolled farms were 36 and they were of the multiplier type (when sows and piglets are in the farm). The analysis was conducted in 18-months long period with four sampling times. The tested animals per farm were 60, grouped in pool per age. Out of the 16 positive farms positive at the beginning, 10 were positive at the end of the study. Seven pigsties became negative in the meanwhile of the analysis. The overall ESBL-producing *E. coli* prevalence dropped from 27% to 13% at the end of the 18 months. Farrow-to-finish open farms were the most colonised. The higher amount of ESBL- positive samples was collected in the category “suckling piglets” (24.2 %), with a reduction during the later phases (“weaned piglets” 17.2%, “finishing pigs” 16.9%). The Authors also found that the occurrence of positive animals was significantly unlikely when the source of water for pigs was public and not private. Furthermore, the presence of a hygiene lock as the only entrance of the farm, and pest control made by an expert, were associated to an unlikely presence of ESBL-producing *E. coli* carriers in the farm. Contrary, presence of goats in the farm and external supply of animals were considered risk factors. *bla*$_{\text{CTX-M-1}}$ was the most prevalent ESBL- associated gene detected among swine samples [54].

Conversely to the previous study, Herrero-Fresno et al. identified *bla*$_{\text{TEM}}$ as the most common gene identified in swine faecal samples collected from the gut of healthy nursery pigs. The Authors found less frequently AmpC related genes, *bla*$_{\text{CMY}}$ and *bla*$_{\text{ACT}}$. The overall prevalence of β-lactamase genes was 59.5%. These data are important because commensal *E. coli* can be a reservoir of antibiotic-

resistant determinants, that can be spread to pathogenic strains present in the gut [67].

In a retrospective study of Luppi et al. analysis on multidrug resistant pathogenic F4+ *E. coli* in Italian sick pigs found that the occurrence of cefquinome (fourth generation cephalosporin) resistance arose from 3.8% to 44% during the tested period. This increasing percentage highlights the importance of testing also clinical samples to monitor antibiotic resistance in pigs towards cephalosporins [18].

Another retrospective survey in Spain, in the period 1999-2018, found that 200 pathogenic *E. coli* strains, sampled from neonatal and post weaned pigs, often carried $bla_{\text{CTX-M}}$ genes (13.5%). The most frequently detected type of enzymes were CTX-M-14, CTX-M-1 and CTX-M-32, while CTX-M-27, CTX-M-9 and CTX-M-3 were less frequently identified. AmpC genes ($bla_{\text{CMY-2}}$) were also recovered in 3% of samples [68].

The last reports of EFSA and ECDC, considering the interval 2016-2018, reported a reduction of ESBL/AmpC- producing *E. coli* in farm animals (fattening pigs, calves, broilers and fattening turkeys) in 40% of European countries. The survey also highlighted that prevalence was much lower in meat of fattening pig than in the animal caecal content sampled at the slaughterhouse. This ultimate founding shows that, although animals carry high load of ESBL-producing *E. coli*, these bacteria do not contaminate carcasses, maybe for the "decontamination effect" of the slaughter workflow [69,70].

Considering mandatory surveillance on *E. coli* in the United Kingdom, the most frequently detected ESBL-associated gene was $bla_{\text{CTX-M-1}}$ for the years 2015 and 2017. The genes *sul2*, *tet(A)* and *dfrA17* were the most frequently detected among non-beta-lactamases. The contemporary presence of *aadA5,* $bla_{\text{CTX-M-1}}$, *sul2* and *dfrA17*, was the most often recovered genes' association found in ESBL-producing isolates in 2013, while in 2015 and 2017, $bla_{\text{CTX-M-1}}$, *sul2* and *tet(A)* were found frequently in combination [44].

Reflecting on all these reports about ESBL-producing *E. coli* occurrence in pigs, farmers and even environment (air dust and manure), it is clear that these resistant microorganisms are widespread in European swine farms. Horizontal transmission of ESBL-associated genes, through mainly plasmids, may worsen the epidemiological situation; thence, plans to reduce and hinder the appearance of these resistant bacteria at farm level are urgently needed. This is more relevant assuming the elevated risk of ESBL- producing *E. coli* transmission from colonised animals to humans. Strategies to combat these AMR bacteria, primarily the reduction of the antibiotic usage at farm, especially the oral group therapy, improvement of the biosecurity measures and global farm hygiene will be discussed in the next chapters.

1.4. Extended-spectrum β-lactamase (ESBL)- producing *Escherichia coli* in poultry farming

In broiler farms, the ESBL-associated genes more frequently identified in ESBL-producing *E. coli* from poultry and farm workers, that are at strict contact with animals, are bla_{CMY-2} bla_{CTXM-1} and bla_{SHV12} [71,22,72]. In the study of Huijbers et al., ESBL-producing *E. coli* were isolated in different productive phases from the Netherlands' broiler farms and from farmers. The ESBL genes' distribution was similar in farm workers and their broilers. For the Authors, this event was dependent on the direct and continue exposition to animals, carrier of ESBL/AmpC- positive bacteria [71]. The same was observed in the study of Dorado-Garcìa et al., where The Authors observed a high genetic similarity (proportional similarity index= da 0.8 a 0.9) among genes coding for ESBLs enzymes carried by *E. coli* isolated in the human community that inhabits farms and animals (broilers and pigs). Furthermore, genetic pool of ESBL/AmpC associated- genes from farm workers' category was limited with respect to the one from general population. This indicates that there is a more restricted origin of the ESBL/AmpC- associated genes from people that are exposed to farm animals, putative reservoir of these resistant genes. In this survey, bla_{CMY-2} was the most frequently found gene in *E. coli* of broiler source, in people living or working in the farm, in broiler meat and laying hens; moreover, bla_{SHV-12} was identified from the same group, of people and animals [57].

In the study of Huijbers et al., analysis on broilers from organic Dutch farms in two different moments (34 and 68 days) of the productive cycle, revealed high prevalence both after the first sampling (94.3%) and after the second one (80%) [73]. These percentages are higher than European mean (31.9%) and Italian prevalence (63.7%) [74]. From the same farms, 11.1% of farm workers and people residing in broiler organic farms was ESBL/AmpC-producing *E. coli* positive [73]. The percentage of positive farmers from organic farms was not so different from the one detected in farm workers from intensive broiler farms (19.1% [71]). Genes identified in animal and farmer samples were mostly $bla_{CTX-M-1}$ and bla_{CMY-2}. The absence of bla_{SHV-12} in organic farms can be due to diverse breeders that are used for new organic lineages. The study of Huijbers et al. is important because detected an elevated prevalence of ESBL/AmpC- producing *E. coli* from organic broilers, that is similar to the result from intensive farms detected in the same country (96.4% [71]). This high prevalence cannot be explained by the antibiotic usage, as in organic farms antibiotic treatments are not allowed.

In a study conducted in Germany in 2013, Reich et al. found a prevalence of ESBL-producing *E. coli* of 88.6 % in broiler carcasses, while was 72.5% in caecal samples. For AmpCs lactamase, positive samples were found in 52.9% of carcasses and in 56.9% of ceacal samples. They detected co-resistance to other non- β-lactams antibiotics, mostly associated to ESBL-phenotype, and less frequently to AmpC-positive samples. Resistance to nalidixic acid, cloramphenicol and tetracycline were detected in ESBL-positive samples too. The Authors supposed that the use of some non- β-lactams antibiotic classes can have a selective pressure that supports the acquisition of the ESBL/AmpC associated genes; indeed, many ESBL/AmpC- producing bacteria are often multiresistant [24,75].

In 2014, a Norwegian survey, that analysed the presence of ESBL/AmpC-positive samples in the poultry productive chain, detected a very high prevalence for AmpC- positive samples (43%), especially in the broiler category. This was considered whimsical as Norway do not use generally a large amount of antibiotics, and there are no cephalosporines sold for food-producing animals. This result can be explained by the breeders' import from countries where antibiotics are largely used. bla_{CMY-2} was the most frequently detected gene, like Sweden and Denmark broiler productive chain. This type of resistance was found along the productive chain until the final meat byproduct (32%) [76].

In 2015, ESBL-producing *E. coli* was recovered in broiler semen by Mezhoud et al. This finding supports the possibility to disseminate resistant strains through reproductive route, and this is supported by the use of a limited number of male broilers selected as breeders [77].

bla genes can be present in pathogenic *E. coli* too. This was observed in Italy, in colibacillosis-affected animals from 200 intensive farms of turkeys, broilers and laying hens. It was discovered that broilers were the animals with the highest prevalence of ESBL/AmpC- associated genes (9%, with respect to 7% in turkeys and 4% in laying hens). The isolation of ESBL/AmpC producing-bacteria was made from colibacillosis lesions, following CLSI guidelines [78]. $bla_{\text{CTX-M-1}}$ was the genetic ESBL-determinant most frequently found in all animal categories, instead the genes less often recovered were $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{SHV-12}}$ e $bla_{\text{CMY-2}}$. All the *bla* genes were plasmid-associated and IncI1-Iγ was the most frequently reconstructed plasmid [79]. The resistant to β-lactams antibiotics was associated to aminoglycosides, florfenicol, nalidixic acid, sulfonamides and tetracyclines resistance.

In 2013, a French study discovered that ESBL-positive *E. coli* were more frequently found in young hens than in adults and that the prevalence of positive isolates changed with the type of incubator. The Authors hypothesise that the administration of antibiotics like tetracyclines or trimethoprim-sulfonamides is able to co-select for the resistance to third-generation cephalosporins, which is transmitted vertically in the hens' lineage [80]. Even in this case, $bla_{\text{CTX-M-1}}$ was the most frequently detected gene, as in French broilers [81]; $bla_{\text{CMY-2}}$ was found in some samples. Although this study was conducted in laying hens, this result supports the hypothesis that administration of antibiotics in incubator can elicit the resistance ESBL/AmpC mediated; to corroborate this, a recent study observed ESBL- associated resistance already *in ovo*, from which originate all the broilers lineages [82].

The vertical transmission of ESBL/AmpC-mediated resistance is sustained by Baron et al., who recovered the same genetic markers, especially $bla_{\text{CMY-2}}$, in chicks from the same incubator [83]. Even in the study of Laube et al. in 7 broiler farms in Germany, it was found the presence of ESBL/AmpC-producing *E. coli* during the initial phase of the productive cycle (from day 1). The survey recovered an increasing level of positive samples during the productive cycle (51% at first sampling, 75% in the second and 76% in the last sampling), that was associated with higher contamination levels of the farm environment. $bla_{\text{CMY-2}}$ was the most frequently recovered gene, often associated with $bla_{\text{TEM-1}}$. $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M}}$ variants, and rarely $bla_{\text{TEM-52}}$, were recovered with less frequency [72].

Vertical transmission of ESBL/AmpC-producing bacteria is also associated to breeders and their lineages. This event is corroborated by the fact that few farms provide these animals to poultry productive chain [77]. The study of Agersø et al. highlighted this transmission route. Indeed, they found 93% of positive parental flocks' farms. The prevalence of positive broilers was reduced (27%) compared to parental animals; the most frequently identified ESBL/AmpC associated genes were $bla_{\text{CMY-2}}$, $bla_{\text{SHV-2}}$ or $bla_{\text{CTX-M-1}}$. For some positive $bla_{\text{CMY-2}}$ broilers' *E. coli* strains, identical PFGE patterns to the parental flocks were identified [84].

Looking to all these surveys, it is evident that the poultry sector can be highly colonized with ESBL-producing *E. coli*, even in the organic productive chain. This can be explained by the limited number of breeders' suppliers, which can facilitate ESBL-producing *E. coli* dissemination, even in low-antibiotics user countries. Assuming this, the same strategies adopted in swine farming (biosecurity, global farm hygiene, low antibiotics usage), should be used in broiler productive chains, to try to tackle these AMR microorganisms, and stop the transmission to humans working at strict contact with animal reservoirs.

1.5. Virulence factors in *Escherichia coli*

Escherichia coli is a Gram-negative bacterium that is considered commensal of humans and warm-blooded animals' gastro-intestinal tract. However, the same microorganism can be an opportunistic pathogen when is equipped with a set of specific virulence factors [85]. These factors are generally located on mobile genetic elements (MGE), as the ESBL-associated genes, so they can be transmitted at the same time among different strains [85]. Certain virulence factors allow the bacteria to resist in difficult environments (e.g. urine) and to cause different types of affections, from gastroenteritis to urinary tract infections in presence of favorable host conditions. Every year, millions of people suffer from the different *E. coli*- related maladies worldwide [86,87]. The pathogenic *E. coli* strains can cause clinical symptoms in animals too; nevertheless, in some cases, it can colonize animals without any sign. In this carrier status, *E. coli* acts as a simple commensal microorganism that inhabits the animal gut and can be spread to humans, other animals and nearby environment.

Pathogenic strains can be generally categorized as either diarrhoeagenic intestinal *E. coli* or extraintestinal *E. coli* (ExPEC). Till now different pathotypes — enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC; including *Shigella*), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) have been fully distinguished as diarrhoeagenic bacteria, while other pathotypes — uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), sepsis-associated *E. coli* (SEPEC), and avian pathogenic *E. coli* (APEC), which cause respiratory infection and sepsis in poultry, are the most common ExPEC isolates [3]. Considering the transmission route, it is noteworthy that *E. coli* can reach human hosts from diverse sources that include the food chain. It has been observed that certain human and animal ExPEC strains are not so different, and they can possibly cause urinary tract infections (UTI) after urethral colonization. This type of infections is now considered as foodborne UTI, or FUTI [88].

To verify the potential pathogenicity of a bacterial strain it is necessary to uncover the presence of particular associated virulence factors, which can be classified in 6 categories:

I. Adherence and colonization factors,
II. Type I to VI secretion systems
III. Immune evasion factors
IV. Toxins
V. Siderophores for iron intake
VI. Invasion genes [89]

It is possible to classify *E. coli* pathotype not only depending on the particular group of virulence factors, but also depending on the resulting clinical symptoms [90]. However, till now there is no consensus on the set of virulence factors to classify an *E. coli* as ExPEC or to differentiate ExPEC subtypes [86]. Some particular and specific groups of virulence genes have been recognized in UPEC and APEC; however, no set of distinct virulence factors have been identified for UPEC, that can allow to differentiate this pathotype from APEC [91]. On the other hand, there are some studies that have recognized pathogenic as well as commensal strains from livestock as putative UPEC strains in humans [92,93,94].

The ExPEC pathotype can inhabit various and highly complex ecological niches like urinary tract and central nervous system (meninges). To be able to colonise these districts, these *E. coli* strains have "weapons" that are represented by highly specialized virulence factors. Typical ExPEC virulence factors comprehend different adhesins (type I and P fimbriae), iron acquisition and utilization systems (aerobactin and salmochelin siderophores), protectins (structural components of the bacterial outer membrane), toxins (e.g. hemolysin), and biofilm forming factor (antigen 43). All these factors allow ExPEC to colonise and invade host organs, and to tackle and evade host immune defence [95]. Johnson et al. classified ExPEC as *E. coli* strains containing two or more of the following virulence markers: *papA/papC* (P fimbriae), *sfa/foc* (S and F1C fimbriae), *afa/dra* (Dr-binding adhesins), *kpsMTII* (group 2 capsules), and *iutA* (aerobactin siderophore system) [96]. Another virulence factor *hlyD*, coding for a cytolytic protein toxin, is generally considered ExPEC specific [97]. As said before, these virulence genes are often located on mobile genetic elements like pathogenicity islands (PAIs) and plasmids, permitting to be transmitted through horizontal gene transfer (HGT) across different *E. coli* strains [96]. Virulence determinants have been detected in *E. coli* strains not only from patients with UTI or community-dwelling humans, but also from Danish and imported pork, and from swine samples (*kpsM* II, *iutA*, *papA*, *papC*, *hlyD*, *sfaS*, *focG*); even in Danish and imported broiler meat and broiler samples *kpsM* II, *iutA*, *papA*, and *papC* were identified [97]. Other virulence genes have been identified and considered specific in human UPEC-related UTI. These are *chuA* (heme uptake), *fyuA* (yersiniabactin siderophore system), *vat* (vacuolating toxin), and *yfcV* (adhesin) [98]. Additional factors that are involved in the iron metabolism (*iroN, iucC* and *sitA*), appeared to be typical of the extraintestinal UPEC pathotype, but they have been identified in APEC strains too [87,99]. *traT, fyuA, chuA, PAI, yfcv* and *vat* have been identified in ESBL-producing *E. coli* sampled from fish, livestock, surface waters, vegetables and poultry meat [85]. *fimH* is also a characteristic determinant of UPEC, but can be found in other extra-intestinal pathotypes like SEPEC, NMEC and avian pathogenic *E. coli* (APEC). *fimH* codes for a type 1 fimbria that is involved in colonization and biofilm formation, particularly in cystitis and meningitis, attaching to the receptor D-Mannose [87]. *fumC* encoding oxidative fumarase enzyme FumC, is also necessary for the colonization of the iron-limited environment of the urinary tract in UPEC pathogenicity [100]. The genes *iss*, *ompT* and *traT* code for virulence factors that are implicated in extraintestinal pathogenicity too, as they increase serum survival, blocking complement activity (*iss* and *traT*) and allow urine survival and resistance to protamine (*ompT*) [87]. The *lpfA* gene has been recovered from human and swine EPEC strains but it is also commonly present in extraintestinal pathogenic or commensal *E. coli* [101,102]. The gene *hlyF* that codes for an hemolysin, has been previously found in UPEC and it is nowadays considered a virulence genetic marker of ExPEC as well as *cvaC* (microcin C) and *etsC* (putative type I secretion outer membrane protein) [102].

Virulence genetic determinants, mainly identified in diarrhoeagenic intestinal *E. coli,* which can be ESBL-producing, are:

-*estA* and *estB*, coding for heat-stable enterotoxins STI and STII, and LT type I and II coding for heat-labile toxins, that are produced by ETEC, causing diarrhoea in humans and animals [93,103];

-*eae* (intimin encoding gene) that can be found in EHEC and EPEC pathotypes, and was identified in EHEC strains of cattle origin [104,105];

-*stx1* and *stx2,* responsible for shiga-like toxins (SLT) expression, main virulence factors of EHEC, that can cause haemolytic-uremic syndrome and, in some cases, death; animal reservoir of this dangerous stx-producing pathotype are cattle, in which the strain can be ESBL-positive [106,107];

-*astA,* coding for enteroaggregative heat-stable toxin, causing secretory diarrhoea, was identified in commensal ESBL-producing *E. coli* of swine origin [108];

-*aagR* (transcriptional activator of aggregative adherence fimbria I) [85,104];

-*pic* (serine protease/mucinase) and *air* (enteroaggregative immunoglobulin repeat protein), commonly present in EAEC strains, that were identified in livestock ESBL-producing *E. coli* [93];

- *fae, fan* and *f17,* coding for ETEC-associated fimbriae F4, F5 and F17, which were also detected in livestock ESBL-producing *E. coli*, as well as the ETEC-related genes *aidA (*AIDA-I-like adhesion protein), *iha* (bifunctional enterobactin receptor adhesin protein) and *tia* (toxigenic invasion protein) [93,108].

The majority of these virulence determinants can move through MGEs across different pathotypes of *E. coli* and need to be molecularly identified in order to understand the potential pathogenicity of the analysed strains. Furthermore, using molecular analysis, like WGS, it will be easier to find out if some virulence markers move together with antimicrobial resistance genes, posing a double threat to the putative host, that can be colonised by *E. coli*. In Chapter 7, WGS analysis is used to characterise a group of ESBL-producing *E. coli*, sampled from swine hosts and farm environment.

Chapter 2- Review of Methicillin-resistant staphylococci (MRS)

2.1. General characteristics of methicillin-resistant staphylococci (MRS)

Staphylococci are Gram-positive bacteria divided into coagulase-positive, like *Staphylococcus aureus* and *Staphylococcus intermedius,* and coagulase-negative staphylococci (CoNS), like *Staphylococcus haemolyticus*. CoNS are considered less virulent than coagulase-positive, and can be detected as commensal on human and animals' skin and environment [109]. Both coagulase positive and negative can carry the *mecA* gene, which is responsible for the methicillin-resistant phenotype. The *mecA* gene encodes for an additional penicillin-binding protein (PBP), called PBP2a (or PBP2'), which, as modified transpeptidase, takes part to the cell wall building process when beta-lactam antibiotics are present. This modified protein causes an overall β-lactam resistance (to penicillins, cephalosporins, and carbapenems), apart from the fifth- generation cephalosporins with anti-MRSA activity, which are ceftobiprole and ceftaroline [109].

The most famous methicillin-resistant *Staphylococcus* spp. (MRS) is methicillin-resistant *Staphylococcus aureus* (MRSA). *Staphylococcus aureus* is the most studied of the staphylococcal category as human and animal opportunistic pathogen, and it can cause different morbidities ranging from mild to severe symptoms. It can be generally found as commensal on skin and respiratory tract in both animals and humans [109]. In 1961 MRSA was first isolated [110]. After this finding, MRSA strains and lineages were detected worldwide. They caused numerous epidemics, especially in hospital environment [111]. The MRSA associated to healthcare settings was defined HA-MRSA. However, MRSA infections are also linked to community, and so, it was created another acronym CA-MRSA [112]. This MRSA can be differentiated from HA-MRSA when the MRSA infection is diagnosed in the outpatient setting or within 48 hours of hospitalization. Other variants need also to be considered to define a CA-MRSA infection; in fact, these HA-MRSA associated risk factors need to be absent: hemodialysis, surgery, residence in a long-term care facility, or hospitalisation during the previous year, presence of an indwelling catheter or a percutaneous device at the time of culture [113]. Furthermore, the *Staphylococcal cassette chromosome mec* (SCC*mec*) type is useful to distinguish between CA-MRSA and HA-MRSA. The SCC*mec* type present in CA-MRSA are generally IV or V [114], but there are some non-typeable SCC*mec* cassette or SCC*mec*IV that were detected in HA-MRSA lineages (ST22-IV and ST5-IV), so this method is not always exact [115]. The MRSA sequence type 398 (ST398) has been recognized worldwide as the most diffuse in farm animals and it has been found among pigs, bovines, and poultry. However, there are other MRSA lineages (e.g., ST1, ST5, ST9, ST97, ST130, and ST433) that are associated to livestock [116]. Lineages that are linked to farm animals are called "livestock-associated MRSA" (LA-MRSA). In Italy the second most frequently detected LA-MRSA is the clonal complex 97 (CC97). Apart from swine finishing farms, this MRSA was recovered in clinical bovine samples (mastitis) and from cattle bulk tank milk [117].

In the meantime, a growing proportion of CoNS is becoming resistant to methicillin (MRCoNS), with *S. epidermidis* being the most frequent isolate in human medicine [109]. After the penicillin introduction in human medicine, around 10% of *S. epidermidis* isolates resulted resistant to methicillin (called as "celbenin" or "staphcillin") [118]. Nowadays, most of the clinical CoNS strains carry the SCC*mec* cassette.

Till now other *mec* types have been recognized. The strain *Macrococcus caseolyticus*, identified from chicken meat, is able to code a *mec* homolog, that is called *mecB* [119]. In 2011, a new *mec* type (*mecC)*, similar to *mecA* and located on a *SCCmec* type XI cassette was first reported in MRSA strains of both human and bovine source. Afterwards, it was detected from diverse animals, like companion animals and wild small mammals [120,121,122,123,124]. The *mecC* gene is similar to *mecA* for around 70% of the nucleotide sequence and for the 63% of the aminoacidic structure. Through *in silico* analysis, zoonotic transmission of *mecC* MRSA have been reported in Denmark in two human

patients, that lived on cows' and sheep farms respectively. The same MRSA strains were present in humans and livestock, since they shared the MLST types (ST130), *spa* types (t843), fingerprints (MLVA and PFGE), and antimicrobial resistance patterns [125]. Interestingly, in Italy in 2012 a *mecC*-positive MRSA was detected from bulk tank milk, originated from a sheep farm of Lazio region [126].

2.2. Methicillin-resistant *Staphylococcus aureus* (MRSA) in swine farming

LA-MRSA was first detected in 2005 in the Dutch swine farming chain, and then again in 2007, with growing reports until nowadays [127,128,129,130]. If LA-MRSA from livestock animals is typed with molecular analysis (MLST), the most common among Western European farm animals is ST398 [131]. It is also spread in the American swine sector, although there can be found the type ST9, the most common in Asian countries. ST398 was firstly detected in farm pigs, and then isolated in companion animals, other farms animals, animal food products and humans [132,133,134,135]. This LA-MRSA comprehends different spa-type and can be separated by the clonal complex (CC) 398 human clades for the lack of some virulence determinants like PVL (Panton Valentin leucocidin), encoded by the genes *lukS-PV* and *lukF-PV* and staphylococcal complement inhibitor (*scn*), that are generally more easily found in human adapted strains [136]. Another sequence type, the ST1, has been recognised as one of the more frequently recovered LA-MRSA from pig farms in Italy and in some European countries, like recently Norway [137,138,139]. Recently, it has been demonstrated that CC-1 has a good adaptation, and a great stability on nasal mucosa and skin of experimentally infected piglets [140]. Other less frequent genotypes, specifically ST-97 and ST-9, have been found in a survey in Southern Italy farms [139]. A new MRSA clone belonging to CC30, carrying *lukM* and *lukF-P83* genes, was found in animals, suffering ill-thrift, in farms from Northern Ireland [141]. The latter two genes are considered animal pathogenic markers and have been found in cases of bovine mastitis and exudative dermatitis in squirrels.

Generally, pigs are LA-MRSA carriers and reservoirs without no apparent symptoms [142]. However, in some cases this type of MRSA can become pathogenic and cause lesions in different organs and tissues, particularly joints, and secondary lungs, especially in very young pigs (suckling and weaned) [143].

Pigs can be more frequently temporary MRSA carriers. More specifically, sows can also be positive both for MSSA and MRSA simultaneously [144,145]. They can thus represent a relevant source of contagion for farmers; in fact, a variable percentage (24-86%) of pig farmers has been found CC398 positive in a survey across Europe [146]. In Italy, specifically in Apulia and Basilicata regions, a survey found that 19.2% of 130 tested farmers from 79 farms were MRSA positive, and the risk was higher in fattening farms, where 80% of farmers (8 out of 10) came out to be MRSA colonised [139]. However, from the study of Locatelli et al. it emerged that pigs can be a threat also for other animals, like cattle. In cows, LA-MRSA can cause subclinical and clinical mastitis. The Italian Authors used bulk tank milk to detect the presence of LA-MRSA in bovine herds in high-density pig farms territory, including the provinces of Brescia, Bergamo and Mantova. Results highlighted a significantly higher number of fattening pig herds in the 3 km area surrounding MRSA positive bovine herds. For this reason, The Authors inferred environmental dust as a passive vector of MRSA, especially through wind that can cause colonisation of the nearby dairy farms [142].

The study of Wardyn et al. in a densely populated swine USA state, Iowa, found a more probable carrier status in farmers with livestock exposure (direct contact), especially pigs, for MRSA, TRSA (tetracycline-resistant *S. aureus)* and LA-SA (livestock-associated *S. aureus*), with respect to the workers without exposure. The main risk factor was working in direct contact with live pigs, with a

higher prevalence with more than 10 hours of direct contact per week. Furthermore, they detected some human-related *S. aureus* strains with markers of livestock connection, like the absence of *scn* gene, and/or the tetracycline resistance gene (*tet*). Another important fact was that they detected a higher prevalence of LA-SA in the household members of pig farmers [147]. This study also reported human cases (all previously exposed to farm animals) due to SSTI (skin and soft tissue infections) from ST398 strain [147].

LA-MRSA is not a good human coloniser, and a direct or indirect contact with positive farm animals (live or dead) is needed to be at risk of contagion [148]. Occupational contact is very important, although in some studies, not all the LA-MRSA colonised persons were farm workers or lived in a farm. In fact, out of 55 persons positive for ST398 *S. aureus*, 38% claimed to not have any previous contact with livestock animals [149]. So, there are other ways of contagion for humans. Dust and manure from contaminated farms can be suitable vehicles of contamination of the surrounding areas around the farms. Also contact with a known MRSA carrier can be a risk factor; however, for some of the LA-MRSA positive cases, an occupational cause (for example live in an area with a lot of farms around) could not explain the infection status. Around 50% of the MRSA colonised patients with no occupational exposure, reported that they had been hospitalized during past six months, and this can be considered a risk factor in the occurrence of LA-MRSA colonisation [149]. High density of piggeries around a specific area is considered a probable reason for the higher occurrence of LA-MRSA infections in certain hospitals and may be the cause of the uneven distribution of these human infections in certain regions [150]. However, in Lombardy, the Italian Region with the highest density of swine farms, counting 1000 pigs per km^2, LA-MRSA have been isolated in people, but symptomatic infections were observed infrequently [151].

In a study of Angen et al. the MRSA carriage status was measured in volunteers after visiting positive farms for a short time (1 hour). From the survey, the initial colonisation prevalence resulted 94%; but this percentage declined considerably after just 2 hours. The almost overall clearness of all the participants was obtained after 48 hours from the visit to the pig farm, only one visitor still resulted MRSA positive after 7 days and became negative after 14 days [152]. The presence of positive samples was positively related to high MRSA concentration in air samples. Thence, masks should be considered for long time exposed farmers, that are at risk of being carrier of LA-MRSA. The relevance of dust in transmission of LA-MRSA was evidenced also by the analysis by Feld et al., that highlighted a medium survival time of 5 days for LA-MRSA in highly contaminated piggery dust [153].

Looking at the phases of the swine productive cycle, data from the analysis of Schmithausen et al., showed that early finishing pigs are the most colonised animals by MRSA [66]. Finishing herds were one of the most MRSA colonised even in the study of Broens et al. (2011) [154]. The same productive stage was considered at higher risk of MRSA colonization with respect to breeding farms, for Parisi et al. too [139]; in this Italian study 100% of all the fattening farms were MRSA infected, while in the reproductive sectors, 60% were MRSA positive [139].

The integration of the diet with zinc in post weaning pigs, as growth promoter or in the treatment of diarrhoea, could be the cause of co-selection of the *mecA* gene. In fact, data from a genomic analysis on 100 MRSA (ST398) isolates, from humans and animals (Germany and Austria), evidenced that the most frequent *SCCmec* element in CC398-MRSA strains was *SCC* [*mecVT +czrC*]. *czrC* is the gene responsible for the transcription of the heavy metal translocating P-type ATPase, leading to cadmium and zinc resistance. In this way, zinc usage can be a promoter of methicillin-resistance, and β-lactam antibiotics can select for resistance to this heavy metal, because both resistance genes are on the same mobile element [155].

Another possible driver of antibiotic resistance in the swine sector is animal transportation among farms. Sieber et al. unraveld a higher prevalence of MRSA isolates in farms where animals originated from positive farms with a 4-fold-more elevated incidence rate than the farms taking their pigs from negative farms. On the base of these results, animal movement needs to be considered in the evaluation of the transmission pathways of MRSA in the swine sector [156]. Te Authors also found cadmium/zinc resistance *czrC* genes in the predominant MRSA lineage, with other resistance determinants for aminoglycosides, lincosamides, quinolones (*gyrA*) and tetracyclines (*tetK* and *tetM*).

Non- intensive farms have been shown to have lower MRSA prevalence. In Italy, in the southern region of Calabria, only 9.1% of animals were MRSA positive among autochthonous black (*Calabrese*) breed pigs present in 11 farms [157]. However, higher MRSA positivity can occur in antibiotic-free swine herds [158]; so, other determinants (apart from the continuous usage of antibiotics, zinc integrative diet and animals' movement among diverse farms) need to be identified to understand the successful MRSA diffusion in swine farming worldwide.

2.3. Methicillin-resistant *Staphylococcus aureus* (MRSA) in poultry farming

There are still scarce data on the presence of MRSA in poultry farms, and more studies are necessary to uncover the route of transmission and prevalence at animals and farm environment level.
In Germany MRSA levels were 50-54% in broiler and 62-77% in turkey farms. Environmental samples taken from the MRSA-positive poultry farms were positive in almost all cases [159]. Few data are also available on the factors linked to the dissemination and permanence of MRSA in poultry farming, and it is not clear if domestic avian species, particularly chickens, can be MRSA reservoirs [160].
A more recent study (2019) from German broiler farms (15 farms from 4 German counties), detected 56 MRSA-positive isolates from broiler flocks or farm environment [161]. The majority of them were CC398, in line with previous poultry-associated MRSA lineages found in Europe [162].
Transmission of poultry-related LA-MRSA to humans has been detected. Infected humans were slaughterhouse workers and carried LA-MRSA with clonal lineages (CC398 and CC9) identical to the broilers from the same working place [163]. This study supports the fact that working at strict contact with MRSA colonised broilers, can represent a risk factor to acquire these resistant bacteria. The same risk factor was identified for MRSA positive workers and inhabitants of Dutch broiler farms. People that work or live in these farms are more frequently colonised with LA-MRSA compared to the rest of the population (5.5% vs. <0.1%) [164].
LA-MRSA is able to survive along the production chain in turkey slaughterhouse. Indeed, high

prevalence (32%) of positive samples was found at retail meat in the study of Vossenkuhl et al. The majority of the strains belonged to the lineage CC398 [165]. The fact that there is a high prevalence of MRSA at the end of the productive chain can suggest that transmission of MRSA can be due to cross-contamination during slaughterhouse activities and meat processing. The hands of the workers can represent a vehicle of MRSA transmission too.

The LA-MRSA detected in poultry farming are often multidrug resistant [165,166]. Resistance to tetracycline, clindamycin, erythromycin, trimethoprim, quinupristin/dalfopristin and tiamulin is often found [165]. In the study of Kittler et al. the most common antibiotic resistance detected in CC9 and CC398 strains was against tetracyclines, macrolides, lincosamides, streptogramin B and trimethoprim antibiotics [166].

2.4. Methicillin-resistant coagulase-negative staphylococci (MRCoNS) in swine farming

Coagulase-negative staphylococci (CoNS) are generally deemed as commensal non-pathogenic bacteria, belonging to the skin microbiota of different animals [167,168]. However, they can cause severe conditions, such as exudative epidermitis in piglets [169] and mastitis, endocarditis and osteomyelitis in other farm animals [170,171,172]. Even in human medicine, they have acquired more attention, due to the growing number of hospital-related human infections [173] and the increasing numbers of resistance associated genes. As coagulase-positive staphylococci (CoPS), CoNS can have a methicillin-resistant phenotype, often due to the presence of the *mecA* gene. The evolution of this gene can be correlated to the native *mecA1* present in the CoNS belonging to the *S. sciuri* group, from which it derived after various recombination and mutation events [174,175]. Like the *mecA* gene coded by *S. aureus*, in MRCoNS, *mecA* is located on a mobile genetic element (MGE): the staphylococcal cassette chromosome *mec* (*SCCmec*) [176,177]. In swine farming, study on the *SCCmec* cassette have identified different types (V, IVc, IVa, III and VI) and non-typable cassettes; also, in some cases, diverse staphylococcal species, namely *S. aureus*, *S. epidermidis*, and *S. haemolyticus*, owned the same cassette (*SCCmec* type V), supporting the hypothesis that this MGE can move across various staphylococci carried by pigs [177]. This finding is of concern because implies that CoNS can harbour *mecA* in different *SCCmec* elements, working as reservoirs for *S. aureus* in the swine nasal environment [177].
The first data on MRCoNS in livestock animals is from Japanese healthy chickens in 1996 [178]. Afterwards, few investigations have been carried out on this AMR bacterial group in farm animals. In Belgian pigs, methicillin-resistant *Staphylococcus sciuri,* one of the main CoNS isolated in swine nares [167], was reported in 6.5% of tested animals from sampled farms [179]. In Switzerland, a prevalence of 36.3% of MRCoNS was found at the slaughterhouse [167]. The resistant staphylococcal species were *S. sciuri* (50%) and *Staphylococcus fleurettii* (50%) [167]. A more variegated MRCoNS group (*Staphylococcus cohnii*, *S. haemolyticus*, *S. epidermidis, S. sciuri*, *Staphylococcus pasteuri*, *Staphylococcus equorum*, *Staphylococcus saprophyticus*, *Staphylococcus lentus*, and *S. fleurettii*) was isolated from swine nose and farm dust samples, along with *S. aureus*, in the study of

Tulinski et al. in Dutch farms [177]. Even at farm environment level, *S. sciuri* is the predominant species among detected MRCoNS; indeed, in the study of Schoenfelder et al., 105 out of 344 collected samples from farm manure and dust were methicillin-resistant *S. sciuri* harbouring *mecA* gene [180]. Moreover, from the same farms, MRSA was also recovered. Thence, this study sustains the hypothesis that MRCoNS are potential reservoir of *mecA* for *S. aureus*, considering environment another valid source of these resistant bacteria, and a putative place were horizontal gene transmission of *mecA* can happen across staphylococci [180]. In the same study, 2 *cfr* (multi-resistance gene, mediating oxazolidinone resistance) positive CoNS strains, one *S. sciuri* and one *S. lentus*, and 2 daptomycin-resistant *S. sciuri* were detected too. This finding is alarming as indicates that resistance to last-resort human antibiotics, like oxazolidinones and lipopeptides, can be recovered from swine farm environment. Moreover, it confirms the fact that resistant to these antibiotics can be found in CoNS, that, in human medicine and veterinary field, are becoming more often resistant to last-resort antibiotics with respect to *S. aureus* [181,182].

As for MRSA [147], MRCoNS of swine origin can be transmitted to humans, especially if daily exposed in swine farming activities [183]. The transmission of swine MRCoNS from animals to people, involved in farm work practices, was investigated and it was observed that the human MRCoNS prevalence increased with the frequency and duration of the direct occupational swine contact [183].

2.5. Methicillin-resistant coagulase-negative staphylococci (MRCoNS) in poultry farming

Data on the presence of methicillin-resistant coagulase-negative staphylococci (MRCoNS) in poultry farming are scarce. In recent studies, Authors focused on the methicillin-resistant status of a particular opportunistic staphylococcal species, that is often found in livestock animals and in environment [179,184]. They detected a prevalence of methicillin-resistant *S. sciuri* that ranged from 12.5% to 30% in broilers of Belgian farms [179,184]. A higher prevalence of MRCoNS was recovered (48.6%) in chickens from Swiss farms [167]. This percentage is not so different from the prevalence of MRSA found in broiler farms in Germany (~50%) [159] and highlights that MRCoNS can colonise broilers farms. The general farm practice of oral administration of amoxicillin and tetracyclines, could be linked to the increase of these antibiotic-resistant bacteria, as it was already proved for MRSA [116]. MRCoNS have been recovered in farm environment as well, and this was documented in a recent study, where these bacteria were found in 36.3% of bioaerosol samples taken from hen houses located in China [185]. Recovering this high MRCoNS prevalence in farm environment, strengthens the hypothesis that these bacteria can colonise avian species and farmers that are indirectly exposed through environment and farm aerosol. In the last years, CoNS have gained more interest in human and veterinary medicine for their multidrug resistant status and because they acquire easily oxazolidinones-associated resistance genes [186,187]. However, they are not important only for the antibiotic-resistance profile, but also for the clinical conditions that can cause after humans and animals' infections [173,188]. Indeed, Pyzik et al. observed an enterotoxin genetic marker in 3 resistant staphylococcal species. The gene *see*, which codes for the enterotoxin E, was identified in *S. hominis* (n=2), while the gene coding for the enterotoxin B *(seb*) was recovered only in one strain of *S. epidermidis* [189]. Enterotoxins are usually produced by CoPS, like *S. aureus*; therefore, this study indicates that even CoNS can encode these toxins, posing a threat for human health. The enterotoxin B is classically associated with human food poisoning, and the finding of the

genetic marker in poultry-associated isolate can represent a risk for farmers, that work daily in direct contact with these animals [189]. Furthermore, MRCoNS were identified in USA retail meat, not only of poultry origin but from beef and turkey too [190]. The prevalence in retail poultry meat was 7.9%, and the species were mainly *S. fleuretti* and *S. sciuri* with one *Staphylococcus vitulinus* [190]. *Staphylococcus sciuri* and *S. fleuretti* were the MRCoNS most often identified in bulk tank milk and minced meat from a European study [167].

The presence of MRCoNS in live broiler, farm environment and retail poultry meat, can be considered a health risk not only for farm and slaughterhouse workers, but also for the final consumers. In fact, these bacteria are well-established reservoir of the *mecA* gene, which can be horizontally transmitted to the more pathogenic *S. aureus* [191]. The risk of human exposure to these AMR bacteria is not only related to the *mecA* gene, but also to the virulence genes typical of *S. aureus*, that can be present in some strains of MRCoNS isolated in chickens [189]. Moreover, in the last decades, staphylococci have gained relevance for owning the multi-resistance gene *cfr,* responsible for the resistance to five antibiotic classes, among which the oxazolidinone linezolid, last-resort human antibiotic. Till now, the *cfr* gene has been occasionally detected in livestock worldwide, even in CoNS of poultry and turkey origin [192,193].

Chapter 3 - Review of farm Biosecurity measures, Animal Welfare and Antimicrobial usage (AMU)

3.1. The "ClassyFarm" Italian system to categorise farms based on risk level

Animal welfare, antimicrobial usage (AMU), use of slaughterhouse as an epidemiological tool and biosecurity with associated sanitary parameters (e.g. average daily gain, skin lesions and mortality) are all interconnected in livestock farms [194,195,196,197]; for these reasons, it is necessary to use an holistic perspective that include all these factors, when an evaluation of the global farm management is required [198]. Antibiotic resistance is nowadays considered a prominent issue even at farm level, demanding rapid and specific measures with interventions to counteract this growing problem [199,200]. In this context, the Italian Ministry of Health (Direzione della sanità animale e dei farmaci veterinari) worked in the last years to innovate and ameliorate the animal health system. The main objective of this innovative system, called "ClassyFarm", is the categorization of livestock farms based on the level of risk associated to public health issues [201].
ClassyFarm can be considered an integrated monitoring approach that is new in Europe. Furthermore, it allows to have a more in-depth collaboration among farmers and local veterinary sanitary authority to enhance the overall farm biosecurity level, animal welfare and the quality of the final byproducts.
This system can be consulted by official veterinarians, farm veterinarians and farmers to monitor, analyse and address the farm interventions that are necessary, in agreement with the current European legislation on Animal Health Law and the Official controls.
The system was funded by the Ministry of Health, while the Istituto Zooprofilattico Sperimentale di Lombardia ed Emilia Romagna, with the collaboration of the University of Parma, were in charge to build the overall system.
ClassyFarm is online on the National official veterinary site (www.vetinfo.it), that allows to visualise, collect and elaborate the data associated to the following parameters:

- biosecurity;
- animal welfare;
- sanitary and productive parameters;
- animal nutrition;
- antimicrobial consumption;
- lesions observed at the slaughterhouse

Checklists to collect data on welfare and biosecurity are present on the dedicated ClassyFarm website [201] for the food-producing animals: beef cattle, dairy cattle, dairy buffalos, calves (white meat), dairy goats, dairy sheep, weaning and finishing pigs, breeder pigs, broilers, laying hens and turkeys. A specific checklist on welfare associated to tail-docking was developed for piglets, because the presence of lesions due to the negative behaviour of tail-biting can be considered alone as an overall swine welfare evaluation [202]. With this checklist, farmers and farm veterinarians will understand the specific critical points that can cause the risk of tail-biting in the weaning and finishing sectors.

Biosecurity in veterinary medicine consists of all measures that are applied to limit, to the minimum, the risk of introduction (external biosecurity) and dissemination (internal biosecurity) of infectious agents on farm. The overall aim is to keep animals safe and healthy. Implementing all the biosecurity measures in the daily routine farm management, is paramount to keep away endemic and epidemic diseases from the farm [195,198,203]. Keeping healthy and strong animals on farm decreases the risk for them to acquire infections and consequently becoming sick. This will imply ultimately that

less antibiotic treatments are necessary for animals [204]. Reduced and prudent use of antibiotics is one of the main final aims of the ClassyFarm system in livestock production, to reduce the risk of the onset of new and old antibiotic resistance events.

Looking specifically to the swine productive chain, biosecurity data are collected on farm using the checklist Biocheck.UGent® pig ver. 2.1. This survey was previously developed by the unit for Veterinary Epidemiology from the Faculty of Veterinary Medicine of the University of Ghent [205]. The total biosecurity level on a farm is considered as the average of the external and internal biosecurity scores, which can range from 0 to 100. The maximum score indicates the implementation of all biosecurity measures, thanks to the farmers' compliance to high biosecurity standards. The checklist includes 109 questions divided into six subcategories for external and six subcategories for internal biosecurity (see Table 1).

	External biosecurity	**Internal biosecurity**
Checklist subcategories	• purchase of animals and semen; • transport of animals and removal of manure and dead animals; • feed, water and equipment supply; • personnel and visitors; • vermin and bird control; • environment and region;	• disease management; • farrowing and suckling period; • nursery unit; • fattening unit; • biosecurity measures between compartments and the use of equipment; • cleaning and disinfection;

Table 1: Biocheck.UGent® pig ver. 2.1 checklist subcategories for analysis of external and internal biosecurity.

In this risk-based survey, focus is dedicated on new animals' purchase (breeders or piglets), with particular attention on animal supplier, sanitary level of the animal supplier farm, frequency of animal acquisition and animal quarantine with the presence or not of a hygiene lock. All these are recognised key factors that are able to leverage the introduction of external infectious agents on farm, with negative effects on animal productive indexes [195,198,204]. Transport of animals, removal of carcasses and manure management are important points too, and they are located in *ad-hoc* section of the checklist. In the same part, attention is paid to the drivers that are responsible of pigs' transport among farms or from farm to slaughterhouse. A cleaned transport vehicle, dedicated area for loading animals and providing farm-specific clothing and shoes to the driver and visitors are recognised critical points as highlighted by previous studies [203,206,207,208]. Management of dead animals with a specific refrigerated carcass storage room that needs to be physically- separated from the clean farm area is another important point of the checklist. Critical points of the external

biosecurity are feed and water supply too. Feed should be provided with farm internal pipelines from the dirty area of the farm, to hinder the truck to reach the clean area to deliver it. For water, high hygienic requirements need to be checked periodically through microbiological tests, in order to prevent contamination at different sites from the tank or well to the final nipples where animals drink [207]. Furthermore, feed and water supplies should be in completely closed systems to avoid dust, pests or wild birds' contamination. Vaccination is also addressed in the Biocheck.UGent® survey, as it is notorious that it can be considered not only a way to tackle infectious diseases, but also a welfare-related instrument that allows animals to live free of diseases and with a good immune system. Previous studies highlighted that vaccination is associated to a decreased mortality risk and limited spread of diseases, with positive outcomes on the overall animal life and farm productivity [207,209]. On farm animal management and movements in the different productive stages (farrowing and suckling period, nursery unit, and finishing unit) are addressed in the last parts of the survey. Cross-fostering (transfer) of suckling pigs among different sows is a common practice in swine farming system, however it is associated to transmission of infectious diseases among diverse groups of piglets and increased mortality, so its application should be limited [210,211]. Finally, a cleaning and disinfection section is considered in the survey. Indeed, it is well known three separate steps in the routine cleaning process of the farm facilities are necessary, in order to limit infectious diseases at pig farm and avoid pathogen entry: meticulous cleaning, correct disinfection and sufficient time to dry out farm surfaces [203]. Although Biocheck.UGent® is a long survey to fill in, the checklist-derived scores (for global, external and internal biosecurity) will allow to uncover positive and negative aspects present on farm at that time, recognizing interventions that are necessary to improve biosecurity, but that are also linked to animal welfare amelioration and reduction of AMU.

A practical application of the ClassyFarm schematic protocol used in the evaluation of swine lesions at the slaughterhouse is well described in the article of Ghidini et al. [212]. In this study, the Authors tried to correlate ante-mortem (AMI) conditions in heavy pigs (like skin and ear lesions, lameness and umbilical hernia) with anatomo-pathological findings at the post-mortem inspection (PMI). F or example, they found that manure on more than 30% of the body during AMI was associated with kidney and pulmonary lesions at PMI. The most often recovered swine conditions at the AMI were dirt on more than 30% of the body (37.1), skin lesions (9%), ear lesions (3.3%) and lameness (0.3%). During PMI the most frequently observed anatomo-pathological findings were pleurisy (17.2%), pericarditis (7.8%), pneumonia (8.2%) and skin wounds (6%) [212]. All these data can be used to classify farms, considering also that lesions and conditions detected at the slaughterhouse can be ascribed to welfare indicators (e.g. tail-biting lesions and lameness) [202,213]. Furthermore, this is an excellent example of how slaughterhouse can be used as epidemiological observatory to assess overall animal management and welfare (tail-biting lesions [202]), for the surveillance of the principal swine diseases and to evaluate the effectiveness of the control measures taken to limit them [196].

The ClassyFarm tool has the final aim to strengthen the measures for the prevention of animal diseases and to tackle antimicrobial resistance. To do so, a Defined Daily Dose (DDD)-based metric, DDDvet, was developed in agreement with previous indications from European Medicine Agency (EMA) [214], to calculate the exact quantity of active ingredient used to treat a specific animal stage (e.g. finishing pigs or adult dairy cattle). The defined daily dose animal for Italy (DDDAit) is the standard metric for the Italian livestock farms. It defines the standard amount of active ingredient, in milligrams, that is administered per kg of live weight per day (mg/kg/d), considering the summary

of the product characteristic (SPC) [215]. It was decided to use this metric, in place of the standard DDDvet, because DDDvet is based on dosages of other European countries, and they are still incomplete. Indeed, for the "long-acting" macrolides like gamithromycin, tildipirosin, tulathromycin used in swine species or the injectable dicloxacillin, authorized in Italy, there are no specific dosages for DDDvet calculation. Furthermore, there is another difference between DDDAit and DDDvet: in fact, for DDDAit every drug has its measurement unit, while for DDDvet is used the average or modal value of the dosages of certain drugs. Thus, with DDDAit the calculation for the specific antibiotic dosage is more precise with respect to the DDDvet. All the DDDAit, determined for each active ingredient, are integrated in the ClassyFarm online portal and they will contribute to the classification of the farm based on the real antimicrobial consumption, the most used antibiotic classes (critical or non-critical) and the consequent risk. Understanding the effective consumption of the diverse antibiotic classes is fundamental to counteract the AMR phenomenon and the possible onset of livestock-associated resistance in humans (e.g. fluoroquinolones resistance in *Campylobacter* and *Salmonella* in humans is positively associated to the consumption of fluoroquinolones in food-producing animals) [216]. Third and fourth- generation cephalosporins, fluoroquinolones, macrolides, and colistin are all critical antibiotic classes (CIAs) based on the WHO classification [217], and their administration in farm animals need to be targeted to the microorganisms that result only susceptible to these drugs, after the response of a validated antimicrobial susceptibility test [218]. Overall, data originated from the DDDAit calculation are more precise with respect to the previously used mass-based indicator, milligrams/population correction unit (mg/PCU), that described the proportion of antibiotic sold and not actually administered. DDDAit are used to benchmark the specific farm with others of the same sector and category, and will be useful to indicate possible interventions on the most critical productive stages where more antibiotics are administered and possible bacterial resistance can occur [219,220].

In this context, the official controls will be more efficient, and, in the meantime, farmers will have all the instruments to enhance the overall farm level of biosecurity.
This new online platform elaborates the data that are included by the official veterinarians during the official controls, data that are already elaborated by the previous in-use systems, data that are inserted voluntary by the operator, and are included by the farm veterinarian, as defined by the Italian Health Ministry decree of the 7th December 2017 [221].
In this process, the farm veterinarian represents the link between farmer and the official control authority. He/she supports the farmer in the decisions that involve strategies to combat infectious diseases and procedures to ameliorate the overall animal welfare and health management, basing on the level of risk detected through the ClassyFarm system in the different topic areas.
All the data that are inserted in the ClassyFarm system will be converted, through scientific coefficients, in one indicator that allows the measurement of the current risk for the specific farm. All these calculation procedures used to categorize farms based on their risk will be publicly available.
Through ClassyFarm it will be possible to improve the overall farm productive chain depending on the best farm practices, and all this will be converted in an economic return for farmer, an increased animal welfare and an improved quality of the final byproducts for consumers.
Moreover, this even risk categorization used for livestock farms will allow official authorities to organize in advance the controls with less costs, and farms, even the smallest facility, will have the opportunity to understand their own "*status*" in comparison with other farms of the same category. In the future, this system could be applied at the European scale [201].

Part II – Experimental studies

Chapter 4 - Occurrence of Methicillin-Resistant Coagulase-Negative Staphylococci (MRCoNS) and Methicillin-Resistant *Staphylococcus aureus* (MRSA) from Pigs and Farm Environment in Northwestern Italy

This chapter was adapted from the article: Bonvegna M, Grego E, Sona B, Stella MC, Nebbia P, Mannelli A, Tomassone L. Occurrence of Methicillin-Resistant Coagulase-Negative Staphylococci (MRCoNS) and Methicillin-Resistant Staphylococcus aureus (MRSA) from Pigs and Farm Environment in Northwestern Italy. *Antibiotics 2021* Jun, 10, 676. doi:10.3390/ antibiotics10060676 [269]

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4.1.Abstract

Swine farming as a source of methicillin-resistant *Staphylococcus aureus* (MRSA) has been well documented. Methicillin-resistant coagulase-negative staphylococci (MRCoNS) have been less studied, but their importance as pathogens is increasing. MRCoNS are indeed considered relevant nosocomial pathogens; identifying putative sources of MRCoNS is thus gaining importance to prevent human health hazards. In the present study, we investigated MRSA and MRCoNS in animals and environment in five pigsties in a high farm-density area of northwestern Italy. Farms were three intensive, one intensive with antibiotic-free finishing, and one organic. We tested nasal swabs from 195 animals and 26 environmental samples from three production phases: post-weaning, finishing and female breeders. Phenotypic tests, including MALDI-TOF MS, were used for the identification of *Staphylococcus* species; PCR and nucleotide sequencing confirmed resistance and bacterial species. MRCoNS were recovered in 64.5% of nasal swabs, in all farms and animal categories, while MRSA was detected only in one post-weaning sample in one farm. The lowest prevalence of MRCoNS was detected in pigs from the organic farm and in the finishing of the antibiotic-free farm. MRCoNS were mainly *Staphylococcus sciuri*, but we also recovered *S. pasteuri*, *S. haemolyticus*, *S. cohnii*, *S. equorum* and *S. xylosus.* Fifteen environmental samples were positive for MRCoNS, which were mainly *S. sciuri*; no MRSA was found in the farms' environment. The analyses of the *mecA* gene and the PBP2-a protein highlighted the same *mecA* fragment in strains of *S. aureus*, *S. sciuri* and *S. haemolyticus.* Our results show the emergence of MRCoNS carrying the *mecA* gene in swine farms. Moreover, they suggest that this gene might be horizontally transferred from MRCoNS to bacterial species more relevant for human health, such as *S. aureus*. Further studies are needed to support the transmission of *mecA* gene among diverse staphylococcal species.

4.2. Introduction

Antimicrobial resistance (AMR) is a global concern to human, animals, and environmental health [222]. Livestock can be reservoir of different antibiotic-resistant bacteria. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been well documented in pigs since its first detection in 2005 in the Netherlands [127] and can be found in the intensive swine farm system across Europe, USA, and Asia [223,224,225,226]. The high MRSA colonization rate among industrially raised pigs poses a threat for farm workers and to people living in high farm-density areas [151,227,229].

Coagulase-negative staphylococci (CoNS) are generally considered as commensal non-pathogenic bacteria, belonging to the skin microbiota of different animals [167,168]. However, they have been recently documented as source of severe conditions, such as exudative epidermitis in piglets [169] and mastitis, endocarditis and osteomyelitis in other farm animals [170,171,172]. Furthermore, they are gradually becoming more relevant from a clinical point of view, as cause of hospital-related human infections [173]. Like the coagulase-positive staphylococci (CoPS), CoNS can have a methicillin-resistant phenotype, often due to the presence of the *mecA* gene. This gene is responsible for the expression of a modified penicillin-binding protein (PBP2-a), which has a low affinity for β-lactam antibiotics, like cephalosporins [174]. Indeed, *mecA* gene is responsible for the most clinically relevant antibiotic resistance mechanism in *S. aureus.* Analysis of the evolution of this gene showed that it probably originated from the native *mecA1* present in *S. sciuri* group, which underwent recombination and mutation events [175,230]. The *mecA* gene is located on a mobile genetic element: the staphylococcal cassette chromosome *mec* (*SCCmec*) in *Staphylococcus* spp., and on a *SCCmec*-like element in other species, such as *Macrococcus caseolyticus* [119].

Methicillin-resistant CoNS (MRCoNS) in livestock were firstly reported in healthy chickens in Japan in 1996 [178]. Since then, few investigations have been focusing on these bacterial species in farm animals. For example, methicillin-resistant *Staphylococcus sciuri* were reported in 6.5% of pigs in 10 Belgian farms [179], and ten different MRCoNS species were isolated, along with *S. aureus*, from nasal swabs and farm dust samples in swine farms in The Netherlands [177].

In Italy, scarce information is available on MRCoNS in livestock, and no study has elucidated their presence in swine farms. The initial aim of our study was to unravel the presence of MRSA in pigs at different production stages and in the farm environment, in an area of intensive pig farming in northwestern Italy (Piedmont region, Cuneo province). Due to the massive presence of MRCoNS, we focused on both bacterial groups, sequencing the *mecA* gene to find similar nucleotide mutations in the different staphylococcal strains that can support horizontal gene transfer events. Biosecurity and general farm management were analysed to evaluate possible impacts on the occurrence of MRCoNS and MRSA.

4.3 Results

4.3.1. Biosecurity and Management of Farms

From the analysis of the questionnaires, we appreciated a homogeneous level of general biosecurity in the five visited farms. All the farms claimed an external animal remount from only one gilts' supplier, with the exception of farm G that had an internal remount. All farmers used to isolate new animals in quarantine. Looking to animal management through the production cycle, it emerged that all farmers used to mix different animal groups through the production cycle, especially piglets from different litters. Only the owner of farm T declared to mix diverse animal groups during the finishing stage. Considering the farm hygiene, all the farmers claimed to adopt a cleaning protocol,

however nobody used dedicated clothes and boots to enter the different animal sectors, apart the workers from farm B. Regarding the storage of carcasses, the dedicated refrigerated room was near the animal sectors in all farms, with the exception of farm T. The animals were kept on slatted or partially slatted floor, with the exception of farm S, where pigs were on straw bedding with a minimum slatted part.

4.3.2. Laboratory Analyses

Overall, 127 MRS (mannitol-fermenting on MSA) were recovered from 195 swine nasal swabs (65.1%, 95%CI: 58.0–71.8; Table 1). MRS were recovered in all farms. MALDI-TOF MS bacterial species identification was confirmed by 16S rDNA sequencing; only one post-weaning environmental sample of farm B, initially considered *S. xylosus*, was identified as *S. cohnii* after sequencing.

Table 1. Methicillin-resistant staphylococci (MRS) isolated from swine nasal swabs and the environment in five swine farms in northern Italy, 2019–2020.

Farm ID	**Farm Type**		**MRS**	***Staphylococcus* Species (*n* Positive)**						
			***n* Positive Samples /*n* Tested (%; 95% CI)**	***S. aureus***	***S. cohnii***	***S. equorum***	***S. haemolyticus***	***S. pasteuri***	***S. sciuri***	***S. xylosus***
Farm B	intensive	animals	27/45 (60%; 44.3–74.3)	0	1	2	2	0	22	0
		environment	4/6 (66.7%; 22.3–95.7)	0	1	1	1	0	1	0
Farm G	intensive (antibiotic-free finishing)	animals	31/45 (68.9%; 53.3–81.8)	0	0	0	0	0	31	0
		environment	4/6 (66.7%; 22.3–95.7)	0	0	0	0	0	4	0
Farm P	intensive	animals	17/45 (37.8%; 23.8–53.5)	0	1	0	0	0	16	0
		environment	2/6 (33.3%; 0.4–77.7)	0	0	0	0	0	2	0
Farm S	organic	animals	8/15 (53.3%; 26.6–78.7)	0	0	0	0	0	8	0
		environment	0/2 (0%; 0–84.2)	0	0	0	0	0	0	0
Farm T	intensive	animals	44/45 (97.8%; 88.2–100)	1	0	0	2	5	35	1
		environment	5/6 (83.3%; 35.9–99.6)	0	0	0	0	0	5	0
Total		animals	127/195 (65.1%; 58.0–71.8)	1	2	2	4	5	112	1
		environment	15/26 (57.7%; 36.9–76.7)	0	1	1	1	0	12	0

Staphylococcus aureus was isolated from one nasal swab, in the post-weaning phase of the intensive farm T (Table 1). The other MRS isolated were MRCoNS, of which 88.2% (95%CI: 82.6–93.8) were identified as *S. sciuri*. *Staphylococcus pasteuri (*3.9%, 95%CI: 0.5–7.3), *S. haemolyticus (*3.1%, 95%CI: 0.1–6.2), *S. cohnii (*1.6%, 95%CI: 0.0–3.7), *S. equorum* (1.6%, 95%CI: 0.0–3.7), and *S. xylosus* (0.8%, 95%CI: 0.0–2.3) were isolated from nasal swabs as well. The prevalence of MRS was significantly different among farms (Fisher's Exact test, $p < 0.001$). Indeed, they were particularly abundant in farm T, where 44 out of 45 nasal swabs were positive and one isolate was identified as MRSA. The lowest number of MRS isolates was recovered from the organic farm S (8/15) and at farm P (17/45) (Table 1). MRS prevalence also significantly differed among productive stages ($p < 0.001$). The majority of positive samples was collected from the sows, followed by the post-weaning phase; finishing animals had a lower MRS prevalence, with the lowest number of positives (2/15 swabs) in the antibiotic-free finishing of farm G (Table 2).

Table 2. Methicillin-resistant staphylococci (MRS) isolated from animals (nasal swabs) and the environment in five farms in northern Italy, 2019–2020, by productive stage. All isolates were MRCoNS except from one MRSA in farm T (indicated by an asterisk).

Farm ID	**Farm Type**	**MRS Per Productive Stage**					
		***n* Positive Samples/*n* Tested (%; 95%CI)**					
		Finishing		**Post-Weaning**		**Sows**	
		Animals	**Environment**	**Animals**	**Environment**	**Animals**	**Environment**
Farm B	Intensive	8/15	1/2	8/15	2/2	11/15	1/2
		(53.3%; 26.6–78.7)	(50.0%; 12.6–98.7)	(53.3%; 26.6–78.7)	(100%; 15.8–100)	(73.3%; 44.9–92.2)	(50.0%; 12.6–98.7)
Farm G	Intensive (antibiotic-free finishing)	2/15	0/2	14/15	2/2	15/15	2/2
		(13.3%; 1.6–40.5)	(0%; 0–84.2)	(93.3%; 68.1–99.8)	(100%; 15.8–100)	(100%; 78.2–100)	(100%; 15.8–100)
Farm P	Intensive	5/15	1/2	6/15	0/2	7/15	1/2
		(33.3%; 11.8–61.6)	(50.0%; 12.6–98.7)	(40.0%; 16.3–67.7)	(0%; 0–84.2)	(46.7%; 21.3–73.4)	(50.0%; 12.6–98.7)
Farm S	Organic	8/15	0/2	-	-	-	-
		(53.3%; 26.6–78.7)	(0%; 0–84.2)				
Farm T	Intensive	14/15	1/2	15/15 *	2/2	15/15	2/2
		(93.3%; 68.1–99.8)	(50.0%; 12.6–98.7)	(100%; 78.2–100)	(100%; 15.8–100)	(100%; 78.2–100)	(100%; 15.8–100)
Total		37/75	3/10	43/60	6/8	48/60	6/8
		(49.3%; 38–60.6)	(30%; 6.7–65.2)	(71.7%; 58.6–82.5)	(75%; 34.9–96.8)	(80%; 67.7–89.2)	(75%; 34.9–96.8)

Fifteen out of 26 samples collected from the farms' environment were positive for MRCoNS (Table 1). MRS were recovered in the environment of the farms, except from the organic farm S and the antibiotic-free finishing stage of farm G (Table 2). Again, *S. sciuri* was the main species isolated ($n = 12$), and it was the unique species recovered in the environment in three out of the four positive farms. In farm B, we also identified *S. cohnii*, *S. haemolyticus* and *S. equorum*. In accordance with the animal swabs' results, MRS were mainly identified in the sows and post-weaning environment.
PCR amplicons were confirmed as the *mecA* type of *mec* gene through sequencing.
The analysis of the 527 bp *mecA* amplicons, obtained from 59 selected strains, revealed that all *mecA* sequences were highly related to the reference *S. aureus mecA* sequences (strain N315, COL and

MW2). The percentage of identity to the reference strains was higher than 99% in all the tested sequences. The nucleotide alignment revealed four point-mutations in the non-penicillin binding domain (non-PBD) (see Figure 1).

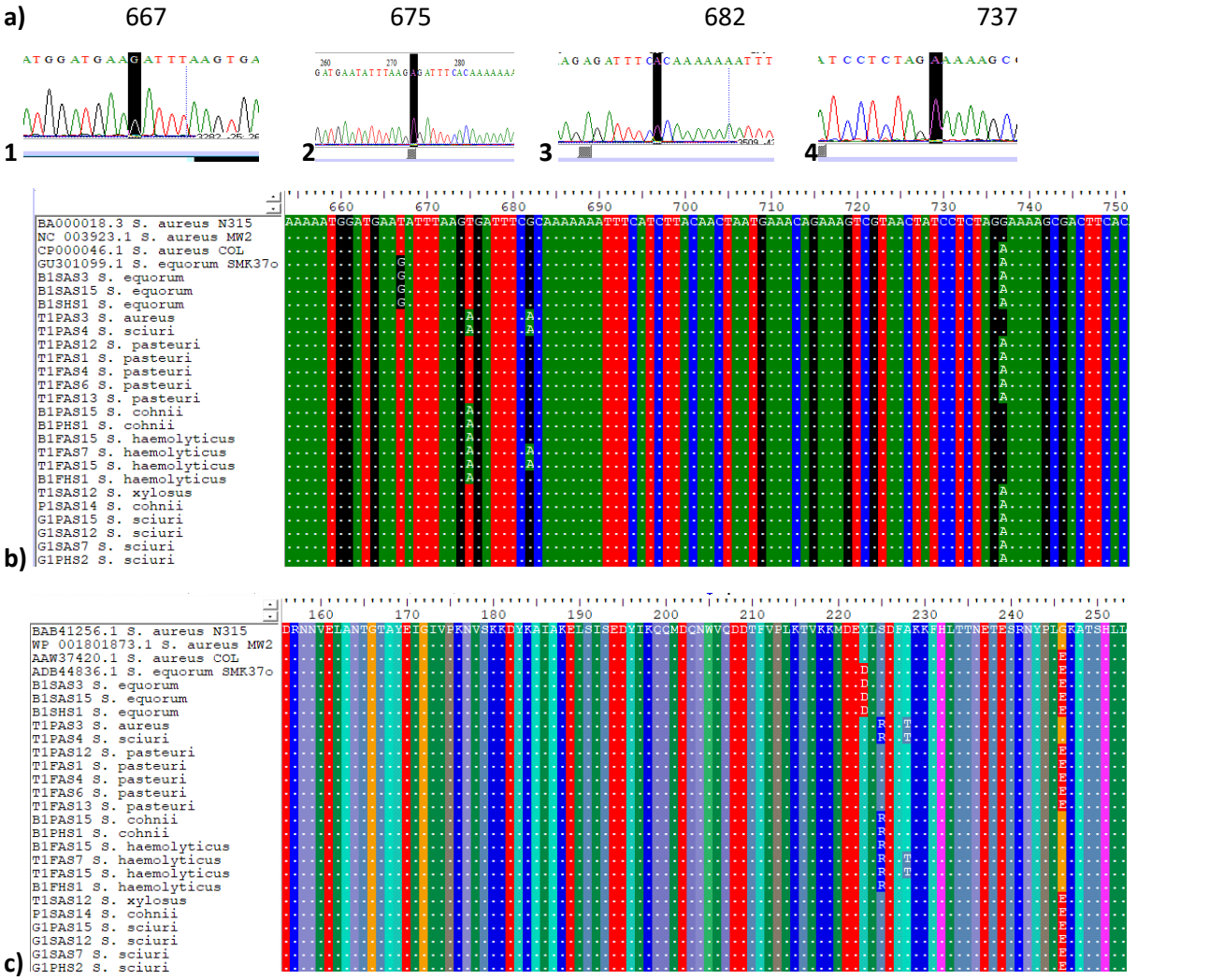

Figure 1. a) Chromatograms of the nucleotide mutations detected in the 527 bp *mecA* fragment: **1)** T667G. **2)** T675A. **3)** G682A. **4)** G737A.

b) Multiple nucleotide sequences alignment of a sample (n=22) of methicillin-resistant staphylococci with the four detected mutations. The reference strains (*S. aureus* N315, *S. aureus* MW2, *S. aureus* COL, *S. equorum* SMK37o) are located at the top of the nucleotide alignment.

c) Multiple aminoacidic sequences alignment of a sample (n=22) of methicillin-resistant staphylococci with the four aminoacidic substitutions (Y223D, S225R, A228T, G246E). The reference strains (*S. aureus* N315, *S. aureus* MW2, *S. aureus* COL, *S. equorum* SMK37o) are located at the top of the nucleotide alignment. Chromatograms, nucleotide and aminoacidic multiple alignments were created using BioEdit 7.2.5 Sequence Alignment Editor© software.

The most frequently detected mutation was at G737A (missense), which was also present in the reference strain COL (CP000046.1). This point mutation was present in all five farms across fifty-one strains of diverse staphylococcal species (*S. cohnii*, *S. equorum*, *S. haemolyticus*, *S. pasteuri*, *S. sciuri* and *S. xylosus*), in all productive steps, and was also recovered from environmental bacterial strains. In farm P and S, we detected only this mutation. Moreover, the nucleotide mutation T675A was

detected in farm B, in two post-weaning *S. cohnii* strains (B1PAS15 and B1PHS1) and in two *S. haemolyticus* samples from the environment and from a finishing pig (B1FHS1 and B1FAS15). *S. equorum* strains (two from animals: B1SAS3, B1SAS15; one from environment: B1SHS1) from farm B had two nucleotide variations: T667G and G737A; this double mutation was detected in the reference *S. equorum* strain SMK37o (GU301099.1). Finally, in farm T we detected two point mutations, T675A and G682A, in four identical *mecA* fragments from animals' samples, namely a *S. aureus* (T1PAS3), a *S. sciuri* (T1PAS4) and two *S. haemolyticus* (T1FAS7 and T1FAS15).

The analysis of the aminoacidic sequences revealed the presence of four mutations: Y223D, S225R, A228T and G246E (see Figure 1). The mutations Y223D and G246E were detected together only in *S. equorum* strains (B1SAS3, B1SAS15 and B1SHS1), such as is in the reference *S. equorum* SMK37o sequence (ADB44836.1). The mutations S225R, A228T were detected in four strains from farm T, one *S. aureus* (T1PAS3) and one *S. sciuri* from post-weaning (T1PAS4) and two *S. haemolyticus* from finishing (T1FAS7 and T1FAS15). In farm B, four bacterial strains presented the aminoacidic substitution S225R: two *S. haemolyticus* from finishing (B1FHS1 and B1FAS15) and two *S. cohnii* from post-weaning (B1PAS15 and B1PHS1) (Table 3). The mutation G246E was the most frequently detected in bacterial strains from all five farms and all the productive stages in animals and environment (86.4%, 95%CI: 75.0–94.0). S225R was detected in 13.6% (95%CI: 6.0–25.0) of the 59 selected strains, while A228T in 6.8% (95%CI: 1.9–16.5) and Y223D in 5.1% (95%CI: 1.1–14.1) (Table 3).

Table 3. Point mutations and PBP2-a aminoacidic substitutions in MRS isolated in five farms in northern Italy, 2019–2020, by productive stage (sows, post-weaning and finishing). In the strain column, the first on the left, sometimes results of more than one strain (separated by commas) are provided. Reference strains MRSA COL, MW2, N315 and MR-*S. equorum* SMK37o were used.

Strain	Organism	Farm	Productive Phase	Sample	Point Mutation				PBP2-a Mutation			
					T667G	T675A	G682A	G737A	Y223D	S225R	A228T	G246E
B1SAS3	*S. equorum* (MW768099)	B	sows	animal	X			X	X			X
B1SAS5,9,11	*S. sciuri*	B	sows	animal				X				X
B1SAS15	*S. equorum* (MW768100)	B	sows	animal	X			X	X			X
B1SHS1	*S. equorum* (MW768101)	B	sows	environment	X			X	X			X
B1PAS10	*S. haemolyticus*	B	post-weaning	animal				X				X
B1PAS15	*S. cohnii* (MW768093)	B	post-weaning	animal		X				X		
B1PHS1	*S. cohnii* (MW768094)	B	post-weaning	environment		X				X		
B1PHS2	*S. sciuri*	B	post-weaning	environment				X				X
B1FAS9	*S. sciuri*	B	finishing	animal				X				X
B1FAS13	*S. haemolyticus*	B	finishing	animal				X				X
B1FAS15	*S. haemolyticus* (MW768103)	B	finishing	animal		X				X		

B1FHS1	*S. haemolyticus* (MW768102)	B	finishing	environment		X				X		
G1SAS7,12	*S. sciuri*	G	sows	animal				X				X
G1PAS6,15	*S. sciuri*	G	post-weaning	animal				X				X
G1PHS2	*S. sciuri*	G	post-weaning	environment				X				X
P1SAS1,3	*S. sciuri*	P	sows	animal				X				X
P1SAS14	*S. cohnii* (MW774905)	P	sows	animal				X				X
P1SHS1	*S. sciuri*	P	sows	environment				X				X
P1PAS6,12,13,15	*S. sciuri*	P	post-weaning	animal				X				X
P1FAS2,3,9	*S. sciuri*	P	finishing	animal				X				X
P1FHS1	*S. sciuri*	P	finishing	environment				X				X
S1FAS2,7,10,14	*S. sciuri*	S	finishing	animal				X				X
T1SAS2,4,7,10,14	*S. sciuri*	T	sows	animal				X				X
T1SAS12	*S. xylosus* (MW768096)	T	sows	animal				X				X
T1SHS1,2	*S. sciuri*	T	sows	environment				X				X
T1PAS3	*S. aureus* (MW768098)	T	post-weaning	animal		X	X			X	X	
T1PAS4	*S. sciuri* (MW732662)	T	post-weaning	animal		X	X			X	X	
T1PAS7,9,14	*S. sciuri*	T	post-weaning	animal				X				X
T1PAS12	*S. pasteuri*	T	post-weaning	animal				X				X
T1PHS1,2	*S. sciuri*	T	post-weaning	environment				X				X
T1FAS1,4,6,13	*S. pasteuri* (MW768095)	T	finishing	animal				X				X
T1FAS5	*S. sciuri* (MW768105)	T	finishing	animal				X				X
T1FAS7,15	*S. haemolyticus* (MW768097)	T	finishing	animal		X	X			X	X	
T1FHS2	*S. sciuri* (MW768104)	T	finishing	environment				X				X
COL	*S. aureus* (AAW37420.1)			human				X				X
MW2	*S. aureus* (WP_001801873			human								

	.1)											
N315	*S. aureus* (BAB41256.1)			human								
SMK37o	*S. equorum* (GU301099.1)			cat	X			X	X			X

Letters are used in the table to indicate amino acids (A = alanine, D = aspartic acid, E = glutamic acid, G = glycine, R = arginine, S = serine, T = threonine, Y = tyrosine) and nucleic acid bases (A = adenine, G = guanine, T = thymine).

4.4. Discussion

The objectives of our study were to investigate the presence of MRSA and MRCoNS in pigs at different production stages and in their farm environment, in an area of intensive pig farming of Italy (Piedmont region, Cuneo province). The detection of only one MRSA positive sample out of almost 200 tested animals was unexpected. Indeed, the prevalence of MRSA in finishing pigs in another region of northern Italy, Lombardy, was recently estimated at 17.5% [231]. Furthermore, in the south of Italy, prevalences higher than 45% were reported, with the majority of positive samples from intensively reared animals [139,157].

Although our limited sample size may have led us to underestimate the prevalence, our results indicate a rare presence of MRSA in pig farms in our study area. On the other side, we highlighted the massive presence of MRCoNS in pigs and in their environment, especially in the non-antibiotic-free farms. MRCoNS prevalence in intensive and organic farms' animals was 64.6% overall, much higher than in previous studies from other European countries, where prevalence varied from 36.3% in Switzerland to 6.5% in Belgium [163,179]. The breeding stage (sows) showed the highest MRS prevalence (80%, Table 2). This may be explained by the older age of these animals compared to the other productive categories, so that they are possibly subjected for a longer time to antibiotic treatments. During their lifetime, sows can indeed manifest different clinical problems at the respiratory and reproductive system and suffer from joint diseases, requiring antibiotic treatments [232]. Post-weaning was the second most colonised phase by MRS, with 71.7% of positive samples. Colonization in this stage could be determined by different reasons: 1. the young age of the animals, which are probably more susceptible to MRS due to their immature nasal microflora [233]; 2. the fact that piglets from different sows are mixed after the farrowing stage and can exchange bacteria [233,234,235]; 3. post-weaning stress; 4. environmental contamination [233]; 5. antimicrobial treatment [234,235]. All the farmers stated that animal mixing, especially piglets, was a common practice, and this could be considered a risk factor for MRS spread, especially in the post-weaning phase. Another contributing factor could be the use of the same clothes and boots to visit the different productive phases in the farm. This habit was common in almost all our farms and could contribute to the dissemination of bacteria across the farm sectors. For example, in farm P, *S. sciuri* with the *mecA* gene G246E mutation was detected in all three animal sectors, while *S. pasteuri* harbouring the same mutation was sampled on finishers and in one weaned animal in farm T. The general use of slatted floor among farms was another potential risk factor for the dissemination of MRS among animals, as was elucidated in previous studies regarding MRSA [236].

In our research, *S. sciuri* was the predominant species among MRCoNS in animals and in the farm environment. This is in agreement with other studies on animals, sewage and dust in swine farms in Europe and Asia [180,237]. We hypothesize that the predominant presence of MR-*S. sciuri* in the farm environment is linked to *S. sciuri* nasal colonisation of the animals, due to their natural nuzzling behaviour. Indeed, this bacterium is a well-fitted free-living microorganism, that can be found in a wide range of hosts [238].

The massive presence of MR-*S. sciuri* on the nasal mucosa might negatively affect MRSA colonisation. Indeed, a natural inhibition of *S. aureus* in humans with a previous nasal colonisation

by a commensal CoNS, *Staphylococcus epidermidis*, was demonstrated [239]. However, additional studies are needed to understand if the presence of MRCoNS may inhibit a successful nasal colonisation by MRSA even in pigs. This natural inhibition could explain the extremely rare presence of MRSA among our study animals, together with management practices in the farms, such as the animal remount system. In fact, previous studies have documented that using multiple animal suppliers is a risk factor for MRSA colonization in the farm animals [240]. All our farmers instead declared to have only one gilts' supplier, or to have an internal remount (Farm G), and this could positively influence the animal negative MRSA status.

Contrary to the high prevalence in animals' samples, MRCoNS prevalence in the farm environment, in all the production phases, was in line with other European studies, where a prevalence up to 64% was found (e.g., in Germany [180]).

Finishing environment, like finishing animals, was the less contaminated with MRS, with only three bacterial strains isolated from a total of 10 samples (Table 2). This can be explained by the infrequent antibiotic treatments during this productive stage, due to the approaching slaughtering.

Although we did not recover the simultaneous presence of MRCoNS and MRSA in the same animal, we found them in the same productive stage of one farm (farm T). This can be considered a risk for the horizontal gene transfer of *mecA* from one staphylococcal species to *S. aureus*, that is a well-established human pathogen. The mobilization of *SCCmec* cassette, including the *mecA* gene, between CoNS and *S. aureus* was demonstrated by previous studies; the same nucleotide sequence was detected in various staphylococcal strains and species, indicating that this genetic element can move among staphylococci [191,241,242,243,244]. In vivo *mecA* mobilization from a CoNS species to *S. aureus* was demonstrated in a neonate, with the detection of the same *mecA* restriction patterns in a MRSA and a MR- *S. epidermidis* isolate [245].

In farm T, the same mutated *mecA* fragment was recovered from different staphylococcal species from two diverse productive phases: MRSA (T1PAS3) and MR-*S. sciuri* (T1PAS4) from weaned animals and two *S. haemolyticus* strains from finishers (T1FAS7 and T1FAS15). To our knowledge, this mutation had never been reported in *S. haemolyticus*; it was only previously detected in pigs in *S. aureus* strains from Denmark (CP028163.1 and CP028190.1) and China (CP065194.1). Moreover, the mutated *mecA* fragments of all *S. pasteuri* strains (T1FAS1, T1FAS4, T1FAS6 and T1FAS13) in this same farm, had never been reported from swine.

Farm B displayed the highest diversity of MRS species in animals and in the environment. The mutated *mecA* gene found in *S. equorum* strains (B1SAS3, B1SAS15 and B1SHS1) was identical to the one recovered from a cat in the Netherlands in 2005 (GU301099.1).

The *mecA* genes sequenced in *S. haemolyticus* (B1FHS1 and B1FAS15) and *S. cohnii* (B1PHS1 and B1PAS15) strains were identical to two MR-*S. haemolyticus* isolated in China from pathological bovine milk (KM369884.1 and KM369884.1) and from a swine nasal swab sampled in China (CP063443.1). The same *mecA* sequence was found in MR- *S. haemolyticus* isolated from human blood (AB437289.1) and urine (CP052055.1) samples. From our knowledge, this *mecA* nucleotide sequence is here first reported in *S. cohnii*.

The identified nucleotide mutations led to aminoacidic substitutions that had been previously recovered from human clinical specimens and had been correlated, with other variations in the non-PBD of the PBP2-a protein, to resistance to fifth generation cephalosporins [246,247,248]. The highly recurrent G246E mutation was also the most frequently reported in a study from Algeria, where it was recovered in *S. aureus, S. sciuri, S. saprophyticus* and *S. lentus* strains collected from human nasal samples [249]. Considering the available scientific literature, we here first report this mutation in the PBP2-a from *S. xylosus* (T1SAS12) and from *S. cohnii* (P1SAS14) of animal origin; this variation in *S. cohnii* has been hitherto detected from human clinical samples (ADM43473.1).

The S225R mutation, that we highlighted in *S. cohnii* and *S. sciuri* strains, had been previously

recovered in *S. haemolyticus* and *S. aureus* strains [247].

In conclusion, our study highlights an unexpected number of mutations in the *mecA* gene from swine MRS, some of which had never been detected in staphylococcal species from pigs. Consequently, monitoring of MRS at farm level is relevant to understand the risk for farmers to acquire these bacteria. The possibility for swine farmers to be colonised with MRCoNS, due to occupational exposure, was documented in a recent study [250]. The finding of the same MRCoNS in the animals and environmental samples suggests that the farm environment can be a source of animal contamination, or that animals can contaminate the environment, due to their massive colonisation with these bacteria [177]. Indeed, in Italy, MRSA colonised pigs seem to be the principal vehicle of transmission of MRSA to the environment [251].
Although we sampled a small number of farms, our results prove a significant colonization of pigs with MRS in different productive stages in the study area, where our farms represent the standard production typology. We will further investigate whether the antibiotic usage in the farms can be related to the MRS prevalence detected. Indeed, the misuse of antibiotics, in particular extended-spectrum cephalosporins and aminopenicillins, can contribute to the selection of methicillin-resistant bacteria in swine farms [116,252].
The unpredicted massive detection of MRCoNS in this area of Italy in pigs and their farm environment, with the contemporary presence of MRSA and MRCoNS, underline the need of monitoring both bacterial groups, since they can possibly transfer the *mecA* gene between them and can colonise human hosts. Furthermore, the finding of the same *mecA* genes in *S. aureus* and other swine-related species such as *S. sciuri*, support the role of these last bacteria as reservoir of *mecA* gene. Further studies are necessary to understand the possibility of horizontal gene transfer among staphylococci at farm level and the possible negative effect of MRCoNS on MRSA nasal colonization in pigs.

4.5. Materials and Methods

4.5.1. Farm Samples' Collection

The study was carried out in an industrial farming area in Cuneo province, Piedmont region [253]. Farms were representative of the standard pig farms present in the area, according to the veterinarians of the Local Veterinary Health Service (ASL CN1). Farms were chosen based on a convenience sampling, considering the willingness of the farmer to collaborate, the production type (intensive, organic and antibiotic-free) and cycle (close, farrow-to-finish; or open, finishers only). We selected five farms: three intensive close (farrow-to-finish) farms (named B, P, T), one intensive close farm, antibiotic-free at finishing (G), and one organic finishers-only farm (S). Samples were collected between October 2019 and September 2020, in occasion of routine veterinary checks. The sample size was calculated to detect at least one MRSA positive sample per farm, based on a minimum expected MRSA prevalence of 10% and considering a 95% confidence level. During each sampling, we collected 15 nasal swabs (Microbiotech s.r.l., Maglie, Italy) from each animal category present in the farm (post-weaning, finishing and sows); within each pen, the sampled pigs were randomly chosen. Moreover, we sampled two environmental swabs for each productive stage, from sites in tight contact with animals like bed pavements, troughs, and barriers, and on places around the animals like the pigsty walls, floor corners and tubes. Each sample was identified with a code indicating the farm name (B,G,P,S and T), number of sampling (1), productive phase (F = finishing, P = post-weaning and S = sows), source (A = animal and H = environment), bacterial genus (S = *Staphylococcus*) and a progressive number (1–15 for animals, 1–2 for environment). In the organic farm S, only 15 animals and 2 environmental samples were taken, since it was a finishers-only farm. Samples were kept in a refrigerated box till the arrival in the laboratory and were processed within

24 h from the collection.
Prevalence of positive samples was calculated, with 95% confidence intervals (95%CI). Fisher's Exact test was used to assess differences in MRS prevalence among different farms and productive stages. Statistical analyses were performed with R software (R Core Team, 2020 [254]).

4.5.2. Biosecurity and Management Data Collection

A questionnaire about general farm biosecurity was compiled when farms were visited to evaluate animal flow in the different farm sectors, remount, piglets mixing from different litters, gilts' quarantine, use of dedicated clothes and boots to enter the different animals' sectors, cleaning protocol, floor type and the carcasses management (see Questionnaire 1).

Farmers' questionnaire about biosecurity and general management
1) Productive cycle: a) open
b) close
c) only finishing
2) Remount: a) internal
b) external: how many gilts' suppliers do you have?
3) Are new animals quarantined (gilts or new weaned for finishers' only farm)?
4) Animal mixing a) Is the animals' flow unidirectional?
b) Are herds composed only by animals from the same group?
c) During which productive phase do you mix animals from different groups?
5) Are farm workers using dedicated clothes and boots for each animal sector?
6) Is there a specific cleaning protocol during sanitary stop?
7) Which type of floor is present in the different productive sectors?

Questionnaire 1: general farm biosecurity checklist, that was compiled for every visit in the five sampled swine farms.

4.5.3. Phenotypic Analysis

Each swab was subjected to an enrichment stage in a liquid medium. Tryptic soy broth (TSB) (Oxoid, Wade Road Basingstoke, UK) with 2.5% of NaCl [255] was used with the addition of two antibiotics: cefoxitin (3.5 mg/L) (Sigma-Aldrich, St. Louis, MO, USA) and aztreonam (20 mg/L) (Sigma-Aldrich, St. Louis, MO, USA). Cefoxitin was added to select MRS, while aztreonam to inhibit Gram-negative bacteria growth. Swabs were immersed for 5 min in 4 mL of this broth; afterwards, broth samples were placed in a shaker incubator for 24 h at 35–37 °C at 220 rpm. After the enrichment step, a loop

of 10 µL of the liquid samples were spread on a selective solid medium; the medium was Mannitol Salt agar (MSA) with 6% NaCl ([256]), plus cefoxitin (3.5 mg/L) (Sigma-Aldrich, St. Louis, MO, USA). MSA was prepared with phenol red, 0.025 g/L (Sigma-Aldrich, St. Louis, MO, USA), bacteriological peptone 10 g/L (Oxoid, Wade Road Basingstoke, UK), mannitol, 10 g/L (Sigma-Aldrich, St. Louis, MO, USA) NaCl, 60 g/L, cefoxitin 3.5 mg/L (Sigma-Aldrich, St. Louis, MO, USA) beef extract powder 1 g/L, and agar 15 g/L (Oxoid, Wade Road Basingstoke, UK) with a final pH of 7.4 +/− 0.2. Catalase test, Gram staining and oxidase test were used as supportive tests in staphylococcal identification on round and yellow colonies that presented mannitol fermentation on MSA. After collecting 1-2 yellow colonies with the same size, bacterial strains were stored in 500 µL of TSB plus 15% glycerol at −80 °C.

Phenotypic bacterial identification was performed using matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) Microflex™ LRF (Bruker Daltonik GmbH, Bremen, Germany). A modified direct transfer-formic acid method was used for sample preparation as described previously [257]. Briefly, one colony from a fresh pure culture was taken with a disposable loop and spread on a single well of the microplate reader, to have a thin layer. Then, 0.9 µL of formic acid (diluted at 70%) was added on the well. After formic acid was dried, 1 µL of the saturated α-cyano-4-hydroxy-cinnamic acid (HCCA) matrix (Bruker Daltonik GmbH, Bremen, Germany) was added over the well. Finally, after HCCA matrix was left to dry, bacterial samples on the microplate were analysed with MALDI-TOF MS within 24 h. MALDI Biotyper® (Bruker Daltonik GmbH, Bremen, Germany) software was run to classify bacteria at genus and species level. Following Bruker recommendations, specimens with a similarity log-score threshold between >1.7 and <1.999 were classified for presumptive genus, while a score > 2 and < 2.299 were used for secure genus identification, and probable species identification. Results < 1.7 were considered not reliable for bacterial genus identification.

4.5.4. Genotypic Analysis

DNA was extracted from bacterial colonies using a modified boiling method: briefly, one or two colonies were picked with a sterile loop and immersed in 1 mL of PBS in a 1.5 mL Eppendorf tube; then, samples were centrifuged for 5 min at 13,500 rpm. Supernatant was discarded and the remnant bacterial pellet was mixed with 100 µL of sterile deionized water and vortexed for some seconds. Afterwards, samples were placed in thermoblock for 8 min at 95 °C, and then, stored at −20 °C [258]. Quantity of extracted DNA was measured with a spectrophotometer NanoDrop™ 2000 (Thermo Scientific, Waltham, WA, USA).

Polymerase chain reaction (PCR) was used to confirm phenotypic methicillin resistance (*mecA* gene) and to verify bacterial identification (16S rDNA gene). To confirm MRSA identity, we used a multiplex PCR protocol, targeting the *mecA* and 16S rDNA genes and the *S. aureus*-specific *nuc* gene [259]. Simplex protocols were used to amplify the *mecA* (527 bp) and 16S rDNA genes (500 bp) for nucleotide sequencing. The 16S rDNA gene was tested to confirm genus in samples with a MALDI-TOF MS log-score between 1.7 and 2. Positive controls (from Turin University Culture Collections) and negative controls (deionised DNA-free water) were added to every PCR reaction.

Amplified fragments of a group of 59 strains, randomly chosen from all the farms and productive stages, were purified with ExoSAP-IT™ PCR Product Clean-up Kit (GE Healthcare Limited, Chalfont, UK) and sequenced in an external laboratory (BMR Genomics, Padua, Italy). Obtained nucleotide sequences were analysed using BioEdit 7.2.5 Sequence Alignment Editor©software; multiple alignment with reference sequences was carried out with ClustalW tool. The same software was used to convert nucleotide sequences in aminoacidic sequences. To compare sequences with available sequences in GenBank, we used BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed

on 18 March 2021). Reference strains used in the nucleotide alignment were the MRSA N315 (BA000018.3), MW2 (NC003923.1), COL (CP000046.1) and the methicillin- resistant *S. equorum* SMK37o (GU301099.1).
We deposited *mecA* sequences in GenBank with the accession numbers: MW732662, from MW768093 to MW768105, and MW774905.

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Chapter 5 - Assessing The level of exposure of Workers to Methicillin-Resistant staphylococci (MRS) And Extended-spectrum β- lactamase (ESBL)- Producing *Escherichia coli* In An Intensive Broiler Farm In Northwestern Italy

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Assessing The level of exposure of Workers to Methicillin-Resistant staphylococci (MRS) And Extended-spectrum β- lactamase (ESBL)- Producing Escherichia coli In An Intensive Broiler Farm In Northwestern Italy. (under preparation)

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5.1. Abstract

Methicillin-resistant staphylococci (MRS) and extended-spectrum β-lactamase (ESBL)-producing *E. coli* are emerging public health concerns. Both antibiotic-resistant bacteria can be transmitted from livestock to farm workers, posing a threat to community dissemination of antimicrobial resistance. In this study, we investigated MRS and ESBL-producing *E. coli* colonisation in broilers (90 animals) and farm environment from a farm in northwestern Italy, in two different productive cycles, separated by a rest period of 17 days. Phenotypic percentages varied in animals' samples, ranging from 0% upon the arrival of chicks, to 73.3% at 27 days for MRS. For ESBL-producing *E. coli,* the highest prevalence was registered at 6 days (23.3%), with a high reduction of positive animals' samples at 27 days (3.3%) in the same cycle. ESBL-producing *E. coli* in the environment was recovered only in one sample at 6 days, while MRS were always detected, even after the rest period, upon the arrival of chicks. The MRS positive samples were mainly *Staphylococcus lentus*. No MRSA was recovered. These laboratory data were used to assess the exposure of farm workers to the resistant bacteria during different working practices, using a modified, semi-quantitative method, FMEA (*Failure Modes and Effect Analysis*). The analysis revealed that "carcasses removal" and "litter removal" are characterised by the greatest levels of exposure of farmers to ESBL-producing *E. coli*, and to MRS, respectively. Our study highlighted the contemporary presence of MRS and ESBL-producing *E. coli* in broilers and in farm environment, posing a threat for farm workers, that are daily exposed to these antibiotic-resistant bacteria.

5.2. Introduction

Broiler chickens have demonstrated high prevalence of intestinal resistant bacteria, especially extended spectrum β-lactamase (ESBL)-producing *Escherichia coli (E. coli)*, that in Italy is present in 86% of sampled broilers [260]. Considering methicillin-resistant staphylococci (MRS) colonisation, there are few studies that document the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) in broilers [164,261,262], while less are about methicillin-resistant coagulase-negative staphylococci (MRCoNS) [179,189]. Previous European studies highlighted that ESBL-producing *E. coli* and MRSA can be transmitted from pigs to farm workers [66,263]. In broilers farming, some studies underlined the risk of acquiring these antibiotic resistant bacteria during occupational exposure to animals and farm environment [22,73,264,265]. For human medicine both ESBL-producing *E. coli* and MRS carriage represent a relevant risk of therapeutic inefficacy and mortality increment, because they carry resistances to last-resort antibiotics [266]. Furthermore, ESBL-related genes are mainly located on mobile genetic elements, specifically plasmids, and thus they can easily be transmitted among the same bacterial species or even different species, facilitating their dissemination [11,267].
Similarly, the methicillin gene *mecA*, can be horizontally transmitted to different staphylococcal species, like *Staphylococcus aureus*, facilitating the spread of this resistance to more human pathogenic bacteria [191].
In this study, we investigated the presence of MRS and ESBL-producing *E. coli* in healthy broilers and their environment, in a broiler intensive farm of southeast Piedmont, in different moments of two productive cycles. Furthermore, we used prevalence estimates in a semi-quantitative exposure assessment method, to classify farm working practices, in terms of the probability level of exposure of workers to AMR determinants. On farm antimicrobial usage (AMU) was also considered, to evaluate possible correlations with microbiological results.

5.3. Materials and methods

5.3.1. Farm

The study was carried out in a broiler intensive farm in southeast Piedmont region, northern Italy. The farm was chosen since it was representative of agistment intensive broiler farms in the area and based on the willingness of the farmer to collaborate. Each production cycle in the farm lasts around 69 days with a rest period of around 17 days; females are slaughtered at 31 days and males at 42-45 days. Broilers are bred in three barns, with a density of around 19 animals/m^2 and 33 kg/m^2, in accordance with current national legislation [268]. Mechanical ventilation is present in all barns and sterile rice hulls are used as litter. Farm workers involved in farm procedures were generally one or two, with the exception of particular farm practices, such as disinfection of barns and tools, and animals loading, when workers were five.

5.3.2. Samples' collection

Between January and March 2019, we sampled animals in two different productive cycles. The first cycle was sampled on day 6 (T2) and day 27 (T3) (see Table 3); the next productive cycle was sampled at the arrival of chicks on farm (day one, T1). In each sampling, we collected 30 faecal samples for

the research of ESBL-producing *E. coli* and 30 skin swabs (Microbiotech s.r.l., Maglie, Italy) for MRS (90 swabs in total for each bacterial group). The sample size was chosen based on an expected bacterial prevalence of 10% with 95% of confidence interval and 5% of error (α). Sampled animals were randomly selected, taking 10 swabs in each of the three barns. At each sampling (T1, 2 and 3), we also took environmental swabs, 3 for each bacterial group, on the surfaces of each barn (9 swabs in total for each bacterial group). Moreover, additional environmental samples from litter (pool of 5 g taken in 5 different places from the centre and corners of the barn) were collected for the research of ESBL- producing *E. coli*. We collected three samples per barn, at T0 (clean litter, before chicks' arrival) of the second productive cycle, and at T4 (dirty litter) of the first productive cycle (immediately after animals were sent to the slaughterhouse).

5.3.3. Laboratory and statistical analyses

We carried out phenotypic analysis on faecal, skin and environmental swabs.
To detect ESBL- producing *E. coli,* we used a first enrichment step in 5 ml of Peptone Water (PW) to incubate bacteria overnight at 37°C at 220 rpm. After this, a loopful of broth was streaked on solid medium, MacConkey 3 (MCC3) agar (Oxoid, Wade Road Basingstoke, UK) with the addition of the antibiotic cefotaxime (1 mg/L; Sigma-Aldrich, St. Louis, MO, USA). A phenotypic test was used to confirm the presence of ESBL enzymes, "cefpodoxime combination disk test" (Oxoid, Wade Road Basingstoke, UK), after the isolation of pure bacterial cultures.
To identify MRS, we used a first enrichment step (Tryptic soy broth 2.5% of NaCl, plus cefoxitin 3,5 mg/L and aztreonam 20 mg/L) and a selective solid agar (Mannitol Salt agar with 6% of NaCl plus cefoxitin 3,5 mg/L) [269].
Agar plates were incubated for 18/22 to screen ESBL-producing *E. coli*, while MRS plates were kept for 24 hours at 37°C. Presumptive MRS round and yellow colonies were selected to proceed with further biochemical tests (catalase and oxidase tests) and Gram staining, while for presumptive ESBL- producing *E. coli*, we considered positive samples, colonies with round shape and red/purple colour, with a pinkish halo [270]. To test ESBL-producing *E. coli* contamination from environmental samples taken at 53 days (T4), 1 g of litter was taken and diluted in 9 ml of PW, vortexed and placed in an oscillator for 18 hours at 220 rpm and 37°C. After this incubation, 10 µl of supernatant were placed on MCC3 agar with cefotaxime 1 mg/L.
Phenotypic prevalence of MRS and ESBL-producing *E. coli* was calculated using R software [254], considering a confidence interval of 95%.
After phenotypic colonies' characterizations, DNA was extracted from presumptive MRS and ESBL-producing *E. coli* pure colonies with a modified boiling method [269]. The spectrophotometer NanoDrop™ 2000 (Thermo Scientific, Waltham, WA, USA), was used to measure the quantity of extracted DNA.
A group of MRS presumptive samples (n=6) were analysed by PCR multiplex protocol to test for the specific *S. aureus* nuclease, *nuc*, *mecA* and 16s rDNA [271]. The presence of *cfr* gene in MRS was also investigated [272].
To confirm phenotypic ESBL-producing *E. coli*, we performed three PCR targeting the specific β-lactamase genes $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} were used [273].
For MRS species confirmation, 16S rDNA gene (500bp) was amplified with PCR [274]. In every PCR reaction, positive controls (Turin University Culture Collections) and negative controls (deionised DNA-free water) were used. Amplified fragments of a sample of MRS strains, from animals and

environmental samples, were then purified with ExoSAP-IT™ PCR Product Clean-up Kit (GE Healthcare Limited, Chalfont, UK) and sequenced in an external laboratory (Macrogen, Netherlands). BioEdit 7.2.5 Sequence Alignment Editor© software was run to analyse nucleotide sequences; ClustalW tool was set up for multiple alignment with reference bacterial genome sequences. BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to match our sequences with available sequences in GenBank.

5.3.4. Exposure assessment

To assess which working practices were associated with the greatest level of exposure to AMR in poultry farmers, we applied a semi-quantitative antimicrobial risk assessment (ARRA), as described previously [275]. We combined prevalence estimates of AMR in animals and in the farm environment (release assessment), with the probability of contacts of humans with AMR during each practice in the broiler production process. We applied a modified FMEA (*Failure Modes and Effect Analysis*) methodology to rank working practices, based on the level of exposure to AMR for farm workers [276,277].
We obtained detailed descriptions of phases of the production of broilers, by interviews to key informants: two public veterinarians, with official responsibility for poultry health, and two private veterinary practitioners, working in the assistance to poultry production [277]. Moreover, we directly observed working practices in the farm. Subsequently, we selected 12 practices, which involve direct, or indirect contacts with animals and with the farm environment, potentially leading to exposure to AMR bacteria: 1. Box preparation, 2. Chicks' offload, 3. Carcasses' removal, 4. Implants maintenance, 5. Animals weighing, 6. Loading female broilers on truck for slaughter, 7. Loading male broilers on truck for slaughter, 8. Manure removal at the end of the productive cycle, 9. Barn clean-up, 10. Barn disinfection, 11. Litter aeration by tumbling, 12. Troughs clean-up.
To classify each farm practice in terms of the exposure of farm workers to AMR, four indicators, or criteria, were chosen, by discussion among the authors, based upon the analysis of interviews of key informants, and published literature: 1. Contact, 2. Hours of work, 3. PPE (personal protective equipment) use, 4. Number of animals for single farm worker.
A specific broiler farm model was created, for the attribution of four exposure levels to each indicator, and for each working practice. Levels ranged from 1 (very low exposure level) to 4 (very high exposure level), as reported in Table 1.

Furthermore, an importance level, from 1 (very low importance), to 4 (very high importance) was assigned to each indicator, separately for exposure to MRS or ESBL-producing *E. coli*. The objective was the attribution of greater weight, in the ranking of working practices, to indicators which were considered as most important in each AMR determinant's transmission routes. As shown in Table 2, we attributed greatest importance to work hours and PPE use, including face mask and gloves, for the exposure to MRS, since these agents can be transmitted by aerosol. Type of contact and PPE were considered as most important for ESBL-producing *E. coli*, which are mostly transmitted by the oral-faecal route [52,278,279,280].

Although direct contact with animals was, in general, an important indicator, the number of animals for worker in itself was less important than other indicators. Indeed, the farming environment is considered a major reservoir of AMR determinants, especially MRS [185,281,282].
In addition to the above-described indicators, we included the observed prevalence of AMR determinants into the overall classification of working practices. Prevalence was divided in intervals

(4 levels), based on previous studies [71,159]. For the level 1 the prevalence interval chosen was 0-<20%, for level 2 20- < 40%, for level 3 40-60% and for level 4 the prevalence selected was >60%.

Combining the information from the exposure level, based on the indicators shown in Table 2, with prevalence levels, which we obtained in animals and environmental samples, we obtained the "risk priority code" (RPC). This value allowed us to order working practices based on an increasing level of exposure to MRS and ESBL-producing *E. coli*.
In case two practices had the same RPC, we applied the "Tie break" value, which is based on the number of indicators with high levels.

5.3.5. Antimicrobial usage (AMU)

We qualitatively evaluated the veterinary antibiotics prescriptions in the farm from 2014 to 2019, considering administration route and specific active ingredient.

5.4. Results

5.4.1. Laboratory results

We detected from broiler skin swabs an overall phenotypic MRS prevalence of 43.3%; prevalence ranged from 0% at T1, to 56.7% at T2 and 73.3% at T3. In the environment, a prevalence of 88.9% (8/9) was recovered (66.7% at T0 and 100% at T1 and T2; Table 3). The *mecA* gene was found only in the positive yellow colonies grown on the modified MSA, while the *S. aureus* specific *nuc* gene was not detected in any MRS DNA sample, confirming the absence of *S. aureus*. No sample was positive for the *cfr* gene.
From animals' samples, ESBL-producing *E. coli* were not recovered at T1, while the prevalence was 23.3% at 6 days (T2) and 3.3% at 27 days (T3). For the environmental samples, 1 sample out of 3 was positive at T2, while no positive sample was found during the other samplings (see Table 3). The $bla_{CTX\text{-}M}$ gene was the only β-lactamase type recovered, with the only exception for an environmental sample (at T2) that carried bla_{SHV} gene and $bla_{CTX\text{-}M}$. The $bla_{CTX\text{-}M}$ gene was identified in three animals' samples (2 at T2 and 1 at T3) and in one environmental sample. Five phenotypically positive animals' samples detected at 6 days (T2) were not confirmed with the molecular analysis for the selected β-lactamase genes.
Sequencing of the 16S rDNA gene revealed that the majority of animal samples were *Staphylococcus lentus*, while one was identified as *Staphylococcus sciuri*. At the environmental level, only *S. lentus* was recovered.

5.4.2. Exposure assessment results

We summarized some data obtained from the observation of work practices, which determined the level of exposure as described in Table 4:

1) Box preparation: the rice litter used to arrange boxes is sterile until the outflow from a truck directly inside the farm. This practice involved two farm workers.
2) Chicks' offload. This practice involved three farm workers. Chicks are from the same hatchery. Cases with animals are manually transferred in the broiler farm.

3) Animals' weighing. The first weighing is automatic; however, the next ones are manually carried out every week, and then more frequently till the end of the cycle. During the weighing, 5 animals are in the same case each time.
4) Removal of carcasses. This practice is carried out manually two/three times daily, depending on the period of the productive cycle. The farm worker needs to pass through the animals to remove the dead ones.
5) Litter aeration by tumbling. This practice occurs one time a week (twice at the end of the cycle) through a specific tool that moves, turns and levels the litter. During this practice, the farm worker is driving the bobcat machine.
6) Maintenance. Often it implies repairing drinking and eating lines, which are inside the animal barns or outside when it refers to silos.
7) Broiler loading. This practice at the end of the productive cycle needs more than one farm worker and it is carried out using a specific tracked machine that allows broilers to be pushed in cages, which are on the back of the machinery. At the end of this phase, all the cages are loaded with a forklift on the truck, directed to the slaughterhouse.
   During this loading practice, some farm workers oversee broilers on the conveyor belt. Gloves were the only protective equipment used by farm workers on the tracked machine. Workers help broilers to enter the cages too. The work practice "female loading A" refers to the farm workers without any PPE, while "female loading B" refers to the farm workers using only gloves as PPE.
   Male loading was performed with the same method as the female one, with three farm workers that actively participate to the practice.
8) Litter removal. This practice is carried out at the end of the productive cycle with a machine and manually for the residual with brooms; generally, there are two farm workers.
9) Troughs' clean-up. It is performed manually at the end of the productive cycle.
10) Clean-up. High-pressure water treatment is used to clean the entire barn up with the equipment by a single farm worker.
11) Disinfection. It follows the cleaning phase on the same surfaces. Farm workers have facial masks during this practice. It includes fumigation with formaldehyde on humid surfaces.

These results were considered with respect to the MRS and ESBL-producing *E. coli* prevalence to calculate the RPC value (see Table 5 for MRS and 6 for ESBL- producing *E. coli*). For the environmental and broilers sampling that were not conducted in this study, we considered only the level of exposure for calculating the final RPC.

Using the "Tie-break" analysis, we had a classification of the work practices based on the level of exposure for the farm worker to be in contact with resistant bacteria. The use of the letters (from A=highest level of exposure, to H=lowest level of exposure) just beside the numbers (1=high exposure degree, 2=low exposure degree) are necessary for a better categorization of the level of exposure to the AMR determinants (see Table 7 and 8).
For MRS, litter removal was the work procedure with the highest exposure level for farm workers, while for ESBL- producing *E. coli* the most important practice in terms of exposure to be in contact with these bacteria was considered carcasses'removal. Litter removal resulted an important way of exposure to ESBL- producing *E. coli* as well.
From both analyses, it is clear that practices with lower level of exposure are those involving barn disinfection and cleaning of troughs. In addition, female loading was considered less relevant for the exposure to resistant bacteria.

5.4.3. Antimicrobial usage (AMU) results

All the antibiotics were administered orally. The most used antibiotics were amoxicillin, doxycycline and enrofloxacin. Less frequently, animals were treated with oxytetracycline, thiamphenicol, levofloxacin and different sulphonamides molecules with trimethoprim.
During the first productive cycle, two antibiotic treatments with doxycycline and one with amoxicillin were administered in water for five days after T2.

5.5. Discussion

Our study recovered the contemporary presence of MRS and ESBL-producing *E. coli* in broilers and in the farm environment from a broiler intensive farm in northwestern Italy. These data were used to understand farmers' exposure to AMR bacteria, by applying the FMEA method.
Looking at MRS detected in animals, the percentage of positive samples seems to grow with the age of the tested broilers, from 0% upon the arrival of chicks (T1), after the rest period, to 73.3% at 27 days (T3), which represents more than half of the broiler productive cycle. It is noteworthy to observe that 6 days after the arrival in the farm, young animals are already colonised by MRS (56.7%). These percentages are higher if compared to other European studies; for example, the prevalence of methicillin resistant *S. sciuri* ranged from 12.5% to 30% in broiler Belgian farms [179,184], while 48.6% of animals from 72 Swiss farms were positive to MRCoNS [167]. These findings indicate that MRCoNS are not rare in broilers farms. The growing prevalence of MRCoNS throughout the productive cycle could be due to oral administration of amoxicillin and tetracyclines, that can elicit the increase of these antibiotic resistant bacteria, as it was demonstrated for MRSA [116]. Unfortunately, we did not test antibiotic susceptibility of MRCoNS against the other frequently administered antibiotics, namely fluoroquinolones, phenicols, sulphonamides and tetracyclines. These antibiotic classes were used during tested productive cycles and may have had a selective pressure on staphylococci. Even at the environmental level, the percentage of positive samples in our study increased during the cycle (from 66.7% at time 0, before the chick's arrival, to 100% in the two samplings at 6 and 27 days of the first tested cycle). This high MRCoNS environmental prevalence, even after the rest period, may explain why recently arrived animals are already colonised with MRCoNS. The fact that MRCoNS are widespread in farm environment is well documented in a recent study, where these bacteria were recovered in 36.3% of bioaerosol samples from hen houses in China [185]. High environmental MRS presence, as it was found in our study, poses a level of exposure to these AMR bacteria for animals and farmers, that can be exposed directly (through contact) and indirectly (through environment and farm aerosol). Considering the elevated MRCoNS environmental contamination, it is easy to understand why litter removal is the working practice at higher level of exposure to these bacteria: MRS can spread through aerosol and farm dust, and colonise farmers, if they do not wear personal protective equipment. Another important farm practice for MRS exposure is barn clean up; during this activity, high quantity of dust, dirty litter and animals' residues can be moved from the farm environment and reach farmers and animals. Indeed, we observed the MRCoNS prevalence to grow even over the broilers' skin during the productive cycle (from the arrival of the animals to the last animal sampling, at more

than half of the cycle), posing a threat of transferring these microorganisms to the farm environment. Animals' weighing and chicks' offload are other relevant farm practices for exposure to MRCoNS, because they imply the direct contact with broilers, which can be highly colonised with these staphylococci, especially during the growing phase. The elevated MRCoNS presence at animal and environment level can be considered a double health issue for farmers, as these bacteria are putative reservoir of the *mecA* gene*,* which can be horizontally transmitted to the more pathogenic *S. aureus* [191]. Furthermore, in a previous study, resistance and pathogenicity related genes found in *S. aureus* were detected in methicillin-resistant strains of *S. sciuri* (MRSS) recovered in chickens [238]. Thence, it is important to unveil the methicillin-resistance status in staphylococci isolated from broilers, to understand if these bacteria are reservoir of *mecA* for MRSA. Also, sporadic cases of human infections caused primarily by *S. sciuri* and *S. lentus* have been reported worldwide. In some of these infections the staphylococci strains were *mecA* positive [283,284]. Moreover, recently staphylococci have gained relevance for owning the multi-resistance gene *cfr,* responsible for the resistance to five antibiotic classes, among which the oxazolidinone linezolid, last-resort human antibiotic. This gene was originally detected in the 17.1-kb plasmid, pSCFS1, owned by *S. sciuri* [285]. In human medicine, linezolid resistance is growing more in CoNS than in *S. aureus* [173,286]. Till now, the *cfr* gene has been occasionally detected worldwide more often in CoNS of poultry and turkey origin [192,193]. Although we did not detect *cfr* in our study, it is relevant to continue monitoring this resistance gene in broilers, to understand if they represent a *cfr* reservoir for humans.

A different situation was observed with ESBL- producing *E. coli* that, at time 0, were not detected in animals and environmental samples. This can be due to the limited presence of faecal Enterobacterales in the farm, that are efficiently removed from the environment after the disinfection of the rest period. The highest prevalence of ESBL- producing *E. coli* in animals was recovered at 6 days (23.3%); this prevalence is low if compared to the national data (86% [260]), and to studies from other European countries like Germany (72.5% in broiler caecal samples [24]). The presence of a higher ESBL-producing *E. coli* prevalence during the first part of the productive cycle can be due to the colonization of the animal caeca in the early stage of their life. It was documented that, in the parental broiler, these bacteria can be present in semen [77]; this suggests the possibility to spread resistant strains by reproduction. Furthermore, this way of ESBL-producing *E. coli* transmission is facilitated by the use of a small group of animals as breeders [287]. Our findings are confirmed by a study in fattening broiler farms from Germany, where ESBL-producing *E. coli* were detected in the first stage of broilers' life (from day 1) [72]. Moreover, the use in the incubator of third generation cephalosporins, like ceftiofur, could be linked to ESBL-producing *E. coli* chicks' colonisation. This was reported in the study of Baron et al., that highlighted a difference in the percentages of ESBL- producing *E. coli* in the first week of life in the chicks that had received antibiotics with respect to the ones non-treated *in ovo* [288]. Dierikx et al. suggested that the introduction of one-day-old chicks in the farm represents a risk factor for introducing ESBL-producing *E. coli* strains [22].

Unfortunately, we could not confirm genotypically all the ESBL samples that were phenotypically positive. This could be due to alteration in the membrane flux of these ESBL-positive bacteria [289] or to the presence of other β-lactamases, not tested in this study, like the plasmid mediated AmpC $bla_{\text{CMY-2}}$, that can be detected in poultry stool samples [290]. The fact that $bla_{\text{CTX-M}}$ was the most frequently recovered gene in animals' and environmental samples is in line with previous studies;

indeed, *bla*$_{\text{CTX-M}}$ is the ESBL-associated gene more frequently found in *E. coli* strains in livestock and in broilers too [59,73]. In the Netherlands, *bla*$_{ctxm\text{-}1}$ and *bla*$_{shv12}$ were recovered in broilers and in farm workers in tight contact with these animals [22]. Due to the faecal route of transmission of ESBL-producing *E. coli*, the farming practices at higher level of exposure to these resistant bacteria are those in which farmers are in tight contact with animals (dead and alive). Carcasses' removal was identified as the most dangerous practice, due to the direct contact with the highly contaminated body of the animal. Litter removal was the second most important practice, due to the high level of exposure to animal excreta, especially if the farm worker does not wear PPE like gloves and masks. Maintenance was also considered a practice at risk for the contamination of the eating and drinking lines that are in direct contact with animals. As for MRCoNS, chicks' offload and animals' weighing resulted farm practices important for the tight contact with animals, carriers of resistant *E. coli*.

Recently, the same ESBL-producing *E. coli* strains were identified in farmers and animals from a poultry farm [280]. Unfortunately, in our study we could not test the farm workers for the presence of AMR determinants. This could have enabled us to evaluate the possible transmission of ESBL-associated genes between animals and workers, which can especially occur during farm practices implying the direct contact with animals or cleaning animal barns contaminated with broiler faeces. A limit in our study was the sampling of broilers and farm environment in two different productive cycles, considering different moments in each cycle. However, this sampling scheme enabled to test animals and environment after the cleaning and disinfection of the farm barns, between cycle 1 and 2, and to evaluate the importance of the rest period and hygiene practices to reduce environmental bacterial contamination, especially by ESBL-producing *E. coli*.

5.6. Conclusions

Although in this study we sampled only one farm, we provided the evidence of the simultaneous colonisation by ESBL-producing *E. coli* and MRCoNS in a broiler farm in the north of Italy. To our knowledge, this is the first time that this carrier status is confirmed in Italian healthy broilers. Our results indicate the need of microbiologically monitoring broiler farms to understand if this co-occurrence is an exceptional finding, or, if this is common among broiler farms from different Italian regions. Furthermore, our findings highlight the need to inform farm workers on the different level of exposure to AMR determinants during daily farm work, and the necessity to use PPE especially in the most dangerous farm practices like carcasses' and litter removal. In this way, it will be possible to effectively reduce the level of exposure to infection, from farm-to-farm workers, and eventually outside the farm, to all the human community.

5.7. Acknowledgments

We thank the broiler farmer for the willingness to take part to this analysis.

Tables

Score	Indicators	Work hours	Personal Protective Equipmentuse (masks, gloves, goggles)	Number of animals for worker
1	Practices without animals	<2	3 out of 3	<20.000
2	Practices far from animals	2-4	2 out of 3	20-40.000
3	Contact with manure	4-6	1 out of 3	40-60.000
4	Direct contact with animals	>6	No PPE	>60.000

Table 1. Scores of the four selected indicators (practices without animals, practices far from animals, contact with manure and direct contact with animals) to evaluate the farm worker daily tasks in broiler farm.

Indicator	**Relevance**	
	MRS	**ESBL-producing *E. coli***
Contact	3	4
Working hours	4	2
PPE	4	4
Number of animals for worker	1	2

Table 2. Importance (with a score from 1, very low, to 4, very high) for each indicator for exposure to AMR determinants, depending on the presence of MRS and ESBL-producing *E. coli*.

Time (day of the cycle)	**MRS**		**ESBL-producing *E. coli***	
	n positive samples/n tested (%; 95% CI)			
	Animal	*Environment*	*Animal*	*Environment*
0 (before chicks' arrival, 2nd cycle)	-	-	-	0/3[0%; 0.0-70.7]
1 (day 1, 2nd cycle)	0/30 [0%; 0.0- 11.6]	2/3 [66.7%; 9.4-99.1]	0/30 [0%; 0.0- 11.6]	0/3[0%; 0.0-70.7]
2 (day 6, 1st cycle)	17/30 [56.7%; 37.4- 74.5]	3/3 [100%; 29.2-100.0]	7/30 [23.3%; 9.9-42.3]	1/3 [33.3%; 0.8-90.6]
3 (day 27, 1st cycle)	22/30 [73.3%; 54.1-87.7]	3/3 [100%; 29.2-100.0]	1/30 [3.3%; 0.1-17.2]	0/3 [0%; 0.0-70.7]
4 (after chicken departure, 1st cycle)	-	-	-	0/3 [0%; 0.0-70.7]
0verall	39/90 [43.3%; 32.9-54.2]	8/9 [88.9%; 51.7-99-7]	8/90 [8.9%; 3-14.8]	1/15 [6.7%; 0.2-31.9]

Table 3. Phenotypic results for MRS and ESBL-producing *E. coli* detected in animals' and environmental samples in different phases of the two sampled productive cycles. During T0 and T4, only environmental samples (litter) were collected for the research of ESBL-producing *E. coli*.

Practice	Contact	Working hours	PPE	Number of animals for farm worker	**Exposure level**	
					MRS	**ESBL-producing *E. coli***
Box preparation	1	2	4	1	**2**	**1**
Chicks' offload	4	1	4	2	**1**	**3**
Carcasses' removal	4	1	3	2	**1**	**3**
Maintenance	2	1	3	2	**1**	**2**
Litter aeration by tumbling	3	1	4	2	**1**	**3**
Animal weighing	4	1	4	2	**1**	**3**
Female loading (A)	2	1	4	1	**1**	**2**
Female loading (B)	2	1	3	1	**1**	**2**
Male loading	2	2	4	1	**2**	**2**
Litter removal	3	2	4	1	**2**	**3**
Barn clean-up	1	3	4	1	**2**	**1**
Barn disinfection	1	1	3	1	**1**	**1**
Troughs' clean-up	1	1	4	1	**1**	**1**

Table 4. Working practices in the farm, with scores indicating the relative level of exposure to MRS ed ESBL- producing *E. coli* (1: low, 4: high).

Work practice	Level of exposition	Animal prevalence	Environmental prevalence	**RPC (MRS)**
Box preparation	2	Not included*	Not included*	**2**
Chicks' offload	1	1	4	**1**
Carcasses removal (<20 days)	1	3	4	**1**
Maintenance (<20 days)	1	3	4	**1**
Litter aeration by tumbling (<20 days)	1	3	4	**1**
Carcasses removal (>20 days)	1	4	4	**1**
Animal weighing (>20 days)	1	4	4	**1**
Female loading (A)	1	4	4	**1**
Female loading (B)	1	4	4	**1**
Male loading	2	4	4	**2**
Litter removal	2	4	4	**2**
Barn clean-up	2	Not included*	Not included*	**2**
Barn disinfection	1	Not included*	Not included*	**1**
Troughs' clean-up	1	Not included*	Not included*	**1**

Table 5. *Risk priority code* (RPC) for MRS. *Since we did not conduct animal and environmental sampling during these phases, the RPC calculation was based only on the level of exposure.

Work practice	Level of exposition	Animal prevalence	Environmental prevalence	**RPC (ESBL-producing E. coli)**
Box preparation	1	1	1	**1**
Chicks' offload	3	1	1	**1**
Carcasses' removal (<20 days)	3	2	2	**2**
Maintenance (<20 days)	2	2	2	**2**
Litter aeration by tumbling (<20 days)	3	2	2	**2**
Carcasses' removal (>20 days)	3	1	1	**1**
Animals' weighing (>20 days)	3	1	1	**1**
Female loading (A)	2	1	1	**1**
Female loading (B)	2	1	1	**1**
Male loading	2	1	1	**1**
Litter removal	3	1	1	**1**
Barn clean-up	1	Not included*	Not included*	**1**
Barn disinfection	1	Not included*	Not included*	**1**
Troughs 'clean-up	1	Not included*	Not included*	**1**

Table 6. *Risk Priority Code* (RPC) for ESBL-producing *E. coli*. * Since we did not conduct animal and environmental sampling during these phases, the RPC calculation was based only on the level of exposure.

Work practice	**MRS**
Litter removal	2A
Male loading	2B
Barn clean-up	2C
Box preparation	2D
Chicks' offload	1A
Animals' weighing	1A
Carcasses' removal	1B
Litter aeration by tumbling	1B
Maintenance	1C
Female loading (A)	1D
Female loading (B)	1E
Troughs' clean-up	1E
Barn disinfection	1F

Table 7. Classification of the different work practices with respect to the level of exposure to be in contact with MRS. Number (1=high exposure degree, 2=low exposure degree) and letters (from A=highest level of exposure, to F=lowest level of exposure) are used for a better classification of the level of exposure to the AMR determinants.

Work practice	**ESBL-producing *E. coli***
Carcasses' removal (<20 days)	2A
Litter aeration by tumbling	2A
Maintenance	2B
Chicks' offload	1A
Animals' weighing	1A
Carcasses' removal (>20 days)	1B
Litter removal	1B
Male loading	1C
Barn clean-up	1D
Female loading (A)	1E
Box preparation	1E
Female loading (B)	1F
Troughs' clean-up	1G

Table 8. This table shows the classification of the different work practices with respect to the level of exposure to be in contact with ESBL-producing *E. coli.* Number (1=high exposure degree, 2=low exposure degree) and letters (from A=highest level of exposure, to H=lowest level of exposure) are used for a better classification of the level of exposure to the AMR determinants.

Chapter 6 - Detection of MRCoNS, MRSA and Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in one intensive swine farm from northern Italy and exposure of farm workers to these resistant bacteria

6.1. Abstract

Domestic swine is a well-known reservoir of methicillin- resistant staphylococci (MRS) and extended-spectrum β- lactamase (ESBL)- producing *E. coli*. In this study, we investigated MRS and ESBL-producing *E. coli* colonisation in pigs (~100 animals) and farm environment from an intensive farm of northern Italy; overall farm management, vaccinations and antimicrobial usage (AMU) were also considered for the selected five productive sectors: weaning piglets, gilts, sows in parturition, sows in gestation and sows in fecundation. ESBL- producing *E. coli* related prevalence varied in animals' samples, ranging from 50% in parturition sows' sector to 93.3% in gilts; in the environment of the same sector, we detected a 100% of positive samples, while no ESBL-producing *E. coli* was recovered in gestation barns. High percentages of MRS were detected in all animal sectors; particularly among weaning piglets all animal and environmental samples resulted positive. Different staphylococcal species, including *S. aureus*, were found. Phenotypic prevalence data were employed to evaluate the exposure of farm workers to MRS and ESBL-producing *E. coli*, during different daily working activities; FMEA (*Failure Modes and Effect Analysis*) was used for the exposure assessment, which revealed that removal of dead pigs >25kg (gilts), fecundation of gilts, and piglets' tattooing and castration were the most dangerous activities for ESBL-producing *E. coli* exposition. Even for MRS exposition, piglets' tattooing and castration resulted risky for farmers. Our study highlighted the simultaneous presence of MRS and ESBL-producing *E. coli* in pigs and farm environment from diverse animal sectors. The high percentages of positive animals and the associated environmental contamination need to be considered in order to find effective strategies to protect farmers.

6.2. Introduction

Looking to the previous chapters, we understood that domestic swine species can be reservoir of Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, methicillin-resistant coagulase negative staphylococci (MRCoNS) and methicillin-resistant *Staphylococcus aureus* (MRSA). Thence, it is extremely important to assess the colonization by these AMR bacteria in the different animal productive categories, which can be reservoir at different level. These resistant bacteria can be present across diverse productive stages in the same farm, and, they can be transmitted to farm workers [66]. For these reasons, we investigated the prevalence of methicillin- resistant staphylococci (MRSA+ MRCoNS) and ESBL-producing *E. coli* in healthy pigs, in different productive phases and farm environments, in an Italian intensive farm of Lombardy, where pigs are sold to produce Parma ham. The prevalence found in the diverse animal sectors was used in a semi-quantitative exposure assessment method, to categorize daily farm working practices, to understand the level of exposure of workers to these AMR microorganisms. On farm antimicrobial usage (AMU), vaccination protocols, information on farm structure (e.g. ventilation and floor) and management, animal movements and density, were also evaluated, to find out potential drivers of AMR onset and dissemination across farm.

6.3. Materials and Methods

The intensive swine farm is located in Lombardy, and it is an agistment. It was selected because farm workers were willing to help during samplings and to give information on farm management. Farm workers daily involved in farm practices were 5. This study started in November 2018 till February 2021. Data collection was carried out by the veterinarian Enrico Riva, including:

1. Internal and external measurements of the farm structure
2. Observations of animal movements
3. Observations of farm management
4. Collection of information directly from farm workers
5. Observation of daily farm practices
6. Animal and environmental samplings to detect AMR bacteria (MRS and ESBL- producing *E. coli*).

6.3.1. Collection of data on farm buildings and compartments

Data of farm building was collected through indirect observation of external compounds through Google Maps, while to collect data directly on internal measurements of boxes (animal compartments) it was used a measuring tape. Observations on the diverse types of animal compartments, calculation of the number of boxes in the single building, observation on the type of pavement, wall, ceiling, type of enclosure of the single box, the presence of windows and the location of doors were carried out. Air and ventilation, light and temperature management were evaluated, while direct observation of drinking trough and eating troughs and the type of food supply (automatic or not), and of environmental enrichments, was performed. 2D and 3D reconstruction of the various farm buildings were carried out through the software *Sweet Home* 3D® [291].

6.3.2. Animal movement

Direct observations of animal movements in the 3 directions were carried out for:

- Boar (intact, sexually mature male pig). Activities of the farm worker to sign in heat (oestrus period) sows and gilts were considered too. During this phase the observation of how animals are inseminated were carried out.
- Sows. From the arrival on farm to the diverse farm sectors
- Piglets. Observation of suckling piglets in farrowing room after cross-fostering (exchange of offspring between litters) and in other farm sectors, till the farm leaving. By using *Sweet home* software, the animal movements were be graphically highlighted on the farm planimetry.

6.3.3. Demographic analysis on swine population

Taking in consideration data given by the farm owner, it was created an Excel file to keep track of animal movement in and out the farm for 3 months. To calculate on farm animal population, it was sum up the numbers of breeders plus piglets (nursing pigs and weaned) at G0 (considered the day of post-weaning). This calculation was carried out for 90 days (3 months). Afterwards it was

calculated the mean, that will be used to compare the different prevalence (MRS and ESBL-producing *E. coli*) found at different moments of sampling.

6.3.4. Animal treatments

Pharmacological treatments and route of administration were evaluated through farm paper registries analysis. Disinfectants were also annotated. Vaccines were evaluated through the farm vaccination protocol for sows and piglets.

6.3.5. Laboratory analysis

Five samplings were performed in different animal sectors:

- sows in parturition
- sows during fecundation,
- gilts
- sows in gestation
- weaning piglets

To identify samples, a code was used with the initial capital letter "E", to identify the farm, followed by the number of the sampled productive phase, (1. parturition; 2. fecundation; 3. gilts; 4. gestation; 5. weaning;), the letters "A/H" were added to indicate if the sample was of animal or environmental origin, "S/E" to indicate if the sample was taken to detect MRS or ESBL-producing *E. coli*, and, at the end, a progressive number. For instance, E4AS11 indicated the sample number 11, taken from the gestation compartment to detect MRS.

The sample size in each animal sector was calculated through WinEpi© Tool [292]. We considered a population of 1500 subjects, a minimum expected prevalence for MRS and ESBL-producing *E.* coli of 30% in the environment and 10% among animals. For animals, we thus obtained a sample size of 29, rounded to 30 animals, and for environmental samples we obtained 9, rounded to 10.

Since during the first two samplings (sows in parturition sector and sows in fecundation) a high AMR bacteria prevalence (~75%) was found, and due to economic issues, the last samplings were performed on half of the total number. Samples taken from sows in gestation were 15 for MRS and 15 for ESBL-producing *E. coli*, while for the environmental analysis we took 5 samples for each bacterial group. The same quantity of samples was collected in the gilts sector. For weaners we collected 15 animal samples and 5 environmental samples for each bacterial group.

Nasal (for MRS detection) and faecal (for ESBL-producing *E. coli* identification) swabs were collected using sterile swabs with Amies transport medium (Microbiotech s.r.l., Maglie, Italy). For further details on the laboratory protocols used to isolate these resistant bacteria see previous Chapters 4 and 5. A total number of 99 nasal swabs were collected, while 107 faecal swabs were taken. For ESBL-producing *E. coli* environmental isolation, it was decided to use a wet swab in Amies transport medium through a paper frame of 100 cm^2 area, privileging highly faeces dirty areas like pavements, and sites less contaminated, like walls, troughs and enrichment tools. Swabs were rolled four times on contaminated surfaces. For MRS, sterile swabs were spread using the same technique of four passages on non-faecal contaminated areas, like walls, ceiling, tubes, lamps, blackboards and windows, using a dedicated paper frame. In total 37 environmental swabs were collected for ESBL-producing *E. coli* and MRS respectively.

After phenotypic analysis, DNA was extracted using a modified boiling method (see Chapter 4 paragraph 4.5.4. Genotypic Analysis). Bacterial strains from pure cultures (1 or 2 colonies with the same aspect) were stocked with 15% of glycerol at -80 °C for further tests. PCR was performed on MRS phenotypic positive sample to detect *mecA* gene (527 bp) and to confirm the presence of MRSA through a triplex PCR protocol (16S rDNA*, mecA, nuc*). A simplex PCR for species identification was performed targeting 16S rDNA (500 bp).

To confirm the presence of ESBL-associated genes $bla_{CTX\text{-}M}$ and bla_{TEM}, two different PCR were performed separately for each phenotypic positive sample. To confirm PCR results, Sanger sequencing was used on a group of positive amplicons. For further information on protocols see Chapter 5 paragraph 5.3.3. “Laboratory and statistical analyses”.

6.3.6. Exposure assessment of farm workers- Failure Modes and Effect Analysis (FMEA)

To assess the exposure of farm workers to MRS and ESBL-producing *E. coli* from animals and farm environment, the *Failure Modes and Effect Analysis* (FMEA) method was used, using four main indicators: type of contact, contact hours, use of PPEs (personal protective equipment) and number of animals for farm worker. These indicators were chosen considering expert opinions from public and private veterinarians, professors of the Department of Veterinary Sciences and swine farmers. To categorise the risk to be in contact with selected AMR bacteria, we used a score from 1 to 4 for each of these indicators. It was decided to proceed through phases:

Phase 1: risk categorization through setting up of a specific table with the different scores for the selected indicators (Table 1).

Score	**Type of contact**	**Hours of work (h)**	**PPEs**	**Number of animals for worker**
1	Farm entry without any contact	<2	3/3	<200 animals
2	Potential contact with dejections	2-4	2/3	200-400 animals
3	Minimum contact	4-6	1/3	400-600 animals
4	Direct contact	>6	0/3	>600 animals

Table 1: Categorization of the risk indicators identified in swine farming sector.

Phase 2: finding farm practices to be evaluated. Observation of farm activities involving farm workers in contact with pigs and dejections was carried out. Afterwards, a score was assigned to the different indicators of that farm procedures. A total of 18 farm practices were identified. Data were collected using a specific scheme (Figure 1).

Phase:

Score	**Type of contact**	**Hours of work**	**PPE**	**Number of animals for worker**
1				

2				
3				
4				
Description:				

Figure 1: schedule used to collect data on farm.

Phase 3: after the identification of the different farm practices, a level of importance was attributed to the related indicators (ranging from 1 to 4) based on the MRS or ESBL-producing *E. coli* prevalence.

For MRS, levels of importance were:

- type of contact: 3
- hours of work: 4
- PPEs: 4
- number of animals for operator: 1

For ESBL-producing *E. coli,* the following are the levels of importance:

- type of contact: 4
- hours of work: 2
- PPEs: 4
- numbers of animals for operator: 2

We referred to expert opinions and scientific literature to assign these levels. The use of PPEs, specifically face mask, and hours of work were considered more relevant for MRS, due to the air transmission route [293,294,295]. Looking to ESBL-producing *E. coli*, type of contact and PPEs were more important, for the high faecal load of this microorganism and the elevated animal and farm environment contamination [296,297]. Thence, the use of gloves was considered paramount, while the hours of work on farm less important, due to the fact that the privileged transmission route of ESBL-producing *E. coli* is direct.

Phase 4: evaluation of the level of exposition. The RPC (Risk Priority Code) was calculated after collecting information of phase 2 and levels of importance of phase 3, through the following equation:

RPC (ai)=Minj[Max{Neg(I(gj)),gj(ai)}]

"RPC (ai)" is the Risk Priority Code with the modality of potential risk "ai". "I(gj)": importance associated to each indicator, identified as "gj"; "Neg(I(gj))": negation of the importance associated to each evaluation criterion; "gj(ai)": association of the potential risk with each indicator [275].

Phase 5: set-up of a scale with 4 levels to define the prevalence intervals in animals and environment for both bacterial groups. - Score 1: prevalence < than 30%; - Score 2: prevalence between 30 and 60 %; - Score 3: prevalence between 61 and 80 %; - Score 4: prevalence > than 80 %.

Phase 6: calculation of the raw prevalence of the two AMR bacterial groups in the different productive phases for animals and environment, based on the lab analysis and prevalence intervals calculated in phase 5.

Phase 7: set-up of a table to stratify the risk based on the exposition to AMR, where RPC of the farm practice is associated with the RPC values originated from the animal and environmental prevalence detected during that farm practice.

Phase 8: set-up of a table for each AMR bacterial group considering other three new indicators obtained from previous phases: intensity of exposition of the specific farm practice, relative animal and environmental prevalence of MRS and ESBL-producing *E. coli*.

Phase 9: through the association of the latter three indicators, calculating the relative RPC and the attribution of the same level of importance to the indicators, to obtain a priority risk code related to the farm practice in each productive phase.

6.4. Results

6.4.1. *Farm structure and management description*

The intensive swine farm raised finishing pigs for the production of Parma ham. The farm had no physical barriers to entry, just a sign of access denied for non-authorised people. At the entrance, a dedicated area with disinfectant for trucks' wheels was present. Pigs from DanBred hybrid (obtained from DanBred Landrance and Danbred Yorkshire) swine cross-bred were raised, and gilts were imported from a Danish DanBred farm. This type of breed was associated with large vital offspring, with an average of 20 piglets per litter in this farm. The piggery hosted 750 sows with 2 boars that were used to show in heat sows and to identify the best moment for sows' insemination. Semen was bought from an external producer.

The farm consisted of four buildings, composed by 6 sectors that represented a particular productive phase of swine farming. Other two parts were present: the infirmary and a section dedicated to sows no longer involved in farrowing.

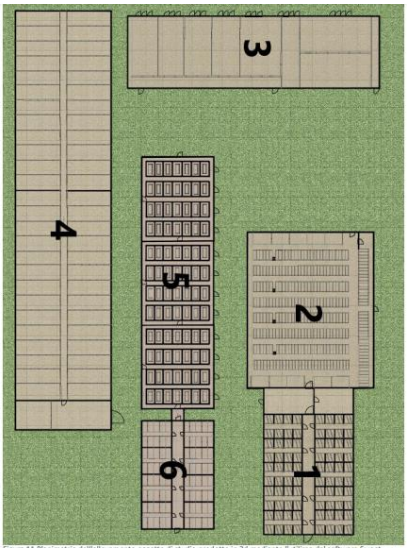

Figure 2: farm planimetry obtained using the Sweet Home 3D® software (Image created by Enrico Riva).

Sector 1 included the “farrowing room”, which was divided in 10 rooms, isolated from each other with a door. Every room had eight boxes, which were sub-grouped in 2 parts; the central was dedicated to sows, while in the external part hosted nursing piglets. The sow-associated floor was iron-slatted, while plastic-slatted for piglets. Gas lamps were activated for heating and an external thermometer was used to indicate internal temperature (generally 27 °C). Fans were present for ventilation. Sows were fed automatically, while piglets had little troughs and the recently added automatic feeding cups for milking. Windows were only on one side of the room to let light enter the room and give positive effects on piglets’ growth.

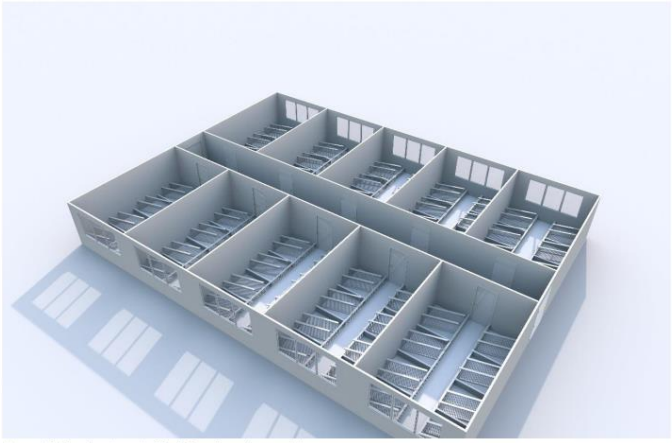

Figure 3: sector 1, namely “farrowing room” (Image created by Enrico Riva with Sweet Home 3D® software).

Sector 2 was associated to sector 1, and is dedicated to sows’ fecundation. There were four rows with 30 single boxes per sow, four rows with 29 single boxes, another row with 42 single boxes and 10 boxes for four sows each. Boundaries dividing boxes were in iron, and sows were located on concrete slatted floor. Active ventilation and automatic feeding were present.

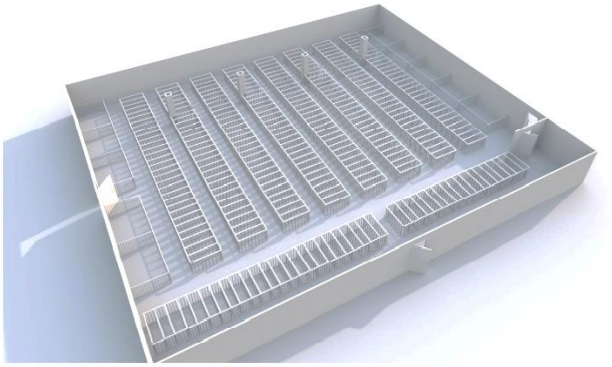

Figure 4: sector 2, namely “fecundation room” (Image created by Enrico Riva with Sweet Home 3D® software).

Sector 3 was dedicated to gilts and was divided in two rooms. Floor was partially slatted. Passive ventilation was present. In the largest boxes a maximum of 20 gilts could be located, in the smaller a maximum of 10. Number of animals was limited based on Animal Law on welfare. Animals were fed manually.

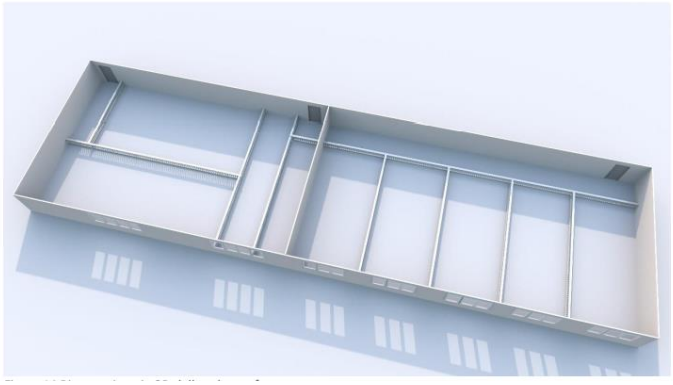

Figure 5: sector 3, namely gilts' rooms (Image created by Enrico Riva with Sweet Home 3D® software).

Sector 4 had 52 boxes, that could host until seven sows in gestation. There were enrichment tools, like wall chains, in each box. Concrete boundaries separated boxes. A concrete slatted floor was present and animals were fed automatically. There were windows on both sides of this sector to allow 12 hours of light during the day. There was no ventilation but just passive air flow through opened windows. At the entrance, in the first box a boar was present.

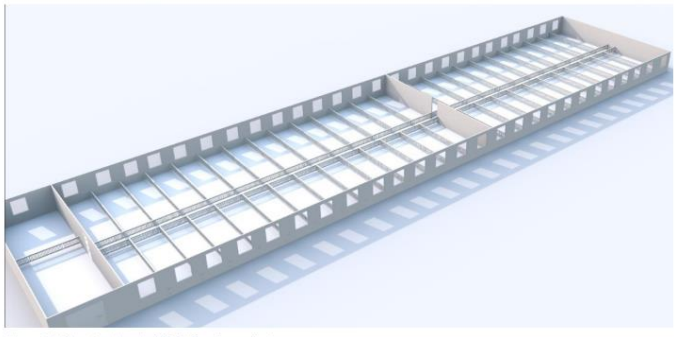

Figure 6: sector 4, namely gestation room (Image created by Enrico Riva with Sweet Home 3D® software).

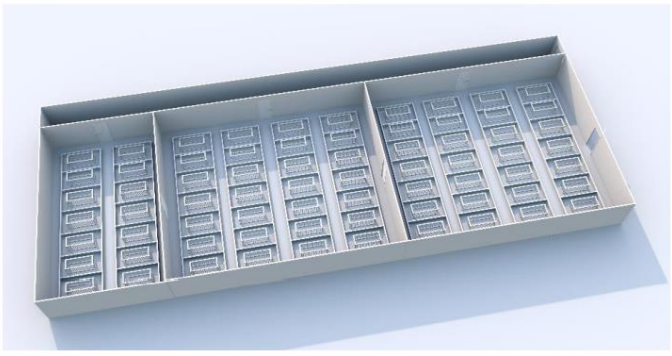

Figure 7: sector 5, namely new farrowing room (Image created by Enrico Riva with Sweet Home 3D® software).

Sector 5 comprised two rooms for 28 animals, and one room for 14 animals located in box; they were further divided in two for the presence of one part for sow and the other for offspring. Floor was iron slatted while for the offspring was plastic slatted. There were no windows, and light was guaranteed with a neon lamp. Like the other farrowing sector, this new farrowing sector had a ventilation system. Infrared lamps were present for heating and a thermometer was located outside

every room to monitor temperature variations. In the first two rooms the average temperature was 24 °C, while for the other was 20°C.

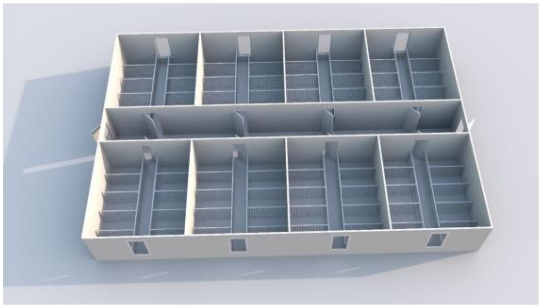

Figure 8: sector 6, namely weaning rooms (Image created by Enrico Riva with Sweet Home 3D® software).

Sector 6 had eight rooms with 10 boxes each; in each box there were a maximum of 20 piglets for a total of 1600 animals of this sector. Boxes were higher with respect of the central corridor. A window was present for each box and neon lamp guaranteed light of the sector. A system of ventilation was present and gas lamps were used to heat single rooms, while temperature was not monitored.

6.4.2. *Animal movement*

There were essentially three types of animal movements: boar movement that occurred daily; sows' movement that occurred once a week, the day before sows' weaning; piglets' movement that happened once a month.

In swine farming at least one boar is necessary to evoke immobility response in sows during oestrus; for this reason, male pigs when present on farm, were located outside sows' sectors to avoid sows getting used to their presence and odour. Boar started his movement daily from sector 2 (Figure 9), namely fecundation room, in which there were 2 farm workers, one was in charge of boar's movement across the corridor and sows' boxes, while the other tried the immobility response on sows' back. When this response was present, the farm worker signed a keyboard associated to the sow's box and the positive sow could be inseminated. On the sow's keyboard, date of insemination, morning or afternoon, owner's initials and a progressive number were reported (Figure 10). Afterwards, the boar visited sector 3, where gilts were located. Here the boar was free to walk inside gilts' boxes (for animals > 120 kg and > 8 months). If gilts were positive for the immobility response, they were marked and divided from the negative gilts. Gilts found in heat for the first time, did not receive the insemination; however, they were marked with a blue or red line. Contrary, for the gilts that were found in heat at the second time, insemination was performed, and a double red/blue line was used as mark. The last step of boar's movement was across the gestation sector. Here, finding positive sows to the immobility response was a negative sign, that indicated that the animal had not been inseminated correctly; thence, based on the presence of lesions, body condition score (BCS) and previous litters' size and vitality, it was decided if inseminate again the animal or not, excluding her from breeding purposes.

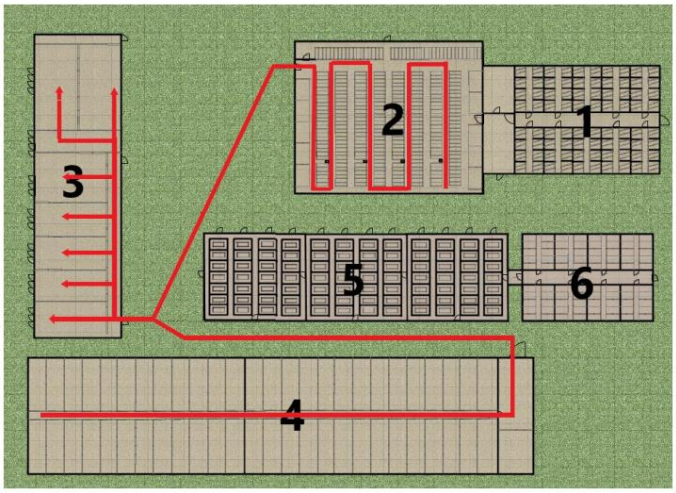

Figure 9: boar movements across diverse farm sectors (Image created by Enrico Riva with Sweet Home 3D® software).

Figure 10: sow box's keyboard to identify the in heat sow and for tracking the date of insemination.Photo by Enrico Riva.

Considering sows' movement on farm, gilts came from external remount (Denmark) on a regular basis, twice each trimester. Productive cycle started in the gilts' sector with insemination. After this event, they stayed in this farm sector until 3-7 days before parturition. Then they went to sector 1 or 5 (farrowing sector), depending on boxes availability. In these sectors they stayed at least 2 weeks to allow a correct piglets' milking and uterine involution. Once a week the sows' weaning occurred, during which 28-32 animals were weaned. After this event, they went to sector 2 (fecundation) to stay in a single box. The first oestrus (after parturition), that was useful for fecundation, generally happened 4-8 days after weaning, with a peak at 5 days. After 3 weeks from fecundation, abdominal echography was performed to diagnose pregnancy. Afterwards, animals were located in gestation sector, where boxes could host seven sows (Figure 11).

Piglets were moved daily when cross-fostering was necessary among diverse boxes in farrowing rooms (sector 1 and 5). Then, they left these sectors to reach sector 6 for weaning phase. The optimal weaning weight (7 kg) was reached between 21 and 28 days (Figure 12). With a periodic interval, twice a month, they were moved from the farm to get to a finishing farm in Lombardy.

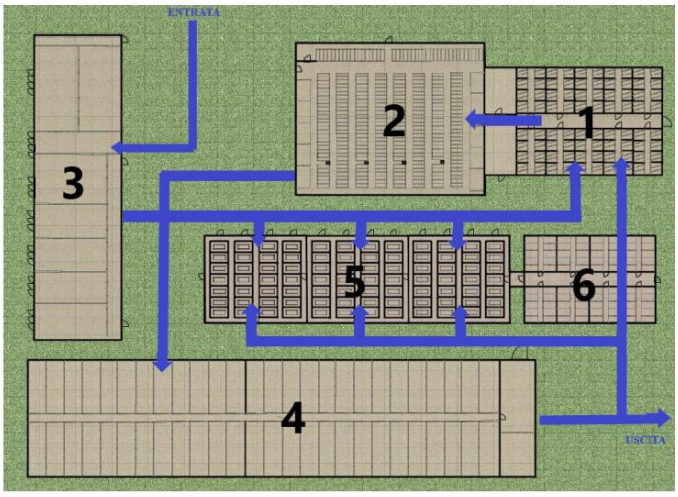

Figure 11: on farm sows' movement (Image created by Enrico Riva with Sweet Home 3D® software).

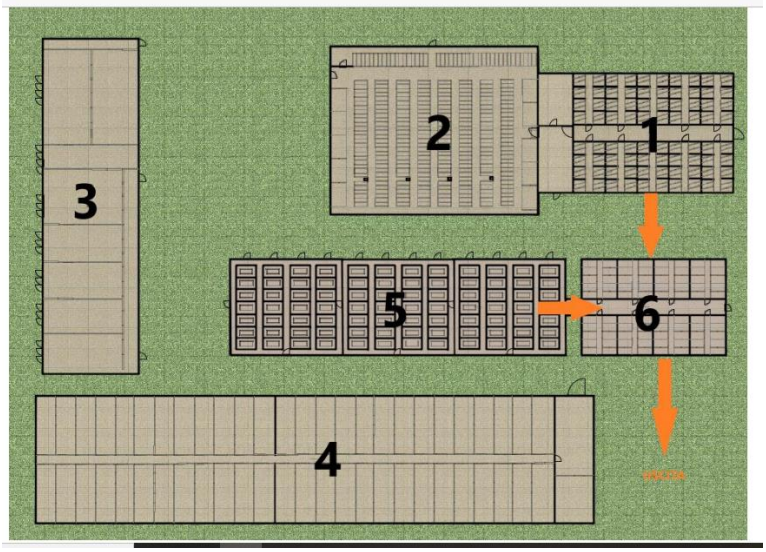

Figure 12: on farm piglets' movement (Image created by Enrico Riva with Sweet Home 3D® software).

6.4.3. Demographic analysis on swine population

The demography of this type of swine farm was very fluctuating; indeed, daily birth of new piglets and income of new gilts, imported once a month and half, increased the number of animals present on farm; however, a reduction was determined by dead piglets, sows no longer able to farrow, and, mostly, by the export of weaned piglets every 15 days.

Breeders were a stable population of about 750 animals, while all the other categories (nursing pigs and post weaning piglets) were extremely variable, due to the continuous new-borns and piglets (700-1000 animals) sold twice a month. Thus, it was really difficult to analyse a farm like a steady population, and arithmetic average was used to calculate the number of animals. Using this tool, 1500 animals were considered as the population daily present on farm.

6.4.4. Animal treatments

Antibiotic treatments were generally administered to piglets and sows (see table 2). In piglets they were mostly used during castration and weaning period. Ceftiofur was regularly used for castration and was administered intramuscularly (IM) with a multidose syringe (5 mg/kg). The same antibiotic was used in female piglets, even if they were not neutered. The use of this 3rd generation cephalosporin ceased in November 2020; for the same prophylactic purpose amoxicillin, through a multidose syringe, was administered IM to neutered and non-neutered piglets. An oxytetracycline spray was applied locally on surgical wound after castration.

During the weaning period amoxicillin with clavulanic acid was administered IM when there were cases of exudative epidermitis generally caused by *Staphylococcus hyicus* [298]. A medicated feeding, Nutrilac Baby Med®, with amoxicillin was administered all the weaning period for prophylactic reasons. This feeding was added with zinc in a concentration of 171mg/kg. The macrolide tulathromycin, was used intramuscularly with a multidose syringe (2,5mg/kg), in the treatment and prevention of the swine respiratory disease (SRD) associated to *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Mycoplasma hyopneumoniae* and *Haemophilus parasuis*. This type of administration can be considered a metaphylaxis because all the pen's animals were treated even if not all of them manifested respiratory symptoms. Against respiratory syndrome sustained by *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni* infections, tildipirosin was also used IM with a multidose syringe, when animals were symptomatic.

In sows, amoxicillin was administered IM when animals present lesions to limbs or for respiratory-associated conditions. In this case, treatment is individual. The fluoroquinolone marbofloxacin was administered at 2 mg/kg, when respiratory disease was thought to be caused by *Pasteurella multocida* and *Mannheimia haemolytica*, and also for metritis and mastitis. Even in this case, treatments were individual.

The only disinfectant used on farm was Virkon®S, which contains potassium peroxymonosulfate and sodium chloride. It has a detergent action associated to bactericidal, virucidal and fungicidal effects. It was generally used to disinfect farm environment and to treat *Staphylococcus hyicus* related exudative epidermitis, disinfecting piglets' barns. After a dilution in water of the solid product, this disinfectant was sprayed in weaning and parturition sectors. A rest period of 3 days was applied after the disinfectant nebulisation.

Antibiotic	**Antibiotic class**	**Animal category**	**Route of administration**	**Type of administration**
Amoxicillin	Aminopenicillin	Sows, piglets	IM sows, oral and IM piglets	Individual in sows, group in piglets (methaphylaxis)
Ceftiofur	3rd generation cephalosporin	Piglets	IM	Group (prophylaxis)
Marbofloxacin	Fluoroquinolone	Sows	IM	Individual
Oxytetracycline	Tetracycline	Piglets	Skin (local)	Group
Tildipirosin	Macrolide	Piglets	IM	Group
Tulathromycin	Macrolide	Piglets	IM	Group (methaphylaxis)

Table 2: antibiotic treatments used in sows and piglets. "IM" indicates intramuscular administration.

There were specific vaccinations protocols for piglets and sows. Considering piglets, the first vaccine was administered during tattoo application. The needleless intradermic monovalent vaccine Porcilis® PRRS (MSD Animal Health) was used against porcine reproductive and respiratory syndrome (PRSS), while the bivalent Porcilis® PCV M Hyo was administered against porcine Circovirus type 2 and *Mycoplasma hyopneumoniae*.

Sows were vaccinated with a multidose syringe using the following products:

- UNISTRAIN® (HIPRA), for immunisation against PRRS, with a dose of 2 ml every 75 days.
- Porcilis® BEGONIA (MSD Animal Health), for immunisation against Aujezky's disease, with a dose of 2 ml every 120 days.
- Porcilis® AR-T ® (MSD Animal Health), for immunisation against *Bordetella bronchiseptica*, responsible of atrophic rhinitis. The protocol was the following: a first vaccination 60 days after the arrival on farm and a second injection 90 days after the pregnancy diagnosis.
- Porcilis® Glässer (MSD Animal Health), for immunisation against *Haemophilus parasuis* serotype 5, etiological agent of Glässer's disease; a dose (4 ml) was administered at 80 days after the pregnancy diagnosis.
- Porcilis® ERY-PARVO (MSD Animal Health), for immunisation against porcine Parvovirus and *Erysipelothrix rhusiopathiae*; a single dose (2 ml) was administered at 7-8 days post-partum.

6.4.5. Laboratory analysis results for ESBL-producing E. coli

In parturition sows' category, we detected the lowest prevalence (50%) of phenotypic positive animals. In fecundation sector 70% (95%CI: 50.6%, 85.3%) of tested sows were positive; however, in the environment almost all samples were negative (16.7%). Among gilts the highest number of positive animals (93.3%) and positive environmental samples (100%) were found (Table 3). A total prevalence of 64.5% (95%CI: 55.4%, 73.5%) was detected among tested animals (n=107)

As regards molecular analysis, almost all positive phenotypic samples possessed at least one *bla* gene or both (bla_{CTX-M} + bla_{TEM}):

- In parturition sows' category, all positive samples had bla_{CTX-M} (16/32), among these 11 were positive for bla_{TEM} too (11/16). The identification of bla_{CTX-M} occurred in all phenotypic positive environmental samples from this sector, only one was not positive for any *bla* gene. bla_{TEM} was detected in 5/12 samples.
- In fecundation sows' category, 14 out of 20 phenotypic positive samples possessed bla_{CTX-M}, while bla_{TEM} was always identified in association with bla_{CTX-M} (n=8). In the environment the unique positive sample had both *bla* genes.
- In gestation sow's category, bla_{CTX-M} (4/15) was the only identified gene in positive animal samples.
- In gilts' sector, bla_{CTX-M} was detected in all positive animal samples (12/15); in one case it was associated to bla_{TEM}. bla_{CTX-M} was the only gene detected in the environmental samples.
- Among piglets in weaning sector, bla_{CTX-M} was recovered in all phenotypic positive samples

(12/15), while *bla*$_{TEM}$ was detected in only 6 samples, associated to *bla*$_{CTX-M}$. In the environment only one sample was positive to both *bla* genes.

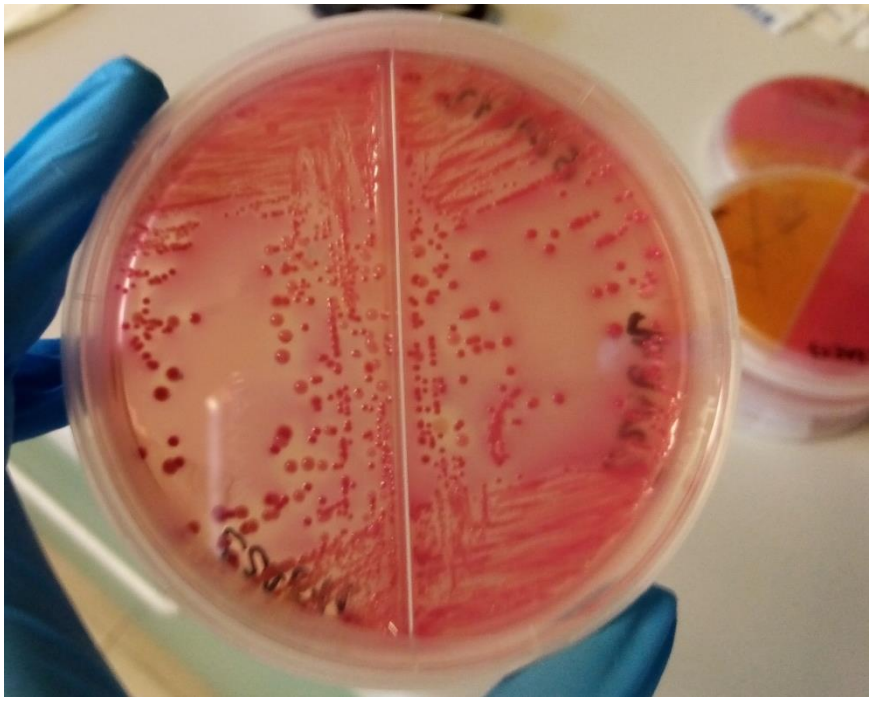

Figure 13: *Escherichia coli* on MCC3 agar. Isolated colonies are red/purple and round-shaped with a fuchsia halo around. Photo by Miryam Bonvegna.

6.4.6. Laboratory analysis results for methicillin-resistant staphylococci (MRS)

We detected high prevalence in each tested sector; particularly among sows in fecundation sector, a prevalence of 83.3% (95%CI:70%, 96.7%) was found in animals' samples, with a slightly higher prevalence in the environment (90%). In gestation sector was detected almost the same prevalence (86.7%) found in fecundation sows. In younger animals, namely gilts, a prevalence of 60% (95%CI: 32.3%, 83.7%) was detected; the environment resulted more contaminated with MRS (80%). The highest prevalence was detected in weaning sector, where all the animals resulted positive (Table 3). A total animal prevalence of 80.8% (95%CI:73%, 88.6%) was recovered.

Farm sector	**ESBL-producing *E. coli***		**MRS**	
	(95%CI)			
	Animals	*environment*	*animals*	*environment*
Parturition sows	50% (32.7%, 67.3%)	83.3% (51.6%, 97.9%)	79.2% (57.8%, 92.9%)	75% (42.8%, 94.5%)
Gestation sows	53.3% (26.6%, 78.7%)	0% (0%, 52.2%)	86.7% (59.5%, 98.3%)	80% (28.3%, 99.5%)
Fecundation sows	70% [50.6%, 85.3%)	16.7% (55.6%, 2.5%)	83.3% (70%, 96.7%)	90% [55.5%, 99.7]
Gilts	93.3% (68%, 99.8%)	100% (47.8%, 100%)	60% (32.3%, 83.7%)	80% (28.3%, 99.5%)

Weaning piglets	73.3% (44.9%, 92.2%)	20% (0.5%, 71.6%)	100% (100%, 100%)	100% [100%, 100%]
Total	64.5% (55.4%, 73.5%)	48.6% (32.5%, 64.7%)	80.8% (73%, 88.6%)	83.8% (71.9%, 95.6%)

Table 3: phenotypic prevalence related to ESBL-producing *E. coli* and MRS detected on animals and environment in different animal sectors: parturition sows, gestation sows, fecundation sows, gilts and weaning piglets.

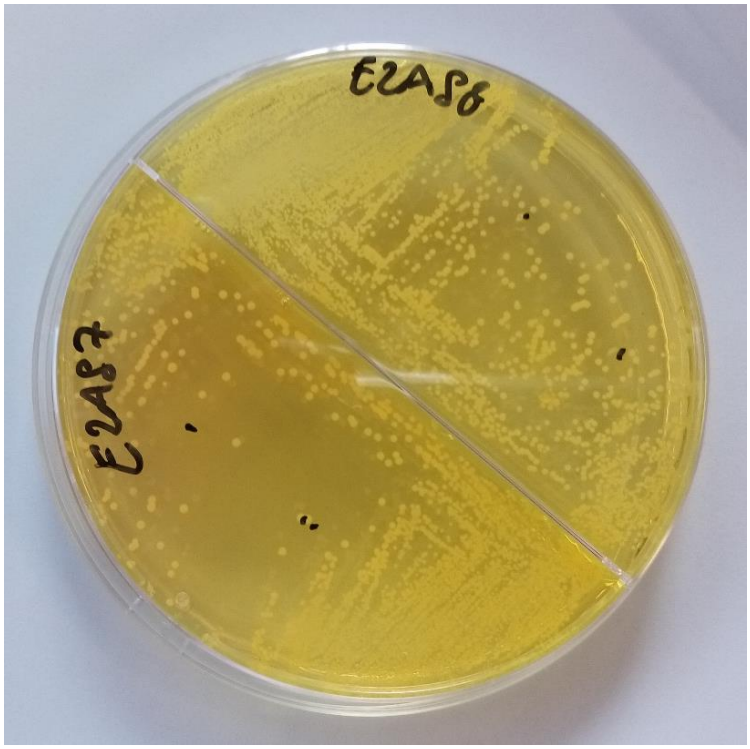

Figure 14: *S. aureus* on MSA. Colonies are characteristically mannitol-fermenting (yellow) on this selective medium. Photo by Miryam Bonvegna).

Almost all methicillin resistant staphylococci possessed the *mecA* gene. Only in 10 animal samples and 5 environmental from gestation sector, this *mec* type gene was not detected through PCR. Staphylococcal species confirmation through 16S rDNA and MALDI-TOF MS indicated that *S. aureus* was mainly present during weaning phase (Figure 15), where almost all animals carried MRSA, with the exception of one piglet that carried a methicillin-resistant *S. sciuri*. MRSA was also present in animals tested in fecundation sector, where the predominant species was however *S. sciuri*. In parturition sector *S. sciuri*, *S. cohnii* and *S. saprophyticus* were detected, while among sows in gestation *S. sciuri* was the most often staphylococcal species recovered. In gilts *S. sciuri*, *S. haemolyticus* and *S. saprophyticus* were detected (see Table 4.)
Considering the farm environment, *S. cohnii* and *S. haemolyticus* were detected in parturition sector, while in gestation sector the majority of staphylococci were *S. sciuri*. In fecundation, *S. haemolyticus* was the predominant environmental species, instead in gilts' room *S. saprophyticus* was most often recovered. A highest staphylococcal variability was found in the weaning sector, where *S. aureus*, *S. sciuri*, *S. cohnii* and *S. saprophyticus* were identified (see Table 4).

SAMPLE	**SOURCE**	**MALDI-TOF**	***mecA***	**16S rDNA seq**
E1AS2	Parturition sow	na	pos	*S. sciuri*
E1AS9	Parturition sow	na	pos	*S. cohnii*

E1AS13	Parturition sow	na	pos	*S. saprophyticus*
E1HS4	farm	na	pos	*S. haemolyticus*
E1HS5	farm	na	pos	*S. cohnii*
E1HS6	farm	na	pos	*S. cohnii*
E1HS8	farm	na	pos	*S. haemolyticus*
E1HS10	farm	na	na	*S. cohnii*
E2AS1	Fecundation sow	*S. sciuri*	pos	na
E2AS2	Fecundation sow	*S. sciuri*	pos	na
E2AS3	Fecundation sow	*S. aureus*	pos	*S. sciuri*
E2AS4	Fecundation sow	*S. sciuri*	neg	na
E2AS5	Fecundation sow	*S. sciuri*	pos	na
E2AS9	Fecundation sow	*S. sciuri*	pos	na
E2AS10	Fecundation sow	*S. sciuri*	pos	na
E2AS11	Fecundation sow	*S. aureus*	pos	na
E2AS12	Fecundation sow	*S. sciuri*	pos	na
E2AS13	Fecundation sow	*S. aureus*	neg	na
E2AS14	Fecundation sow	*S. sciuri*	pos	na
E2AS24	Fecundation sow	*S. aureus*	pos	na
E2AS27	Fecundation sow	*S. sciuri*	pos	na
E2HS1	farm	*S. haemolyticus*	neg	na
E2HS2	farm	na	pos	*S. sciuri*
E2HS3	farm	*S. haemolyticus*	neg	na
E2HS4	farm	na	pos	*S. saprophyticus*
E2HS5	farm	*S. sciuri*	neg	na
E2HS6	farm	*S. haemolyticus*	pos	na
E2HS7	farm	*S. haemolyticus*	neg	na
E2HS8	farm	*S. sciuri*	neg	na
E2HS9	farm	*S. saprophyticus*	pos	na
E4AS2	Gestation sow	*S. sciuri*	pos	na
E4AS3	Gestation sow	*S. sciuri*	pos	na
E4AS4	Gestation sow	*S. sciuri*	pos	na
E4AS5	Gestation sow	*S. sciuri*	pos	na
E4AS6	Gestation sow	*S. sciuri*	pos	na
E4AS7	Gestation sow	*S. sciuri*	pos	na
E4AS8	Gestation sow	*S. sciuri*	pos	na
E4AS9	Gestation sow	*S. xylosus*	pos	na
E4AS12	Gestation sow	*S. sciuri*	pos	na
E4AS13	Gestation sow	*S. sciuri*	pos	na
E4AS14	Gestation sow	*S. sciuri*	pos	na
E4AS15	Gestation sow	*S. sciuri*	pos	na
E4HS1	farm	*S. haemolyticus*	pos	na
E4HS2	farm	*S. sciuri*	pos	na

E4HS3	farm	*S. sciuri*	pos	na
E4HS5	farm	*S. sciuri*	pos	na
E5AS1	gilt	*S. sciuri*	pos	na
E5AS4	gilt	*S. saprophyticus*	pos	na
E5AS5	gilt	*S. sciuri*	pos	na
E5AS6	gilt	*S. haemolyticus*	pos	na
E5AS7	gilt	*S. saprophyticus*	pos	na
E5HS3	farm	*S. saprophyticus*	pos	na
E5HS4	farm	*S. saprophyticus*	pos	na
E3AS1	Weaning piglet	*S. aureus*	pos	na
E3AS2	Weaning piglet	*S. aureus*	pos	na
E3AS3	Weaning piglet	*S. aureus*	pos	na
E3AS4	Weaning piglet	*S. aureus*	pos	na
E3AS5	Weaning piglet	*S. aureus*	pos	na
E3AS6	Weaning piglet	*S. aureus*	pos	na
E3AS7	Weaning piglet	*S. aureus*	pos	na
E3AS8	Weaning piglet	*S. aureus*	pos	na
E3AS9	Weaning piglet	*S. aureus*	pos	na
E3AS10	Weaning piglet	*S. aureus*	pos	na
E3AS11	Weaning piglet	*S. sciuri*	pos	na
E3AS12	Weaning piglet	*S. aureus*	pos	na
E3AS13	Weaning piglet	*S. aureus*	pos	na
E3AS14	Weaning piglet	*S. aureus*	pos	na
E3AS15	Weaning piglet	*S. aureus*	pos	na
E3HS1	farm	*S. aureus*	pos	na
E3HS2	farm	*S. sciuri*	pos	na
E3HS3	farm	*S. cohnii*	pos	na
E3HS4	farm	*S. cohnii*	pos	na
E3HS5	farm	*S. saprophyticus*	pos	na

Table 4: identification of a group of methicillin-resistant staphylococci sampled from animals and farm environment in the 5 different productive sectors: parturition sows, gestation sows, fecundation sows, gilts and weaning piglets.
na=not available

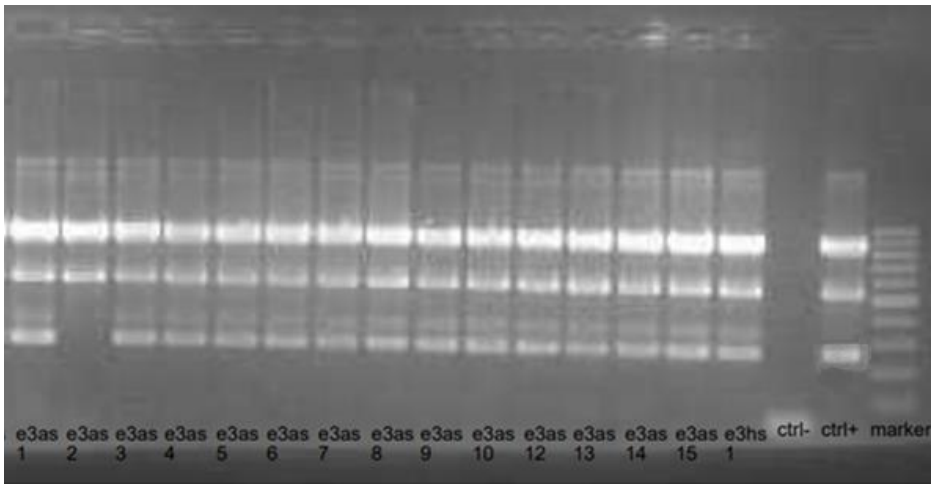

Figure 15: triplex PCR, specific for MRSA detection. All the samples are MRSA with the exception of E3AS2 (*S. sciuri*), which lacks *nuc* positive band, specific for *S. aureus*. A negative control, (ctrl-) (DNA-free water) and a positive control (ctrl+) were used. Marker of 100 bp was used (on the right). Image by Miryam Bonvegna.

6.4.7. *Analysis of farm practices and exposure assessment through Failure Modes and Effect Analysis (FMEA)*

Observing the daily farm activities, we found out that farm workers rarely used PPEs. Surgical face masks were used since the start of Covid-19 pandemics. Cleaning of farm sectors was performed daily by farm workers which wore generally disposable plastic coats and protective gloves as PPEs. However, no filtrating face mask (FFP2 or FFP3) was used. During the procedure of cleaning in gilts' sector, animals were washed too by farm workers. Sector 1 and 5 were cleaned after piglets' weaning. To do this, a pressure washer was used for all the hard surfaces present in the animal sectors (slatted floor, boxes, troughs, walls and ceiling). Afterwards, disinfection was performed using Virkon®S, that was left over the surfaces for 30 minutes. A rest period of three days was guaranteed, only if the number of sows next to the parturition was low.

In the gestation sector, cleaning through pressure washer was performed only once a week when sows were moved to farrowing rooms. No disinfectant could be used because this sector had not a real rest period.

In gilts' sector, pressure washer was used for cleaning and then disinfectant was sprayed on the hard surfaces (Virkon®S). In the fecundation sector, cleaning was difficult, and sometimes it needed more than one day, using only pressure washer, without disinfection. In the weaning sector, cleaning and disinfection with Virkon®S was regularly performed.

During fecundation procedure in sector 2, and animal manipulation in gestation sector, all the activities, that consisted in touching more animals in a short period, were performed without gloves and filtrating face masks. Farm practices involving nursing piglets, like castration, injection of antibiotics, tattoo application, cross-fostering and animal loading were all performed with only disposable gloves. Considering the previous 4 indicators (type of contact, hours of work, PPEs and number of animals for worker), a risk priority code associated to MRS and ESBL-producing *E. coli* was calculated for each farm practices previously observed (Table 5).

Farm practices	Type of contact	Hours of work	PPEs	Number of animals for worker	RPC MRS	RPC ESBL-producing *E. coli*
Dejections' cleaning	2	1	4	1	1	2
Farm sectors' cleaning and disinfection	2	2	2	1	2	2
Manual feeding	1	1	4	2	1	1
Maintenance	1	1	4	1	1	1
Animal movement (sows and boar)	2	1	4	2	1	2
Animal movement (piglets)	4	2	4	2	2	3
Animal inspection	1	1	4	4	1	1
Piglets' care	4	2	3	2	2	3
Removal of placenta and dead piglets	4	2	3	3	2	3
Removal of dead pigs <25kg	4	1	3	1	1	3
Removal of dead pigs >25 kg	4	1	3	1	1	3
Piglets' activities (castration, antibiotic treatment…)	4	4	3	3	3	3
Piglets' tattooing	4	4	3	4	3	3
Sows' treatments	2	1	3	1	1	2
Piglets' treatments	4	2	4	3	2	3
Fecundation	4	3	3	4	3	3
Piglets' loading	4	2	3	4	2	3
Gilts' unloading	2	1	3	1	1	2

Table 5: calculation of the value attributed to the single indicators (type of contact, hours of work, PPEs and number of animals for worker) for each selected farm practice is here reported. Also, calculation of the level of intensity of exposition for the various farm practices is reported considering both MRS and ESBL-producing *E. coli*; attributed values refer to Table 1. PPE:personal protective equipment; RPC: risk priority code.

As next step, we used the actual prevalence found in animals and environment concerning each AMR bacterial group (Table 6 and Table 7). In the previous phase, farm practices were evaluated in all sectors, but in this phase only some sectors' activities were considered, based on the relative prevalence investigated in animal compartments. Thence, the activities most dangerous to be exposed to MRS were: cleaning and disinfection (pre- partum sows), cleaning and disinfection (piglets), piglets' movements, castration and tattooing, weaning piglets' treatments, sows' treatments and sows' fecundation (see Table 5). Considering ESBL- producing *E. coli* exposition, the riskiest farm practices were: dejections' cleaning in gilts' sector, cleaning and disinfection (gilts'

sector), gilts' movements, piglets' care (during farrowing), removal of placenta and dead piglets, removal of dead pigs >25kg (gilts), castration and tattooing, gilts' treatments and gilts' fecundation (see Table 7).

Farm practices	**Intensity of exposition**	**Animal prevalence**	**Environmental prevalence**	**RPC MRS**
Dejections' cleaning in gilts' sector	1	2	2	1
Cleaning and disinfection (post-partum sows)	2	3	3	2
Cleaning and disinfection (pre-partum sows)	2	4	4	2
Cleaning and disinfection (piglets)	2	4	3	2
Cleaning and disinfection (gilts)	2	2	2	2
Manual feeding (farrowing room)	1	3	3	1
Maintenance sows' sectors	1	3	3	1
Maintenance gilts' sector	1	2	2	1
Maintenance piglets' sector	1	4	3	1
Sows' movements	1	3	3	1
Gilts' movements	1	2	2	1
Piglets' movements	2	4	3	2
Cross-fostering	2	3	3	2
Piglets' loading	2	4	3	2
Gilts' unloading	1	2	2	1
Piglets' loading	3	4	2	3
Piglets' care (during farrowing)	2	3	3	2
Removal of placenta and dead piglets	2	3	3	2
Removal of dead pigs <25kg (weaning sector)	1	4	3	1
Removal of dead pigs	1	2	2	1

>25kg				
Sows' inspection	1	3	3	1
Gilts' inspection	1	2	2	1
Piglets' inspection	1	4	3	1
Castration and tattooing	3	3	3	3
Farrowing piglets' treatments	2	3	3	2
Weaning piglets' treatments	2	4	3	2
Gilts' treatments	2	2	2	2
Sows' treatments	2	4	4	2
Gilts' fecundation	3	2	2	2
Sows' fecundation	3	4	4	3

Table 6: calculation of the risk priority code (RPC) for selected sectors' farm practices based on the actual MRS prevalence observed in animals and associated farm environment. A scale with 4 levels to define the prevalence intervals was applied: < 30% (score= 1), between 30 and 60 % (score= 2), between 61 and 80 % (score= 3), > 80 % (score= 4).

Farm practices	**Intensity of exposition**	**Animal prevalence**	**Environmental prevalence**	**RPC ESBL-producing *E. coli***
Dejections' cleaning in gilts' sector	2	4	4	2
Cleaning and disinfection (post-partum sows)	2	2	4	2
Cleaning and disinfection (pre-partum sows)	2	3	1	1
Cleaning and disinfection (piglets)	2	3	1	1
Cleaning and disinfection (gilts)	2	4	4	2
Manual feeding (farrowing room)	1	2	4	1
Maintenance sows' sectors	1	2	2	1
Maintenance gilts' sector	1	4	4	1
Maintenance piglets' sector	1	3	1	1

Sows' movements (pre-partum)	3	3	1	1
Sows' movements (post-partum)	2	2	4	2
Gilts' movements	2	4	4	2
Piglets' movements	2	3	1	1
Cross-fostering	3	2	4	2
Piglets' loading	3	3	1	1
Gilts' unloading	2	4	4	2
Piglets' care (during farrowing)	3	2	4	2
Removal of placenta and dead piglets	3	2	4	2
Removal of dead pigs <25kg (weaning sector)	3	3	1	1
Removal of dead pigs >25kg (gilts)	3	4	4	3
Sows' inspection	1	2	4	1
Gilts' inspection	1	4	4	1
Piglets' inspection	1	3	1	1
Castration and tattooing	3	2	4	3
Farrowing piglets' treatments	2	2	4	2
Weaning piglets' treatments	2	3	1	1
Gilts' treatments	3	4	4	2
Sows' treatment (pre-partum	3	3	1	1
Sows' treatments (post-partum)	3	2	4	2
Gilts' fecundation	3	4	4	3
Sows' fecundation	3	2	1	1

Table 7: calculation of the risk priority code (RPC) for selected sectors' farm practices based on ESBL-producing *E. coli* prevalence observed in animals and relative farm environment. A scale with 4 levels to define the prevalence intervals was applied: < 30% (score= 1), between 30 and 60 % (score= 2), between 61 and 80 % (score= 3), > 80 % (score= 4).

6.5. Discussion

The recovery of both AMR bacterial groups in a single intensive swine farm was never reported in Italy, due to the lack of specific surveys targeting both bacterial groups (ESBL-producing *E. coli* and MRS); furthermore, this occurrence was detected in diverse phases of the animal productive chain, with extremely high prevalence especially for MRS (in three productive stages >80%). The occurrence of both MRS and ESBL-producing *E. coli* was previously recovered in the studies of Schmithausen et al. and Fischer et al. in German swine farms [58, 66]. In the study of Schmithausen et al., differences in prevalence were found depending on the animal age. Accordingly, we recovered more ESBL- producing *E. coli* positive animals in the younger stages (weaning piglets and gilts), while in sows, especially during parturition and gestation, the prevalence was reduced (~50%). The total ESBL- producing *E. coli*-related prevalence (64.5%) detected in animals was higher than previous studies conducted in Northern Italian farms, where a prevalence of around 30% was detected [65]. A similar result (27%) was recently obtained by a national survey that included all the Italian regions [299]. The fact that our prevalence is more than double previous Italian findings is of great concern, and may be explained by the high use of antibiotics in our farm [263,300,301]. In fact, looking to the register of the animal treatments, some antibiotics are still used in a metaphylactic or prophylactic way, and the majority of these molecules are critically important antibiotics (CIAs), like ceftiofur and amoxicillin, which can potentially exert selective pressure on *E. coli* [302]. A very high prevalence of MRS (80.8%) was found across all the farm sectors. Our result is more elevated compared to other studies in Italy, reporting MRSA prevalence of 17.5% in finishing pigs [231], and 64.5% across 5 swine farms [269]. Our result refers mainly to MRCoNS, particularly *S. sciuri*, the most detected staphylococcal species in the MRS group.

In the weaning sector, 100% of animals and environmental samples carried methicillin-resistant staphylococcal strains, mostly MRSA on animals. This was the highest carriage among all the different animal sectors. This finding can be linked to the massive antibiotic usage in young piglets; in fact, male suckling piglets are all treated after neutering, and female are treated too, even if they are not neutered. Recurrent exudative epidermitis is also one of the major cause of piglets' antibiotic treatment. For these piglets' treatments, ceftiofur and amoxicillin (IM and oral in medicated feeding) are administered; these drugs are both able to elicit the onset of methicillin resistance in susceptible staphylococci [116]. Moreover, we observed that MRS prevalence tends to decrease with the animal age, probably due to the reduced antibiotic use in adults, and the privileged individual treatment (IM), which is preferred to metaphylactic oral route [116]. However, the prevalence of MRS is generally high on farm, and this can be also linked to the intensive swine farming and the size of the farm too, as was highlighted by previous studies [157,303]. Considering the environment of the specific animal sectors, ESBL-producing *E. coli* were not always present with the same high prevalence as recovered in sampled animals from the same sector. This was the case of weaning, gestation and fecundation environments. Unexpectedly, in the gestation room, no ESBL-producing *E. coli* was isolated; this may be linked to the efficient cleaning procedures and dejections removal. Contrary, high prevalence of ESBL-producing *E- coli* (100%) and MRS (80%) were recovered in the gilts room. This elevated environmental contamination can be associated to the difficulty in effecting cleaning and disinfecting procedures; in fact, in this sector there were a lot of boxes (n=54), and a rest period never occurred, due to the high number of bought animals.

Another sector where there is a conspicuous number of boxes (n=80) is the weaning location. Here, all tested environmental samples were MRS positive, with different methicillin-resistant staphylococcal species identified (*S. aureus*, *S. cohnii, S. saprophyticus* and *S. sciuri*). As said in Chapter 4 and 5, this high variability in MRS species could support the horizontal gene transfer event of the *mecA* gene through diverse staphylococcal species and strains. Indeed, mobilization of

SCCmec cassette, containing *mecA* gene, between coagulase negative staphylococci and *S. aureus* was recognised in previous studies [241,242,243]. Moreover, these MRS can ultimately be reservoir of the *mecA* gene for the human *S. aureus*. Slightly more variability in ESBL- producing *E. coli* prevalence was observed across animal sectors, especially in the associated farm environment. In fact, we detected extremely high prevalence in gilts (93%), while in parturition sows the percentage of positive animals was 50%. A bigger difference in ESBL- producing *E. coli* prevalence was recovered in the farm environment, considering that no positive sample was detected in the gestation sows' environment, while 100% of samples from the gilts' sector were positive. Furthermore, the ESBL-associated genes were predominantly $bla_{CTX\text{-}M}$, and this is in line with previous Italian and European studies in swine farms [65,66,263,297].
Having not sampled the boar is a lack in our study. This animal is in fact the oldest one, and he usually enters different animal sectors (fecundation and gestation) approaching sows and gilts with diverse ages. However, we sampled animals from almost all the productive categories, with the exception of nursing piglets; this multiple sampling across different animal stages, allowed us to observe that MRS are disseminated across sows in different moment (parturition, fecundation and gestation), gilts and especially weaning piglets. This occurrence can be linked to all the previously analysed factors, but also to human movements across the farm. The same high prevalence was detected in the relative farm environment, and diverse staphylococcal species (*S. aureus*, *S. cohnii, S. haemolyticus, S. sciuri* and *S. saprophyticus*) carried *mecA* gene, even in the same animal sector. This highlights that MRS are ubiquitous on farm, and they are difficult to eradicate during cleaning and disinfecting procedures.

Looking to farm practices, removal of dead pigs >25kg (gilts), castration and tattoo application and gilts' fecundation resulted the most dangerous activities associated to ESBL-producing *E. coli* (RPC=3). The risk of exposition to this resistant *E. coli* is related mostly to the strict contact with animals (dead or alive), the high numbers of animals for farm worker (e.g. during piglets' castration) and the lack of specific PPEs (e.g. not using gloves during animal fecundation). It is noteworthy that removal of dead animals is a risky operation for farm workers for the high-level of ESBL-producing Enterobacterales contamination of swine carcasses [304]. Indeed, high rate of ESBL-producing *E. coli* is found in the caecal content of pigs, with possible contamination of the carcasses [305]. Moreover, contaminated carcasses need to be carried through the animal sector until the carcasses storage room, and this can enhance the risk of exposition for farm worker, especially when PPEs are not correctly worn.

Even for the MRS-associated exposition, castration and tattoo application and animal fecundation (sows, in this case) resulted the most dangerous farm practices, from the FMEA analysis (see Table 4). Piglets' loading was also included among the riskiest farm activities, due to the high number of animals in strict contact with farm worker and the long time spent working. The high level of exposition is linked specifically to the lack of filtrating face masks during this farm practice; these types of masks are able to stop the potential MRS transmission through air route and should be worn during indoor farm practices [295,306].

6.6. Conclusion

Our survey highlighted a high prevalence of two important recognised human and animal resistant bacterial groups, that were both disseminated across all animal categories and relative environment too. Moreover, we analysed the real exposition of farm workers to these AMR microorganisms, identifying the more dangerous farm practices in daily farm working in a standard Italian intensive farm.

Our results can be a useful basis for planning preventive measures to hinder the transmission of MRS and ESBL-producing *E. coli* to humans. For example, more efforts should be directed to encourage the correct use of PPEs, especially filtrating face masks for indoor activities, and gloves during direct contact with animals (live and dead) and faecal contaminated environment. Another important factor that needs to be considered to tackle the onset of AMR bacteria, able to colonise humans, is the progressive reduction of antibiotic usage, specifically CIAs, that are still largely used in piglets (e.g. amoxicillin, ceftiofur and tildipirosin) and in sows (e.g. marbofloxacin and amoxicillin) as well.

Only counteracting AMR at farm level, it will be possible to reduce the risk of infection for farm workers, which will not transmit farm-associated resistant bacteria to the rest of human community and to other animals present in the nearby environment. This is really important if we want to act in line with the One-Health perspective, considering that animals, humans and environment are all interconnected.

6.7. Acknowledgment

We thank all the farm workers that helped with animal sampling during daily farm activities.

Chapter 7- Whole Genome Sequencing (WGS) analysis of Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* from animal and environmental samples in Italian swine farming

7.1. Abstract

Whole genome sequencing (WGS) has become a powerful tool to analyse bacterial genome in a short turnaround time. Indeed, it is widely accepted that AMR genes found with this technique can be used with good probability to understand phenotypic antibiotic susceptibility. In this study, we selected a group of previously characterized ESBL-producing *E. coli* (n=30), sampled from 4 pigsties among animals and farm environment, to carry out WGS; 2 x 250bp paired end sequencing strategy on Illumina MiSeq™ was used, while tools from cge.cbs.dtu.dk/services/ like *ResFinder* 4.1, *PathogenFinder* 1.1 and *VirulenceFinder* 2.0 were run to analyse AMR and virulence genes respectively. Bacterial strains were former analysed for phenotypic AST against doxycycline, enrofloxacin, florfenicol, gentamicin, tetracycline, trimethoprim/sulfamethoxazole and meropenem. Data obtained from WGS were compared to phenotypic results; indeed, it was detected a good match between phenotypic and genomic results, and different AMR determinants were identified: *aac(6')-Ib-cr, aac(6')-Ib3*, *aadA*, *aph*, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, *catA*, *cmlA*, *erm*, *dfrA*, *erm*, *floR*, *mdf*, *mph*, *qnrS* , *sul* and *tet.* Potential pathogenicity of these strains was also assessed and different virulence genes were detected (e.g. *etsC, gad, hlyF, iroN, iss, iucC, iutA, kpsE kpsMII, lpfA, mchF, ompT, sitA, terC, traT*); these genes were mostly related to extraintestinal *E. coli* pathotypes (UPEC/APEC). However, enterotoxin genes, like *astA*, *ltcA* and *stb* were also identified in the same strains, indicating a possible hybrid pathogenic nature. To support the fact that our stains were pathogenic, various plasmids, previously recovered in pathogenic bacteria, were identified (e.g. Col156 and IncR). Furthermore, results from *PathogenFinder* 1.1 tool revealed that all strains were potentially human pathogens with a high probability (>80%).

7.2. Introduction

Escherichia coli is a Gram-negative bacterium that can be pathogenic or non-pathogenic depending on the presence of specific virulence factors coded by certain genes. This microorganism is normally catalogued as commensal; however, when it acquires a particular set of mobile genetic elements (MGEs), it can become a dangerous pathogen that is able to cause different types of conditions, from gastroenteritis to urinary tract infections, bloodstream and central nervous system diseases. Every year, millions of people suffer from *E. coli*- related maladies worldwide [87]. Pathogenic *E. coli* strains can cause clinical symptoms in animals too. Nevertheless, animals can also be carriers and be colonized without presenting any clinical sign. In this case, *E. coli* acts as a simple commensal microorganism, that inhabits the animal gut and can be spread to humans, other animals and nearby environment.

Pathogenic strains can be generally categorized as either diarrhoeagenic *E. coli* or extraintestinal *E. coli* (ExPEC). Till now, different pathotypes — enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC; including *Shigella*), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC), have been fully distinguished as diarrhoeagenic bacteria. Other pathotypes — uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), sepsis-associated *E. coli* (SEPEC), and avian pathogenic *E. coli* (APEC), which cause extraintestinal infections in poultry- are the most common ExPEC isolates [87].

Objective of this study was to genotypically characterized 30 antibiotic resistant *E. coli* strains isolated in four farms from environmental and faecal samples of swine origin. All these strains were previously confirmed as ESBL-producing, through phenotypic and genotypic tests. The sampled pigs were healthy at the time of the survey, and they were in various productive phases: finishing (n=3), post-weaning phase (n=14) and sows (n=10). Environmental samples (n=3) were taken from finishing (n=1) and post-weaning sector (n=2).

7.3. Materials and Methods

Swabs in Amies transport medium (Microbiotech s.r.l., Maglie, Italy) were used for sampling pigs and environment from 4 farms (G, P, S and T) located in Cuneo province (Piedmont, Italy). We sampled 10 animals and 2 environmental sites in each productive phase (finishing, post-weaning and sows) of the 4 selected farms. In farm S only finishing stage was present due to the production type. Numbers of samples were chosen based on a minimum expected prevalence of 10% and considering a 95% confidence level. Phenotypic tests were carried out to isolate pure cultures and to test the resistance to extended-spectrum β- lactamases (ESBLs) enzymes (for further details see Chapter 5, paragraph 5.3.3. "Laboratory and statistical analyses"). Antibiotic susceptibility test (AST) was performed through Kirby-Bauer disk method, to detect phenotypic resistance to doxycycline, enrofloxacin, florfenicol, gentamicin, tetracycline, trimethoprim/sulfamethoxazole (CLSI VET 08 [307]) and meropenem (EUCAST v11.0 [308]). After phenotypic characterization, DNA was extracted from pure colonies (1-2 colonies with the same aspect) with a modified boiling method [269]. The spectrophotometer NanoDrop™ 2000 (Thermo Scientific, Waltham, WA, USA), was used to measure the quantity of extracted DNA.

To confirm phenotypic ESBL-producing *E. coli*, we performed PCR targeting the specific β-lactamase genes $bla_{CTX\text{-}M}$ and bla_{TEM}. Sanger sequencing was used to evaluate PCR products. We used BioEdit 7.2.5 Sequence Alignment Editor© software to analyse nucleotide sequences; ClustalW tool was set up for multiple alignment with reference bacterial genome sequences. BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was run to compare our sequences with the database in GenBank.

Whole genome sequencing (WGS) was performed on selected strains (n=30), previously confirmed genotypically ESBL-producing, isolated from environmental and faecal samples of swine origin. The samples were chosen based on the presence of ESBL-associated genes ($bla_{CTX\text{-}M}$ or bla_{TEM} or both) and productive phases where they were formerly isolated. Due to economic reasons and limited time, we chose only 30 samples. We selected animal samples from finishing (n=3), post-weaning phase (n=14) and sows (n=10), and environmental samples (n=3) from finishing (n=1) and post-weaning sector (n=2). Using WGS, we assessed multilocus sequence typing (MLST), *fumC* and *fimH*

(CH)-typing, plasmids, antimicrobial resistance, virulence genes, human pathogenicity and serotype. Briefly, we performed another DNA extraction with a different protocol, using the GeneJet Genomic DNA purification kit (Thermo Scientific™, USA). RNAse treatment was necessary to not influence the library preparation with Nextera XT (Illumina, San Diego, USA). Extracted samples were eluted in sterile DNA-free water. Quality parameters and DNA quantity were assessed with NanoDrop. Quality was equal or higher than 1.8 (A 260:280), while quantity was around 30 ng/µl. To perform WGS analysis, 2 x 250bp paired end sequencing strategy on Illumina MiSeq™ (Illumina, San Diego, USA) was used at the University of Veterinary Medicine of Copenhagen in Denmark. Assembly of raw reads was performed with CLC Genomic Workbench v. 20.0.4 (©Qiagen, Aarhus, Denmark). The cut-off for contig size was 1000, to leave out problematic contigs. Trimming was performed in CLC with 0.01 error probability. Calculation of the output from assembly compared to number of reads gives coverage which should be minimum 30x [309]. After assembly of contigs, we analyzed the *E. coli* genome using Center for Genomic Epidemiology (CGE) tools from cge.cbs.dtu.dk/services/ [310]. ResFinder 4.1 was used to detect acquired resistance genes, with a threshold of identity of 85% and a minimum length of 60%. Virulence genes were identified through VirulenceFinder 2.0. PlasmidFinder 2.1 was run to detect plasmids, while MLST 2.0 was used to type our strains. SerotypeFinder 2.0 was used to understand the *E. coli*-associated serotype. CHTyper-1.0 was used to detect the house-keeping genes *fumC*, coding for fumarase enzyme, and *fimH*, coding for a specific adhesin (type 1 fimbriae), to further cathegorise the *E. coli* strains. To understand the probability of our strains to have human-associated pathogenicity, we run PathogenFinder 1.1. Using MLST results, a minimum spanning tree was produced using PHYLOViZ with the goeBURST algorithm [311].

7.4. Results

All the selected *E. coli* strains were able to grow on selective medium for phenotypic ESBL- resistance detection. Furthermore, AST analysis revealed that our strains were multidrug resistant (MDR), as it was highlighted by the reduced halo detected for more than 3 antibiotic classes; resistance patterns against doxycycline, enrofloxacin, florfenicol and trimethoprim/sulfamethoxazole were the most often detected. Some strains, like G1PAE7, were resistant to all tested antibiotics, with the exception of meropenem. Considering molecular analysis, most of *E. coli* strains resulted positive for both *bla* genes ($bla_{CTX\text{-}M}$ and bla_{TEM}). Sanger sequencing with nucleotide alignment confirmed the presence of both genes.

WGS analysis revealed that these selected strains had more than two types of antibiotic resistant genes ($bla_{CTX\text{-}M}$ and bla_{TEM}). Indeed, we found other 17 acquired resistant genes (see Table 1): *Aac(6')-Ib-cr,* (fluoroquinolones and aminoglycosides resistance), *Aac(6')-Ib3* (amikacin, tobramycin resistance), *aadA* (aminoglycoside resistance), *aph* (aminoglycoside resistance), *catA* (chloramphenicol resistance), *cmlA* (chloramphenicol resistance), *erm* (macrolide resistance), *dfrA* (trimethoprim resistance), *erm* (macrolide resistance), *floR* (phenicol resistance), *mdf* (multiple resistance to benzalkonium chloride, daunomycin, rhodamine), *mph* (macrolide resistance), *qnrS* (ciprofloxacin resistance), *sul* (sulfamethoxazole resistance), *tet* (doxycycline, tetracycline resistance), *sitABCD,* that is able to confers resistance to disinfectants (peroxide hydrogen), *and* bla_{SHV} (3rd and 4th generation cephalosporins resistance).

Moreover, we detected a wide variety of virulence genes (see Table 1); some virulence factors were more often detected: for example, bacteriocins and microcins like *cea* (colicin E1), *celb* (endonuclease colicin E2), *cia* (colicin ia), *cib* (colicin ib), *cma* (colicin M), *cvaC* (microcin C) were found in half of the tested strains; *etsC* (putative type I secretion outer membrane protein), *gad* (glutamate decarboxylase), *hlyF* (hemolysin F), *iroN* (enterobactin siderophore receptor protein), *iss* (increased serum survival), *iucC* (aerobactin synthetase), *iutA* (ferric aerobactin receptor), *lpfA* (fimbrial subunit gene of long polar fimbriae), *mchF (*ABC transporter protein MchF), *ompT* (outer membrane protease), *sitA* (iron transport protein), *terC* (tellurium ion resistance protein), *traT* (outer membrane protein complement resistance) are other common virulence factors, detected across all farms. This set of genes was recovered in almost all post-weaning (n=7) and finishing (n=2) samples from farm P and farm G (n=4). Also, the environmental post-weaning sample taken in farm G (G1PHE2), carried the same group of virulence factors. Other important virulence genes recurrently detected were *kpsE* (capsule polysaccharide export inner-membrane protein) and *kpsMII* (polysialic acid transport protein, Group 2 capsule). These genes were always detected together in different samples from farm G (G1PAE7), farm P (P1PAE3) and farm T (T1SAE6, T1SAE10); moreover, they were recovered in the environmental sample from farm S (S1FHE2).

In our samples, we did not detect any shiga-toxin gene; however, we found some enterotoxins' genes, like *astA* (heat-stable toxin EAST-1), *ltcA* (subunit of the heat-labile enterotoxin- LT) and *stb* (heat-stable enterotoxin II); *stb* and *ltcA* were always associated to *astA* (see Table 1). Samples that carried these genes belonged to various farms: farm G (G1PAE7), farm P (P1PAE3) and farm S (S1FHE2). In farm T, only *astA* was present, in 2 *E. coli* associated to sows (T1SAE7, T1SAE8). Other virulence genes detected across animal and environmental strains were: *chuA*, coding for an outer membrane hemin; *gad* expressing a glutamate decarboxylase; *eilA,* Salmonella HilA homolg; *fyuA* expressing a siderophore receptor; *hra*, heat-resistant agglutinin gene; *ireA*, coding for a siderophore receptor; *irp2,* encoding a non-ribosomal peptide synthetase; *katP*, coding for a plasmid-encoded peroxidase; *neuC,* expressing the polysialic acid capsule biosynthetic protein; *papC*, encoding outer membrane usher P fimbriae; and *vatA* expressing a vacuolating autotransporter toxin (see Table 1).

MLST analysis showed that the majority of strains belonged to ST23 complex (9/30). This sequence type (ST) was recovered in 2 different farms (G and P) from one environmental and 8 post-weaning samples (see table 1). Analysis on the phylogenetic evolution of our strains showed that ST23, the most often detected ST, is phylogenetically close to ST101, that was detected in the same farm of ST23 strains (farm P and G). The other frequently detected type, ST877, which was found in all sows' associated *E. coli* from Farm G, is strictly related to ST10, that was recovered in one post-weaning environmental sample (G1PHE1) from the same farm (Figure 1).
CH-typing revealed that 8 strains, categorized as ST23, belonged to the CH-category 4-35, while one ST23 strain belonged to the subtype 4-402 (see Table 1). The associated O-serotype was often identified as O8 (9/30), while O45 and O153 were less frequently recovered (3/30).
In all *E. coli* strains, we detected more than one plasmid per strain; in some of them we unexpectedly recovered 7 different plasmids. For example, in the farm P, post-weaning strains P1PAE2, P1PAE4, P1PAE6, P1PAE8 and P1PAE9 showed 7 plasmids: IncB/O/K/Z, IncFlB (AP001918), Incl1-l (Alpha), IncX1, IncY, Col156 and Col (MG828). IncFlB (AP001918) was the most frequently recovered plasmid across all our samples (17/30). It was detected in animals' (post-weaning and finishing) and environmental samples from 3 different farms (G, P, S). IncB/O/K/Z was less often recovered (11/30), but it was carried by *E. coli* strains taken from finishing and post-weaning animals located in farm G

and P. Other plasmids (e.g. IncR and IncX3) were detected (see Table 1). Considering the potential pathogenicity in humans, all strains had a probability >80% to be human pathogen (see Table 1).

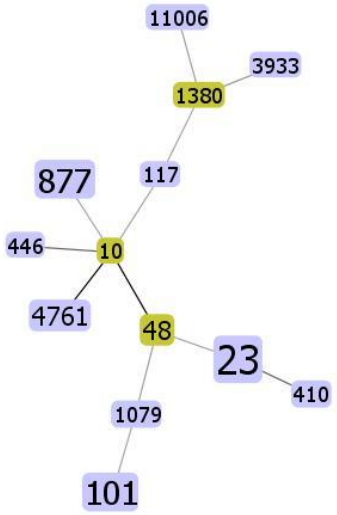

Figure 1: minimum spanning tree with STs detected across our ESBL-producing *E.* coli isolated in animal and environmental samples from 4 swine farms. An eBURST diagram was created through PHYLOViZ with the goeBURST algorithm. The relation between STs is calculated with only one allelic variation (SLV). The STs in yellow squares refer to the "ancestor genotypes". Size of squares increases with the frequency of recovery.

7.5. Discussion

From the results obtained through phenotypic and genotypic analyses, it is clear that our *E. coli* are not just antimicrobial resistant bacterial strains; in fact, they possess a wide range of virulence genes that can be linked to *E. coli* pathogenic ability to colonise and infect human and animal hosts. Furthermore, they can express a variety of bacteriocins and microcins that are involved in microbial competition in bacterial community. These factors can have other functions, for example in the human-tissue colonization; however, their pathogenic function is still to be defined. Some of them, like *cea* and *cvaC*, are more frequently expressed in UPEC strains, that can cause (*cvaC*) pyelonephritis too [312]. Based on the classification of *E. coli* pathotypes, depending on their virulence factors, we can suggest possible virulence mechanisms for our *E. coli* strains; indeed, as it will be highlighted in this paragraph, we detected potential UPEC, APEC and hybrid diarrheagenic pathotypes. Considering all the factors that are involved in the iron metabolism (*iroN, iucC, iutA* and *sitA*), the majority of tested strains appeared to be extraintestinal UPEC pathotype. *fimH* is also a characteristic virulence factor of UPEC, but can be found in other extra-intestinal pathotypes like SEPEC, NMEC and avian pathogenic *E. coli* (APEC). *fimH* codes for a type 1 fimbria that is involved in colonization and biofilm formation, particularly in cystitis and meningitis, attaching to the receptor D-Mannose. *fumC* encoding oxidative fumarase enzyme FumC, is also necessary for the colonization of the iron-limited environment of the urinary tract, to counteract multiple stressors [313]. The genes *iss*, *ompT* and *traT* code for virulence factors that are implicated in extraintestinal pathogenicity too, since they increase survival in serum, blocking complement activity (*iss* and *traT*)

and allow urine survival and resistance to protamine (*ompT*) [93,102,314]. Nevertheless *iroN*, *iss*, *iutA*, *ompT*, and *hlyF* have been considered typical markers of APEC strains too [315]; these 5 virulence determinants were recovered in 13 ESBL-producing *E. coli* strains (G1PAE2, G1PAE3, G1PAE4, G1PAE8, P1FAE1, P1FAE3, P1PAE2, P1PAE4, P1PAE6, P1PAE7, P1PAE8, P1PAE9, P1PAE10), mostly recovered in post-weaning animals from two intensive farms (farm P and G). The gene *hlyF*, coding for a hemolysin, has been previously found in UPEC strains too, and it is nowadays considered a marker of extraintestinal pathogenicity as well as *cvaC* and *etsC*; these two genes were recovered in 13 out of 30 samples, mostly in the post-weaning sector, even at the environmental level (G1PHE2).

lpfA was another frequently detected virulence marker. This gene is involved in the expression of an important fimbria for host-cell adhesion. Previous studies detected it in EPEC, cattle shiga toxin-producing *Escherichia coli* (STEC), extraintestinal pathogenic *E. coli* and commensal *E. coli* [101,316]. Here, we found a widespread distribution of this gene (19/30) in post-weaning, finishing and sows-associated strains; it was recovered also in two environmental samples (G1PHE2 and S1FHE2). All the presumptive APEC strains encoded this gene. Beside APEC-related strains, other presumptive pathotypes were detected. Potential diarrheagenic strains associated to the enteroaggregative heat-stable toxin EAST-1, encoded by *astA,* were G1PAE7, GIPAE8, P1PAE3, TISAE7, T1SAE8 and the environmental S1FHE2. Due to the concomitant presence of UPEC-APEC virulence factors, the strain G1PAE8 could be considered a hybrid APEC strain, that acquired the heat stable toxin. In the study of Maluta et al., the same hybrid APEC strains were found in poultry [317]. EAST-1 has been associated with EAEC strains, that were isolated in humans and animals [318,319,320]. In swine production, this enterotoxin was detected from diarrheagenic and non- diarrheagenic *E. coli* [321], which might explain EAST-1 detection in our *E. coli* sampled from healthy animals. Indeed, the function of this enterotoxin in swine colibacillosis onset is not clear yet.

stb was another enterotoxin coding gene present in 2 animal (G1PAE7, P1PAE3) and one environmental (S1FHE2) *E. coli* strains. The resulting enterotoxin is responsible for secretory diarrhea in human and animal hosts [103]. *ltcA* gene was recovered in two samples, one animal and one environmental (P1PAE3 and S1FHE2); the encoded toxin LT has been previously detected in ETEC strains sampled from pigs [319]. In our strains, *stb* and *ltcA* were always recovered with *astA*. All our presumptive diarrheagenic *E. coli* presented also *terC* gene, that is implied in tellurium resistance and has been associated to ExPEC and UPEC strains [102].

We detected *irp2* gene in two animal strains (G1PAE9, P1FAE3). This gene encodes a siderophore called iron-repressible protein, involved in yersiniabactin synthesis, and it is considered a marker for the detection of high-pathogenicity islands [322], which have been recognised in diarrheagenic swine *E. coli*, [323]. Among the genes found in G1PAE9 and P1FAE3 strains, we also identified *fyuA* (coding for ferric yersinia uptake -yersiniabactin receptor). The couple *fyuA* and *irp2* is the main pathogenicity determinant in the HPI of APEC strains, and is paramount in avian colibacillosis pathogenicity [324,325]. However, some Authors sustain that *fyuA,* when present with *vat*, *chuA*, and *yfcV,* is a determinant of human UPEC strains, and can be considered a distinguishable marker of UPEC, not of APEC [98]. Here we detected *fyuA* with *vat* and *chuA,* only in the finishing-associated *E. coli*, P1FAE3.

Other recurrent virulence markers associated to potential diarrheagenic *E. coli* were *kpsE* and *kpsMII*. These two genes are involved in bacterial capsule formation and they are often sequenced from ExPEC and UPEC strains; for this reason, they are considered genetic markers of ExPEC [102,326]. Surprisingly, in all our potential diarrheagenic strains, these capsule-related determinants were present with enterotoxin-associated genes (*astA*, *ltcA* and *stb*). This finding can indicate that our strains could be considered hybrid, due to the simultaneous presence of ExPEC and diarrheagenic virulence markers.

Another important gene, *neuC*, implicated in capsule biosynthesis, was present in one (T1SAE7) of the 6 enterotoxin-coding *E. coli.*

The pathogenic nature of our strains is well supported by the O-related serotypes detected. Indeed, the serotypes O8 and O45, frequently found in our strains, were previously associated with diarrheagenic pathotypes in piglets and calves [327,328,329]. Moreover, we often detected in Farm G the serotype O153, which was found in human ETEC [328].

The finding of plasmids that were previously detected in human clinical samples, also supports the presumptive pathogenicity of our strains. Indeed, one of the most frequently recovered, Col156, was previously found in a *E. coli* clinical strain isolated in Poland (NC009781). Another recurrent plasmid was IncR. This MGE was recovered from 3 sows' samples, and it has been previously identified in *Klebsiella pneumoniae* strain, isolated in human urine clinical sample (GenBank accession no.: DQ449578). The plasmid IncX3, previously recovered from an Italian *K. pneumoniae* clinical strain (JN247852), was here detected in a sow strain (T1SAE8). Even the plasmid IncFIB(K), is very similar (98.93%) to the IncFIIk-FIB-like plasmid, previously detected in the Italian *K. pneumoniae* ST258, a pandrug-resistant human clinical isolate [330]. Furthermore, we found some plasmids that were livestock-associated. For example, IncX1, a plasmid already detected in Danish pigs (EU370913), was sequenced in *E. coli* strains associated to sows (n=4) from Farm T and post-weaning animals (n=8) from farm P. The conjugative plasmid IncX1 is generally involved in biofilm formation, multidrug efflux and oloquindox (antimicrobial livestock growth promoter) resistance.

Results from PathogenFinder 1.1 analysis showed that all our strains, sampled from animals or environment, could be considered putative pathogens for human hosts. Indeed, we recovered high pathogenicity probability (>80%) and relevant virulence markers like TraF (F plasmid transfer operon, TraF protein), previously sequenced in *Salmonella enterica* Heidelberg (ACF65774), and *Shigella dysenteriae* associated conserved hypothetical protein YhB0 (ABB63325). The highest probabilities of being human pathogens were attributed to sow samples G1SAE7 (95%) and G1SAE2 (94%), from farm G, post-weaning and finishing strains from farm P (P1PAE4, P1PAE2, P1PAE10, P1FAE1, P1FAE3 = 93%), a post-weaning strain from farm G (G1PAE2= 94%) and an environmental *E. coli* from farm G (G1PHE2= 93%).

In conclusion, we can remark that our *E. coli* strains have the potential to be pathogenic for humans and animals. This is indicated not only by the presence of a variety of virulence genes specific for extra-intestinal infections (e.g. bladder infection for UPEC), but also by the presence of enterotoxins' genes (*astA*, *stb*), which can be expressed in human or animal gut and cause the toxin production and ultimately diarrhoea. In this perspective, WGS analysis is a precious tool to understand the real "nature" of confirmed antimicrobial resistant *E. coli*, even when sampled from healthy animals. WGS enables monitoring the dissemination of specific serotypes/ST-types, that can be transmitted to humans through strict contact with animals or farm environmental contamination. However, due the limited number of tested strains and the small group of sampled farms, it will be desirable to broaden this analysis to other farms and other Italian territories, to understand if these *E. coli* strains are common and if they can cause symptoms in reared animals. Furthermore, in further studies it will be beneficial to test simultaneously non-ESBL-producing *E. coli* too, to comprehend if virulence genes' patterns, plasmids, STs and serotypes are the same of ESBL-producing *E. coli*. Thence, surveillance of *E. coli* isolates present at farm level, will be important to identify new or human-related clones that can disseminate in the swine productive chain. Furthermore, in-silico characterization enables the appreciation of potential plasmids' movement among enteropathogenic bacteria, like *E. coli* and other important human pathogen (e.g. *K. pneumoniae*). Indeed, as we found for IncX3 plasmid and other MGEs, the same plasmid previously

characterized in *K. pneumoniae* can be present in *E. coli* too; this event can be alarming if resistance to last-resort antibiotics is carried by these plasmids. For all these reasons, we strongly support to use this new monitoring approach at farm level, especially in intensive production, where high number of animals are present, and spread of certain *E. coli* strains can be facilitated by poor hygiene conditions and lack of biosecurity measures.

Strains	bla genes	MLST type	O-serotype	CH-type	AMR genes	Plasmid replicon	Virulence genes	Probability of human pathogenicity
G1PAE2	ctx-m-1 tem-1C	ST101	O153	41-86	aadA1, aadA2b catA1, cmlA1, mdf(A), sul3, tet(A)	IncFlB (AP001918), IncFIA, IncFIC (FII), Incl1-l (Alpha), IncX1	cma, cvaC etsC hlyF iroN iss iucC iutA lpfA mchF ompT sitA terC traT	88%
G1PAE3	ctx-m-1, tem-1C	ST101	O153	41-86	aadA1, aadA2b catA1, cmlA1, mdf(A), sul3, tet(A)	IncFlB (AP001918), IncFIA, IncFIC (FII), Incl1-l (Alpha), IncX1, Col (MG828)	cvaC etsC hlyF iroN iss iucC iutA	88%

							lpfA mchF ompT sitA terC, traT	
G1PAE4	ctx-m-1, tem-1C	ST101	O153	41-86	aadA1, catA1, cmlA1, aadA2b, mdf(A), sul3, tet(A)	IncFlB (AP001918), IncFIA, IncFIC (FII), Incl1-l (Alpha), IncX1, Col (MG828)	cma cvaC etsC hlyF iroN iss iucC iutA lpfA mchF ompT sitA terC traT	88%
G1PAE7	ctx-m-14, tem-1B	ST11006	O17	3-143	aac(6')-lb-cr, aac(6')-lb3, cmlA1, erm(B), mdf(A), mph(A), tet(B)	IncB/O/K/Z, IncFlB (AP001918), IncFII(pHN7A8)	astA chuA eilA kpsE kpsMII stb	88%

							terC traT	
G1PAE8	ctx-m-1, tem-1B	ST1079	O6	19-32	aacA1, aadA2b, aph(3'')-lb, aph(6)-lb, catA1, cmlA1, mdf(A), sul3, sul2, tet(A)	IncFlB (AP001918), IncFIC(FII), Incl1-l (Alpha), IncY	astA cea, cvaC etsC hlyF, hra iroN iss iucC iutA lpfA mchF ompT sitA terC traT	89%
G1PAE9	ctx-m-1	ST23	O78	4-402	aadA1, catA1, cmlA1, aadA2b, mdf(A), sul3	IncFlB (AP001918), IncFIC(FII), Incl1-l (Alpha)	fyuA, hlyF, irp2 iss iucC iutA lpfA ompT sitA	87%

							terC	
G1PHE1	ctx-m-15	ST10	unknown	11-54	Mdf(A), qnrS1, tet(B)	Col (MG828), IncFIB(pHCM2), Incl1-l (Alpha)	cea gad terC	84%
G1PHE2	ctx-m-1	ST23	O8	4-35	aadA1, dfrA12 catA1, cmlA1, mdf(A), sul3	Col440ll, IncFlB (AP001918), IncFIC(FII), Incl1-l (Alpha), IncY	cea cia cvaC etsC hlyF iroN iss iucC iutA lpfA mchF ompT sitA terC traT	93%
G1SAE2	ctx-m-1	ST877	unknown	175-25	aadA1, catA1, cmlA1, dfrA12, mdf(A), sul3, sul1, tet(B)	IncR, IncFII, Col(pHAD28)	cea, hra ompT terC, traT, tsh	94%

G1SAE4	ctx-m-1	ST877	O45	175-25	aph(3'')-Ib, aph(6')-Id, cmlA1, aadA1, aadA2b, mdf(A),sul3, tet(A)	IncR, IncFII, Col(pHAD28), IncFIB(pHCM2)	lpfA ompT terC, traT, tsh	89%
G1SAE8	ctx-m-1	ST877	O45	175-25	aph(3'')-Ib, aph(6')-Id, cmlA1, aadA1, aadA2b, mdf(A),sul3, tet(A)	IncR, IncFII, Col(pHAD28), IncFIB(pHCM2)	lpfA ompT terC, traT, tsh	89%
G1SAE7	ctx-m-1	ST877	O45	175-25	aph(3'')-Ib, aph(6')-Id, cmlA1, aadA1, aadA2b, mdf(A),sul3, tet(A)	IncR, Col(pHAD28), IncFIB(pHCM2)	lpfA ompT terC	95%
G1SAE10	ctx-m-1	ST877	O45	175-25	aph(3'')-Ib, aph(6')-Id, cmlA1, aadA1, aadA2b, mdf(A),sul3, tet(A)	IncR, IncFII, Col(pHAD28), IncFIB(pHCM2)	lpfA ompT terC, traT, tsh	89%
P1FAE1	ctx-m-14, tem-1B	ST101	O88	41-86	Aac(6')-Ib3, aac(6')-Ib-cr, aadA1, cmlA1ermB, dfrA1, mdf(A), mph(A),sul3, tet(A)	IncB/O/K/Z, IncFlB (AP001918), IncFIC(FII), Incl1-l (Alpha)	cvaC etsC hlyF iroN iss iucC iutA lpfA	93%

							mchF ompT sitA terC, traT tsh	
P1FAE3	ctx-m-14	ST117	O9	45-97	Aac(6')-Ib3, Aac(6')-Ib-cr aph(3'')-Ib, aph(6)-Id, cmlA1, ermB, dfrA1, mdf(A), mph(A),sul2, tet(B)	IncB/O/K/Z, IncFIB (AP001918)	chuA, cia, cvaC etsC fyuA hlyF, ireA iroN, irp2 iss iucC iutA, katP, lpfA mchF ompT papC, sitA terC, traT, vat	93%
P1FAE7	ctx-m-1, tem-1A	ST446	unknown	7-41	Aac(3)-IV, aadA1, aadA2b, aadA5, aph(3')-la, aph(4)-la, catA1, cmlA1, dfrA12,	IncB/O/K/Z, IncFIB (AP001918), Incl1-I(Alpha)	terC	92%

					dfrA17, mdf(A), mph(B),sul3, tet(B)			
P1PAE2	ctx-m-14, tem-1B	ST23	O8	4-35	Aac(6')-Ib-cr, Aac(6')-Ib3, aadA1, aadA5, cmlA1, dfrA17, ermB, mdf(A), mph(A),qnrS1 sul2, tet(A)	IncB/O/K/Z, IncFlB (AP001918), Incl1-l (Alpha), IncX1, IncY, Col156, Col (MG828)	cea, celb cia cib cvaC etsC hlyF iroN iss iucC iutA lpfA mchF ompT sitA terC traT	93%
P1PAE3	ctx-m-14, tem-1B	ST3933	O7	506-544	Aac(6')-Ib-cr, Aac(6')-Ib3, cmlA1, ermB, mdf(A), mph(A),qnrS1 tet(M)	IncB/O/K/Z, IncFlB (AP001918), IncFIA, IncFIC(FII), IncX1	astA chuA eilA kpsE kpsMII_K5 ltcA	89%

							sitA stB terC traT	
P1PAE4	ctx-m-14, tem-1B	ST23	O8	4-35	Aac(6')-Ib-cr, Aac(6')-Ib3, aadA1, aadA5, cmlA1, ermB1, dfrA17, mdf(A), mph(A),qnrS1 sul2, tet(A)	IncB/O/K/Z, IncFlB (AP001918), Incl1-l (Alpha), IncX1, IncY, Col156, Col (MG828)	cea celb cia cib cvaC etsC gad hlyF iroN iss iucC iutA lpfA mchF ompT sitA terC traT	93%
P1PAE6	ctx-m-14, tem-1B	ST23	O8	4-35	Aac(6')-Ib-cr, Aac(6')-Ib3, aadA1, aadA5, cmlA1,	IncB/O/K/Z, IncFlB (AP001918), Incl1-l	cea celb	89%

					ermB, dfrA17, mdf(A), mph(A),qnrS1 sul2	(Alpha), IncX1, IncY, Col156, Col (MG828)	cia cib cvaC etsC hlyF iroN iss iucC iutA lpfA mchF ompT sitA terC traT	
P1PAE7	ctx-m-14, tem-1B	ST23	O8	4-35	Aac(6')-Ib3, Aac(6')-Ib-cr aadA1, aadA5, cmlA1, ermB, dfrA17, mdf(A), mph(A),qnrS1 , sul2, tet(A)	IncB/O/K/Z, IncFlB (AP001918), Incl1-l (Alpha), IncX1, IncY, Col156	cea celb cia cvaC etsC, gad hlyF iroN iss iucC	89%

							iutA lpfA mchF ompT sitA terC traT	
P1PAE8	ctx-m-14, tem-1B	ST23	O8	4-35	Aac(6')-Ib3, Aac(6')-Ib-cr aadA1, aadA5, cmlA1, ermB, dfrA17, mdf(A), mph(A),qnrS1 sul2, tet(A)	IncB/O/K/Z, IncFlB (AP001918), Incl1-l (Alpha), IncX1, IncY, Col156, Col (MG828)	cea celb cia cib cvaC etsC hlyF iroN iss iucC iutA lpfA mchF ompT ompT sitA	89%

							terC traT	
P1PAE9	ctx-m-14	ST23	O8	4-35	Aac(6')-Ib3, Aac(6')-Ib-cr, aadA1, aadA5, cmlA1, ermB, dfrA17, mdf(A), mph(A),qnrS1 sul2, tet(A)	IncB/O/K/Z, IncFlB (AP001918), Incl1-l (Alpha), IncX, Col156, Col (MG828)	cea celb cia cib cvaC etsC hlyF iroN iss iucC iutA lpfA mchF ompT sitA terC traT	89%
P1PAE10	ctx-m-14, tem-1B	ST23	O8	4-35	Aac(6')-Ib3, Aac(6')-Ib-cr aadA1, aadA5, cmlA1, ermB, dfrA17, mdf(A), mph(A),qnrS1 sul2, tet(A)	IncB/O/K/Z, IncFlB (AP001918), Incl1-l (Alpha), IncX1, Col156, Col (MG828)	cea celb cia cvaC etsC hlyF	93%

							iroN iss iucC iutA lpfA mchF ompT sitA terC, traT	
S1FHE2	ctx-m-14	ST1380	O17	35-47	aadA2 cmlA1, dfrA12, dfrA36, floR, mdf(A), qnrS1, sul1, sul2	Col8282, IncQ1, IncFlB (AP001918), IncFlC (Fll), IncFll (pCoo), IncY	astA chuA eilA hra kpsE kpsMII lpfA ltcA stb terC traT	88%
T1SAE6	ctx-m-1	4761	O107	252-27	aadA1, aadA2 cmlA1, dfrA12, mdf(A), mph(A), sul3, tet(A)	IncN, IncX1	kpsE kpsMII terC	83%

T1SAE7	ctx-m-1, tem-1B	ST48	O61	11-0	aadA1, aadA2 cmlA1, dfrA12, mdf(A), mph(A), sul3, tet(A)	IncN, Col (MG28, IncFIA (HI1), Inc FIB (K), IncX1	astA gadA hra iss neuC papC terC	88%
T1SAE8	shv-12, tem-1A	ST48	O8	11-54	mdf(A), mph(B), qnrS1, tet(B)	IncX3, IncY, Col440I	astA gad terC	87%
T1SAE9	ctx-m-1	ST410	O25	4-24	aadA1, aadA2 cmlA1, dfrA12, mdf(A), mph(A), sul3, tet(A)	IncN, IncX1	lpfA terC	88%
T1SAE10	ctx-m-1	4767*	O107	252-27	aadA1, aadA2 cmlA1, dfrA12, mdf(A), mph(A), sul3, tet(A)	IncN, IncX1	gad kpsE kpsMII terC	83%

Table 1: WGS analysis of the 30 ESBL-producing *E. coli* sampled from 4 swine farms located in Piedmont. In the last column on the right side of the table, probability of human pathogenicity is expressed as percentage.

4767*:is the nearest ST found for the strain T1SAE10.

7.6. Acknowledgements

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Chapter 8 - Antimicrobial stewardship and antimicrobial resistance in swine farms from north-western Italy

8.1. Abstract

Considering the elevated AMR level found in livestock, nowadays there is an urgent need of antimicrobial stewardship programs even at veterinary level. Indeed, monitoring of antibiotic usage (AMU) at farm is paramount to understand strategy to limit the overall AMR. Aims of our study were: 1) identify the phenotypic susceptibility to different antibiotic classes (including CIAs, like ceftaroline, linezolid and meropenem) in a group of methicillin-resistant staphylococci (MRS) (n=28), and in ESBL-producing *E. coli* (n=29); 2) analysis of the frequency (total number of prescriptions x year) and route of administration of antibiotics prescribed at farm level. To do this, animals' samples and treatments' registries were taken from 5 farms (B,G,P,S and T) from Piedmont (Italy). Results indicated that all MRS were multidrug resistant, especially to clindamycin, doxycycline, tetracycline and tiamulin. Susceptibility to florfenicol was found in 15/28 tested strains. Few MRS were susceptible to enrofloxacin, while 6 samples were linezolid resistant. From these, 4 carried a genetic marker for linezolid resistance: *cfr* (n=1) and *optrA* (n=3); looking to ESBL-producing *E. coli,* resistance to enrofloxacin (23/29) and florfenicol (27/29) was widespread across all sampled farms and no strain was meropenem resistant. However, a strain was resistant to all tested antibiotics, with the only exception of meropenem. Results about AMU indicated an overall high consumption of antibiotics, particularly oral aminopenicillins (mostly amoxicillin) and penicillins in all farms, apart farm G where macrolides (oral and injectable) were the most often used antibiotics. Fluoroquinolones were scarcely prescribed; only in one farm (B) a high consumption was registered; in the same farm phenicols were administered frequently. Indeed, in this farm *optrA* positive MRS were detected. These results indicate that CIAs (like amoxicillin) are still used orally as first-choice in animal treatment; moreover, they highlight the presence of multiple resistance, that could be due to the elevated quantity of antibiotics used at farm level.

8.2. Introduction

In the last decades antimicrobial resistance (AMR) has gained the level of public health concern. More than half of the total antibiotics consumed worldwide are used to treat farm animals [331]. In livestock farming, the swine productive chain has demonstrated elevated antimicrobial consumption, mostly related to the post-weaning productive stage [332,333,334]. Animals are mainly treated by oral drugs, and among the critical important antibiotics (CIAs), extensive oral use of macrolides is a common practice, particularly to tackle group respiratory infections [334]. Considering that livestock is a recognised reservoir of AMR-associated genes, measures are urgently needed to combat the misuse of antibiotics, the occurrence of resistant bacteria in livestock sector and the transmission to human community [335,336].

The necessity of antimicrobial stewardship programs is nowadays accepted in the veterinary sector [337,338]. However, this practice needs to be fully implemented in the livestock productive chain. In Italy in 2018, Regional guidelines (Emilia-Romagna region) on the prudent use of antibiotics in domestic swines were officially published by Istituto Zooprofilattico sperimentale della Lombardia e dell'Emila-Romagna in collaboration with Istituto Zooprofilattico sperimentale Lazio e Toscana and University Alma Mater Studiorum of Bologna [339]. These guidelines highlight the need of an initial diagnosis that is based on symptoms, lesions, sampling on the site of infection, and laboratory susceptibility tests to choose the right antibiotic. Furthermore, drug selection needs to consider that not all the active ingredients have the same importance in a One-Health view; in fact, there are first-

, second- and third- choice molecules that should be selected based on the laboratory antibiotic susceptibility test (AST), especially for second- and third- choice antibiotics. The routes of administration (oral, intramuscular, intramammary etc..) are also really important, as some of them, like the oral administration, are able to elicit resistance more than others, especially in *E. coli* [340]. To tackle antimicrobial resistance, biosecurity measures and good farm practices are also relevant preventive measures. Moreover, a good management of water and feed distribution systems are important, because the majority of antibiotics are given orally (97.5%) [341].
Objective of our study was to investigate the susceptibility to different antibiotic classes, including CIAs like oxazolidinones and ceftaroline, in methicillin-resistant staphylococci (MRS), and carbapenems in ESBL-producing *E. coli* previously sampled from 5 farms of Cuneo province. Also, analysis of the quantity and frequency of antibiotics consumption at farm level was carried out, to define a possible association between AST results and antibiotic usage (AMU).

8.3. Materials and Methods

8.3.1. Laboratory analysis

As described in the previous chapters, nasal and faecal samples were taken from healthy pigs from four intensive farms (B, G, P and T) and one organic farm (S) located in Cuneo province, Piedmont region (Italy) during 2019-2020. Phenotypic analysis to isolate single pure colonies was previously described in Chapter 5, paragraph 5.3.3 "Laboratory and statistical analyses". The samples' size of MRS and ESBL-producing *E. coli* for antimicrobial susceptibility tests was limited due to economic reasons. Among confirmed MRS, 28 staphylococci were chosen for further susceptibility tests based on an equal representation of each selected farm and considering the different staphylococcal species identified. To detect resistance in MRS, AST, through Kirby-Bauer disk diffusion method, was performed for the antibiotics: clindamycin, doxycycline, erythromycin, enrofloxacin, florfenicol, gentamicin, linezolid, tetracycline, tiamulin and trimethoprim/sulphamethoxazole. Minimum inhibitory concentration (MIC) through test strip (*Liofilchem*®, Roseto degli Abbruzzi, Teramo, Italy) was used to test resistance to ceftaroline, using CLSI breakpoints [342]. Considering ESBL-producing *E. coli*, we selected 29 strains, sampled from 4 farms, with the exception of Farm B, due to problem in bacterial strains' stock. Selection of ESBL-producing isolates was done considering the prevalence of ESBL-producing *E. coli* recovered in each selected farm (see Table 1). We performed AST, using disk diffusion method, for the antibiotics: doxycycline, enrofloxacin, florfenicol, gentamicin, meropenem, tetracycline and trimethoprim/sulfamethoxazole. EUCAST guidelines were followed to assess linezolid and meropenem AST results [308], while CLSI for the other antibiotics [308,342]. After phenotypic AST, linezolid-resistant staphylococcal strains were processed through genotypic analysis with two simplex PCR protocols for *cfr* and *optrA* genes, encoding for resistance to oxazolidinones antibiotics. Primers for *cfr* (746 bp) and *optrA* (1395 bp) were chosen based on previous studies [272,343]. After detection of the amplicons on agarose gel, positive samples were purified with ExoSAP-IT™ PCR Product Clean-up Kit (GE Healthcare Limited, Chalfont, UK) and Sanger sequenced in an external laboratory (BMR Genomics, Padova, Italy). Obtained sequences of *cfr* and *optrA* were analysed with BioEdit 7.2.5 Sequence Alignment Editor©software, while ClustalW tool was launched for multiple alignment with previously detected *cfr* and *optrA* sequences. We used BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for matching our nucleotide sequences with all the accessible in GenBank. Considering *E. coli* strains, WGS was used to confirm genotypically the AST results, as described in Chapter 7.

Farms	Farm type	ESBL-producing *E. coli*
		(overall)
Farm B	intensive	**56.7%** (95%CI: 38.9, 74.4)
Farm G	intensive	**43.3%** (95%CI: 25.6, 61.1)
Farm P	intensive	**33.3%** (95%CI:16.5, 50.2)
Farm T	intensive	**23.3%** (95%CI: 8.2, 38.5)

Table 1: prevalence of ESBL-producing *E. coli* recovered from animal samples in 4 farms from Piedmont, Italy, during 2019-2020. Although, the highest prevalence was detected in farm B, ESBL-producing *E. coli* isolated from this farm were not tested for antibiotic susceptibility, due to problem in bacterial strains' stock.

8.3.2. Antibiotic usage (AMU) analysis

Data recorded in the ClassyFarm 2019 report were used to semi-quantitatively analyse the percentage of antibiotics consumed in four sampled farms (Farm B, G, P, and T). Farm S was not visited by the ClassyFarm team. Antimicrobial usage (AMU) was evaluated in ClassyFarm using a treatment index per 100 days (TI_{100}) through the defined daily dose animal for Italy (DDDAit) parameter [215]. In the meantime, analyses on the frequency and routes of administration of antibiotic were carried out using treatments' registries provided by the veterinarians from the Local Veterinary Service of Animal Health, (Savigliano, Cuneo). The analysis was performed by the Pharmacology sector of the Veterinary Sciences Department, University of Turin, specifically taking into account the number of prescribed treatments for a specific active ingredient for the year 2019-2020 (frequency). Considering farm T, only 2020 data were used, because animal sampling was carried out in September 2020.

8.4. Results

8.4.1. Antibiotic susceptibility test (Kirby-Bauer disk-diffusion test, MIC test strip, cfr and optrA PCR detection)

All selected MRS and ESBL-producing *E. coli* strains were multidrug resistant (MDR), because they were phenotypically resistant to more than 3 antibiotic classes (Table 2 and 3).
Considering more in depth MRS, no strain was resistant to ceftaroline, although two strains P1PAS15 and G1PAS6, could not be considered fully susceptible (Figure 1-2); indeed, P1PAS15 had a MIC of 1/1.5 µg/ml, while G1PAS6 1.5 µg/ml (CLSI breakpoints: S≤1, 2-4 SDD, ≥8 R). All MRS were resistant to clindamycin, doxycycline, tetracycline and tiamulin (Figure 3). Only four staphylococcal strains were enrofloxacin susceptible, while almost all (23/28) were still susceptible to trimethoprim/sulfamethoxazole. More than half of the strains were resistant to gentamicin (23/28)

and erythromycin (20/28) (see Figure 4). Susceptibility to florfenicol was found in 15 out of 28 tested strains. We found 6 samples phenotypically positive for linezolid resistance (Figure 4). The same strains were resistant to florfenicol and tiamulin too. Out of these phenotypically linezolid-resistant staphylococci, 4 presented oxazolidinones' associated resistant genes (*cfr* and *optrA;* Table 4). Specifically, we recovered a positive *cfr* sample (*S. sciuri*) from the post-weaning of an intensive farm (P1PAS6) (see Figure 5). *optrA* gene was detected in 3 samples belonging from the same intensive farm (B), but sampled in different productive phases (finishing, post-weaning and sows) (see Figure 6). No staphylococcal strain carried both linezolid-resistant genes. *optrA*-associated staphylococci were different at species level: *S. pasteuri* in the finishing, *S. cohnii* in post-weaning and *S. sciuri* in sows.

Through Sanger sequencing we confirmed *cfr* and *optrA* presence with 100% identity with available sequences from BLAST®.

Sample	Farm	Doxycycline	Enrofloxacin	Florfenicol	Gentamicin	Meropenem	Tetracycline	Trimethoprim/Sulfamethoxazole
T1FAE5	T	R	R	S	R	S	R	S
T1SAE6	T	R	S	R	S	S	R	R
T1SAE7	T	R	R	R	S	S	R	R
T1SAE8	T	R	R	S	S	S	R	S
T1SAE9	T	R	R	R	S	S	R	R
T1SAE10	T	R	R	R	S	S	R	R
G1PAE2	G	R	S	R	S	S	R	R
G1PAE3	G	R	S	R	S	S	R	R
G1PAE4	G	R	S	R	S	S	R	R
G1PAE6	G	R	S	R	S	S	S	R
G1PAE7	G	R	R	R	R	S	R	R
G1PAE8	G	R	R	R	S	S	R	R
G1PAE9	G	S	R	R	S	S	S	R
G1SAE2	G	R	R	R	S	S	R	R
G1SAE4	G	R	R	R	S	S	R	R
G1SAE7	G	R	R	R	S	S	R	R
G1SAE8	G	R	R	R	S	S	R	S
G1SAE10	G	R	R	R	S	S	R	R
P1PAE2	P	R	R	R	S	S	R	R
P1PAE3	P	R	R	R	S	S	R	R
P1PAE4	P	R	R	R	S	S	R	R
P1PAE6	P	R	R	R	S	S	R	R
P1PAE7	P	R	R	R	S	S	R	R
P1PAE8	P	R	R	R	S	S	R	R
P1PAE9	P	R	R	R	S	S	R	R
P1PAE10	P	R	R	R	S	S	R	R
P1FAE7	P	R	R	R	S	S	R	S
P1FAE3	P	R	S	R	S	S	R	R
P1FAE1	P	R	R	R	S	S	R	R

Table 2: Antibiotic susceptibility results of selected ESBL-producing *E. coli,* isolated from healthy pigs during 2019-2020. “R”= resistant; “S”= susceptible.

Sample	Farm	*Staphylococcus spp.*	Cefta	Clinda	Doxy	Erithro	Enro	Florfe	Genta	Linez	Tetra	Tiamu	Trime/Sulfa	*cfr*	*optrA*
B1SAS9	B	*S. sciuri*	1	R	R	S	R	R	R	R	R	R	S	neg	pos
B1SAS11	B	*S. sciuri*	.75	R	R	R	R	R	R	S	R	R	S	na	na
B1SAS3	B	*S. equorum*	.75	R	R	R	R	R	R	S	R	R	R	na	na
B1SAS15	B	*S. equorum*	.25	R	R	R	R	R	R	S	R	R	S	na	na
B1PAS15	B	*S. cohnii*	.75/1	R	R	R	S	R	R	R	R	R	R	neg	pos
B1PAS10	B	*S. sciuri*	.75	R	R	R	R	R	R	S	R	R	S	na	na
B1FAS9	B	*S. sciuri*	.75	R	R	R	R	S	R	S	R	R	S	na	na
B1FAS13	B	*S. pasteuri*	1	R	R	R	R	R	R	R	R	R	S	neg	pos
B1FAS15	B	*S. haemolyticus*	.75	R	R	S	R	R	R	S	R	R	S	na	na
G1PAS6	G	*S. sciuri*	1.5	R	R	R	R	R	R	S	R	R	S	na	na
G1PAS15	G	*S. sciuri*	1	R	R	R	R	R	R	R	R	R	S	neg	neg
G1SAS7	G	*S. sciuri*	1	R	R	R	R	R	R	S	R	R	S	na	na
G1SAS12	G	*S. sciuri*	1	R	R	R	R	S	R	S	R	R	S	na	na
P1SAS14	P	*S. cohnii*	.38	R	R	R	R	R	S	R	R	R	R	neg	neg
P1PAS6	P	*S. sciuri*	.75	R	R	R	R	R	R	R	R	R	S	pos	neg
P1PAS15	P	*S. sciuri*	1/1.5	R	R	R	R	S	R	S	R	R	S	na	na
P1FAS2	P	*S. sciuri*	.75	R	R	R	R	S	R	S	R	R	S	na	na
P1FAS3	P	*S. sciuri*	.75	R	R	R	R	S	S	S	R	R	S	na	na
S1FAS2	S	*S. sciuri*	.75	R	R	S	R	R	R	S	R	R	R	na	na
S1FAS7	S	*S. sciuri*	.25	R	R	S	R	R	R	S	R	R	R	na	na
S1FAS10	S	*S. sciuri*	.75	R	R	S	R	R	R	S	R	R	S	na	na
S1FAS14	S	*S. sciuri*	.75/1	R	R	S	R	S	R	S	R	R	S	na	na
T1PAS3	T	*S. aureus*	.38	R	R	S	S	S	S	S	R	R	S	na	na
T1PAS4	T	*S. sciuri*	1	R	R	R	S	S	R	S	R	R	S	na	na
T1PAS12	T	*S. pasteuri*	.5	R	R	R	R	R	S	S	R	R	S	na	na
T1FAS7	T	*S. haemolyticus*	.5	R	R	S	S	S	R	S	R	R	S	na	na
TIFAS15	T	*S. haemolyticus*	.75/1	R	R	S	R	S	R	S	R	R	S	na	na
T1SAS12	T	*S. xylosus*	.5	R	R	R	R	S	S	S	R	R	S	na	na

Table 3: Antibiotic susceptibility results of selected methicillin-resistant staphylococci (MRS), isolated in healthy pigs during 2019-2020. Tested antibiotics were: ceftaroline (Cefta), clindamycin (Clinda), doxycycline (Doxy), erythromycin (Erithro), enrofloxacin (Enro), florfenicol (Florfe), gentamicin (Genta), linezolid (Linez), tetracycline (Tetra), tiamulin (Tiamu) and trimethoprim/sulfamethoxazole (Trime/Sulfa). The last 2 columns indicate PCR results for *cfr* and *optrA* detection in linezolid-resistant strains.
"R"= resistant; "S"= susceptible; "na"=not available.

Strains	Staphylococcal species	*cfr*	*optrA*
B1FAS13	*Staphylococcus pasteuri*	-	+
B1PAS15	*Staphylococcus cohnii*	-	+
B1SAS9	*Staphylococcus sciuri*	-	+
G1PAS15	*Staphylococcus sciuri*	-	-
P1PAS6	*Staphylococcus sciuri*	+	-
P1SAS14	*Staphylococcus cohnii*	-	-

Table 4: phenotypic linezolid-resistant staphylococci with oxazolidinone associated-resistant genes (*cfr* and *optrA*) recovered through PCR.

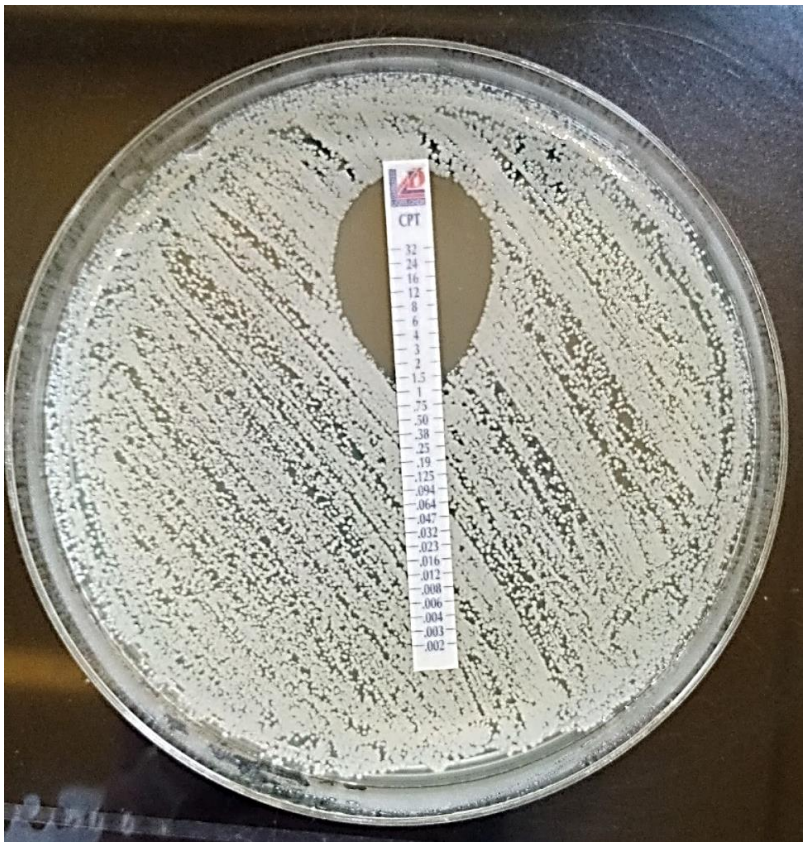

Figure 1: *S. sciuri* (P1PAS15) MIC test strip for the antibiotic ceftaroline (CPT). The eclipse starts between 1 and 1.5 µg/ml indicating that the strain may not be completely susceptible to ceftaroline (CLSI guidelines). Photo by Miryam Bonvegna.

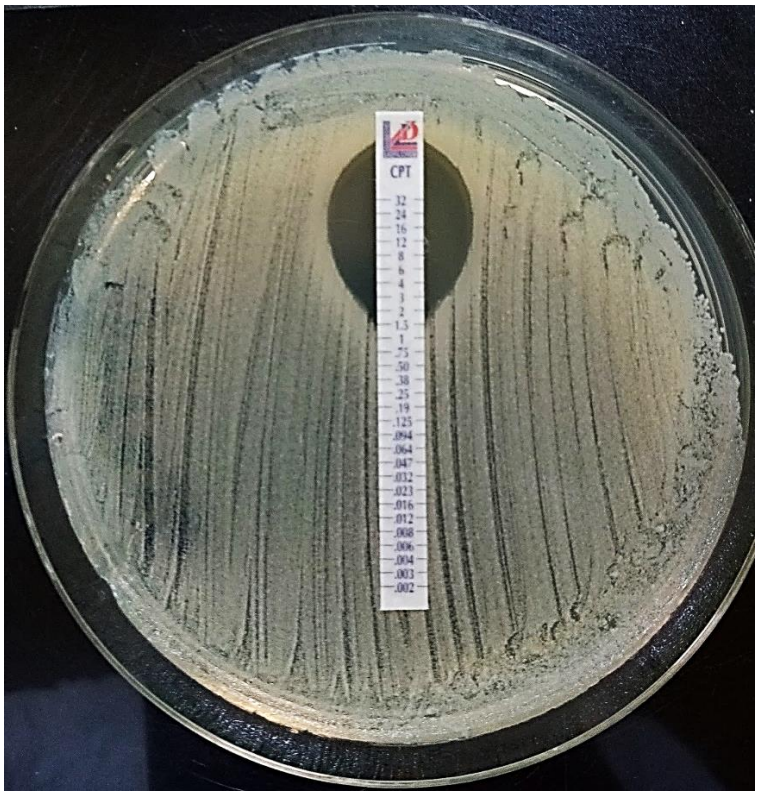

Figure 2: *S. sciuri* (G1PAS6) MIC test strip for the antibiotic ceftaroline (CPT). The eclipse starts at 1.5 µg/ml, indicating an intermediate susceptibility (dose dependent) to this fifth-generation cephalosporin (CLSI guidelines). Photo by Miryam Bonvegna.

Figure 3: Multidrug resistant strain *Staphylococcus cohnii* (P1SAS14) on Mueller-Hinton. No halo is visible for enrofloxacin (ENR), tiamulin (T), clindamycin (DA) and tetracycline (TE). For doxycycline (DO) the halo's diameter is under the range to confirm susceptibility, so it was considered resistant. Photo by Miryam Bonvegna.

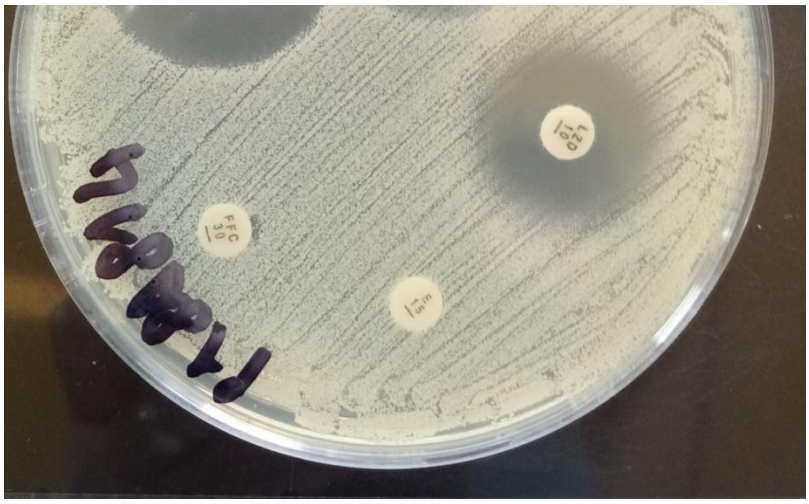

Figure 4: Multidrug resistant strain *Staphylococcus cohnii* (P1SAS14) on Mueller-Hinton. On this plate, the halo's diameter around the linezolid disk (LZD) is under the normal range for considering susceptible a staphylococcus strain (EUCAST guidelines). Moreover, the same strain is resistant to florfenicol (FFC) and erythromycin (E) too, because no halo is visible around the two disks. Photo by Miryam Bonvegna.

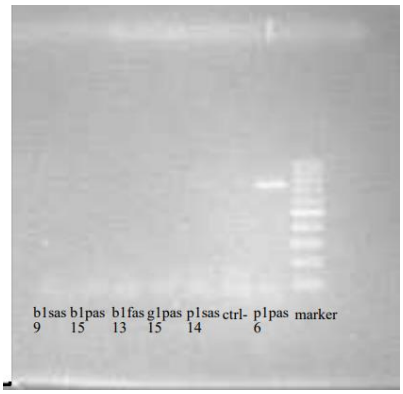

Figure 5: *cfr* positive sample P1PAS6 (amplicon= 746 bp) on agarose gel. Marker (last column on the right) of 100 bp was used.

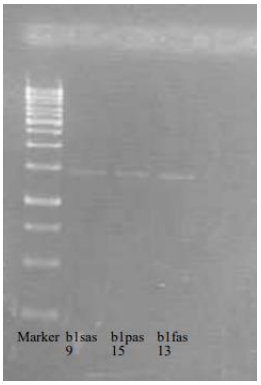

Figure 6: *optrA* positive samples B1SAS9, B1PAS15, B1FAS13 (amplicon=1395) on agarose gel. Marker (first column on the left) of 1 kb was used.

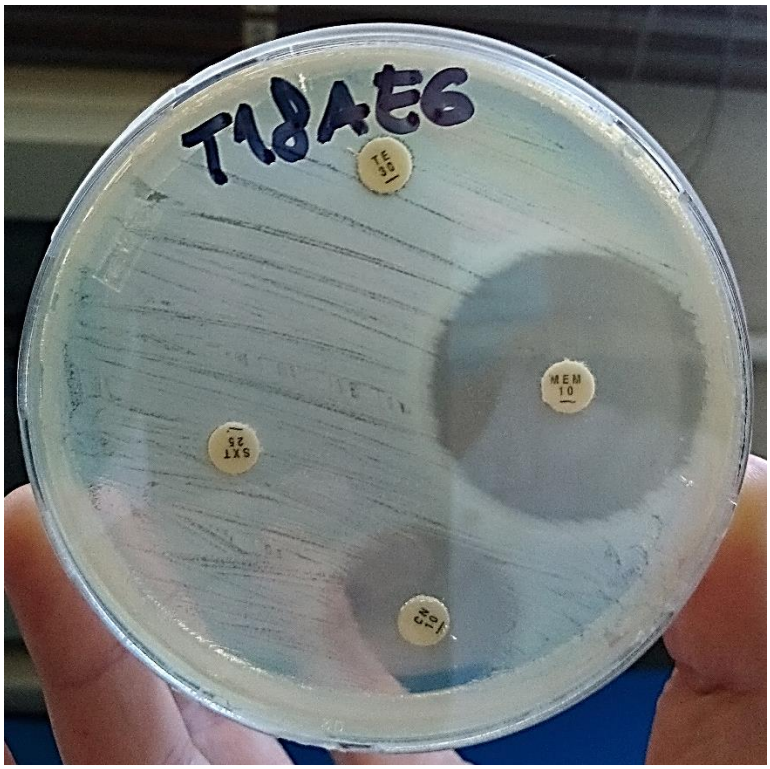

Figure 7: a susceptible *E. coli* (T1SAE6) to meropenem (MEM). The strain is resistant to gentamicin (CN), trimethoprim/sulfamethoxazole (SXT) and tetracycline (TE). Photo by Miryam Bonvegna.

Considering ESBL-producing *E. coli*, all tested strains were multidrug resistant. Resistance to doxycycline was total across all farms, with the exception of the strain G1PAE9. The same strain, together with G1PAE6, was the only susceptible to tetracycline, precursor of the semi-synthetic antibiotic doxycycline [344]. Resistance to enrofloxacin (23/29) and florfenicol (27/29) was also disseminated across all sampled farms. Susceptibility was scarce against trimethoprim/ sulfamethoxazole; in fact, only four samples were not resistant (G1SAE8, P1FAE7, T1FAE5 and T1SAE8). Contrary, susceptibility to gentamicin was present in almost all strains, apart T1FAE5 and G1PAE7. The post-weaning strain G1PAE7, sampled from farm G, was the only one that was resistant to all tested antibiotics, with the exception of the human CIA meropenem. Indeed, all *E. coli* were fully susceptible to meropenem (see Figure 7).

8.4.2. Antimicrobial usage (AMU): DDDAit (ClassyFarm), *frequencies and routes of administration*

Looking to ClassyFarm data (calculated DDDAit per biomass for the year 2019) it is clear that all farms presented elevated levels of antibiotics' consumption (see Table 5). More in depth, the majority of the antibiotics were used in farm P (129.1 DDD), while farm G was the farm where less antibiotics

were administered to animals. However, considering the specific type of DDD (critical, non-critical and pre-critical) it is noteworthy to say that farm P had the lowest number of DDD associated to critical antibiotics, that include 3rd and 4th generation cephalosporins, quinolones and polymyxins (0.1). Moreover, all farms showed very low levels of critical DDD, with the maximum found in farm G (1).

Farm	DDD Biomass 2019	Critical DDD	Non-Critical DDD	Pre- Critical DDD
Farm B	82.7	0.5	80.6	1.6
Farm G	10.4	1	2.9	6.5
Farm P	129.1	0.1	122.9	6,1
Farm T	48.5	0.2	47.5	0.8

Table 5: DDDAit recovered for the year 2019 in the four sampled farms B, G, P and T.

Considering data elaborated from the colleagues in the Pharmacology sector, most of the antibiotics were administered orally through premix or drinking water (see Table 6). However, the injectable way of administration was also frequently used, especially in farm B for the years 2019 and 2020, and farm P for 2020. Skin (subcutaneous) antibiotic administration was only used in farm B. Considering antibiotic classes, aminopenicillins and penicillins were the most frequently administered in all farms, with the exception of farm G, where macrolides were the most often used antibiotics. However, among penicillins/aminopenicillins, the CIA amoxicillin was the most often administered antibiotic. This was observed in farm B and farm T, while farm G preferred to use tylosin and lincomycin, a macrolide and a lincosamide respectively. Even farm P administered more frequently lincomycin. The other two most often used antibiotics of farm P were doxycycline and amoxicillin. Among macrolides, different active ingredients were administered: gamithromycin, tilmicosin, tylosin and tulathromycin. These antibiotics were administered orally (acetyl valeryl tylosin, tilmicosin and tylosin) and intramuscularly (gamithromycin, tulathromycin and tylosin) and were used particularly in farm B, G and P. The highest frequency (51) of the injectable tulathromycin was observed in farm B, especially during 2020. The use of fluoroquinolones was limited. Enrofloxacin was the only fluoroquinolones/quinolones' class active ingredient used in farm P and T, while in farm B, besides enrofloxacin, marbofloxacin and flumequine were rarely administered. The only farm to use a 3rd generation cephalosporin (ceftiofur) was farm B. No polymyxin was used in any of the four tested farms. Aminoglycosides were commonly administered in farm G, where three different active ingredients were prescribed: aminosidine, apramycin and gentamicin. Aminosidine and apramycin were both administered orally. The highest frequency of phenicols' administration was observed in farm B, where two different antibiotics were used: florfenicol and thiamphenicol. Phenicols were used also in farm P and T, while farm G did not treat animals with this antibiotic class. Pleuromutilins were rarely used or administered in association with doxycycline, as observed in farm B. The tetracycline doxycycline was used in farm P with high frequencies (22 for 2019 and 28 for 2020), while in farm G was not used at all. The other two farms consumed less doxycycline or used preferably (farm B) other tetracyclines like oxytetracycline or chlortetracycline. Sulphonamides were particularly used in farm B (see table 6).

Farm P		
Antibiotics (AMU)	**2019**	**2020**
Amoxicillin	22	21
Benzylpenicillin	10	13
Doxycycline	22	28
Enrofloxacin	3	4
Florfenicol	5	4
Gamitromycin	6	12
Gentamicin	9	5
Lincomycin	30	27
Lincomycin + spectinomycin	13	13
Oxytetracycline	3	5
Rifaximin	2	1
Sulfadimetoxina	2	5
Sulfametoxina + chlortetracycline	0	0
Thiamphenicol	3	2
Tiamulin	0	1
Tiamulin + doxycycline	0	0
Tilmicosin	0	0
Tylosin	8	11
Tylosin	7	17
Tulathromycin	2	3

Farm T		
Antibiotics (AMU)	**2019**	**2020**
Amoxicillin	na	54
Ampicillin + dicloxacillin	na	24
Sulfametoxina + chlortetracycline	na	2
Doxycycline	na	12
Enrofloxacin	na	16
Florfenicol	na	11
Sulfametazina + trimethoprim	na	4
Tiamulin + chlortetracycline	na	2
Tiamulin + doxycycline	na	4
Tylosin	na	1
Amoxicillin + lincomycin	na	4
Amoxicillin + clavulanic acid	na	2
Chlortetracycline	na	0
Lincomycin + spectinomycin	na	0
Tiamulin	na	0

Route of administration	**2019**	**2020**
Injectable	58	139
Drinking water	56	30
Premix	32	4
Skin	0	0

Route of administration	**2019**	**2020**
Injectable	na	53
Drinking water	na	41
Premix	na	36
Skin	na	0

Farm G

Antibiotics (AMU)	2019	2020
Acetyl isovaleryl tylosin	18	1
Aminosidine	4	13
Amoxicillin + clavulanic acid	0	1
Amoxicillin	0	1
Ampicillin	2	3
Apramycin	4	1
Gamitromycin	3	14
Gentamicin	0	1
Lincomycin	4	14
Sulfametoxina + trimethoprim	0	1
Tilmicosin	0	1
Tylosin	0	2
Tulathromycin	0	1

Route of administration	2019	2020
Drinking water	8	15
Premix	22	17
Skin	0	0

Farm B

Antibiotics (AMU)	2019	2020
Aminosidine	33	0
Amoxicillin + clavulanic acid	0	6
Amoxicillin	63	94
Benzylpenicillin	0	1
Ceftiofur	5	0
Chlortetracycline	0	17
Doxycycline	0	6
Enrofloxacin	14	0
Florfenicol	33	54
Flumequine	1	1
Gentamicin	1	1
Lincomycin	4	1
Lincomycin + spectinomycin	0	2
Marbofloxacin	1	0
Oxytetracycline	19	13
Sulfametossazina + trimethoprim	1	26
Sulfametoxin + chlortetracycline	0	40
Thiamphenicol	6	19
Tiamulin	0	4
Tiamulin + doxycycline	0	10
Tilmicosin	0	2
Tylosin	2	7
Tulathromycin	32	51

Route of administration	2019	2020
Injectable	147	186
Drinking water	63	30
Premix	0	139
Skin	8	10

Table 6: frequency (number of prescribed treatments for a specific active ingredient x year) and routes of administration of the antibiotics used to treat animals in farm B, G, P and T during 2019-2020. For farm T, data on the antibiotic usage in 2019 were not available (na= not available).

8.5. Discussion

Considering data on AMR on staphylococci and *E. coli*, our selected strains are all multidrug resistant. This information is corroborated by the elevated and frequent antibiotic usage, as reported by ClassyFarm data and our analysis on frequency of antimicrobial administration resulting from the analysis of drug prescriptions registries. However, a direct correlation between resistance found in our isolates and antibiotic administration cannot be established, due to a limited number of tested strains per farm and the lack of statistical analysis. Some antibiotics, like pleuromutilins, are rarely used, but their resistance is widespread (e.g. MRS in all farms and different productive stages). This can be explained by the fact that, in previous decades, pleuromutilins were frequently used in swine farming, and so this phenomenon is not necessarily linked to the actual antibiotic consumption [116]. Another frequently detected resistance is against tetracyclines, antibiotics that are not considered CIA [217]. These drugs were not consumed regularly in our farms, apart from farm B; their diffuse resistance (in MRS and ESBL-producing *E. coli*) can be due to the elevated consumption of previous years. Moreover, in MRS, the resistance to tetracyclines can be associated to the methicillin-resistance phenotype, caused by *mecA*; in fact, this gene is often transmitted with *tet* gene (tetracyclines resistance) with the large MGE called staphylococcal cassette chromosome *mec,* SCC*mec* [345]. The presence of *tet* gene is often considered a marker of LA-MRSA, because of the large tetracyclines consumption in livestock during previous decades [346].

Resistance to macrolides (erythromycin) was also disseminated in our selected MRS. This was observed particularly in intensive farms, where more than one macrolide was prescribed in the same farm. Contrary, in the bacterial strains from the organic farm S, no resistance to macrolides was detected (see Table 3). All the staphylococcal strains were clindamycin (lincosamide) resistant, and this is in line with previous studies, where in LA-MRSA is common to find the resistant gene *erm*, conferring resistance to macrolide–lincosamide–streptogramin B. However, this gene can be easily detected in coagulase negative staphylococci too, and could be the cause of the resistance found in our strains [347].
Another frequently observed resistance in MRS was the one against aminoglycosides. This resistance was found even in the samples from the organic farm. For the intensive farming, it can be linked to the specific antibiotic consumption. In fact, we found a habitual use of aminoglycosides, especially in farm P and G (see table 6). However, where no antibiotic is administered, like farm S, this can be associated to the former dissemination of a methicillin- resistant staphylococcus strain already aminoglycosides' resistant, or can be linked to the phenomenon of co-selection of multiple resistant genes located in MGEs [116,348]. This was observed in a *S. aureus* of poultry origin for the genes encoding resistance to tetracyclines, macrolides, aminoglycosides and lincosamides [348]. Resistance to aminoglycosides was detected in all methicillin-resistant coagulase negative staphylococci tested in the study of Nemeghaire et al. (2014) [179]. Almost all these strains were also clindamycin and tiamulin resistant and this is in accordance with our results. Interestingly, MRS were almost all resistant to the CIA enrofloxacin, with the exception of 4 strains (3 from farm T and 1 from farm B). This result is relevant because we detected resistance to fluoroquinolones even in farms (G and S), where this antibiotic class was not administered. Thence, a direct antibiotic selective pressure cannot be the reason of this event; however, it could be related to the dissemination of some staphylococcal clones that were selected in livestock by a former use of amoxicillin/clavulanic acid, cephalosporins and fluoroquinolones [116]. Unexpectedly, resistance to the last-resort antibiotic linezolid was observed in 6 strains, all coagulase-negative staphylococci (see table 4). Linezolid belongs to the Oxazolidinones' antibiotic class, which includes relatively novel antibiotics,

sold in the pharmaceutical market from the 2000 (linezolid, tedizolid from 2014). They are considered antibiotics of ultimate use in human medicine, in case of resistant pathogens like MRSA, vancomycin-resistant enterococci and penicillin-resistant *Streptococcus pneumoniae* [349]. The resistance mediated by *cfr* gene is against five antibiotic classes: phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A. The *optrA* gene is also able to confer resistance against oxazolidinones. Resistance associated to *optrA* is not only to linezolid, but also tedizolid (second-generation oxazolidinone), and phenicols.

Since the first detection of the oxazolidinone resistance genes *cfr* and optrA in 2000s from *Staphylococcus sciuri* in livestock [285,350], occasional reports have been done from domestic animals worldwide [181].

Considering our linezolid-resistant strains, four presented oxazolidinones' associated resistant genes (*cfr* and *optrA*). *cfr* gene was recovered in *S. sciuri*, and this is in line with previous European and Asian reports in livestock and companion animals [181,193,285,351,352,353]. The gene *cfr* was never recovered in livestock associated staphylococci in Italy; in fact, it was isolated only from an Italian human clinical blood sample *(S. epidermidis)* [354].

For *optrA* gene, positive samples were three, all sampled from the same intensive farm (B), but in different productive phases (finishing, post-weaning and sows) (see table 4). It is noteworthy to say that MRS carrying *optrA* gene were also different at species level (*S. pasteuri* in finishing, *S. cohnii* in post-weaning and *S. sciuri i*n sows*),* supporting a potential horizontal gene transfer (HGT) event through an *optrA*-encoding plasmid [181]. All these strains were multidrug resistant, showing resistance also to phenicols. Indeed, *optrA*, is responsible not only of the oxazolidinones resistance, but also confers resistance to phenicols [181]. Considering the high antibiotic frequency treatment with phenicols (florfenicol + thiamphenicol) in farm B, we suppose a possible "indirect" antibiotic selective pressure of *optrA* gene in MRS, or a potential human to animal transmission of a staphylococcal strain encoding *optrA*. Oxazolidinones are only used in human medicine, thence a direct on farm antibiotic selective pressure is not possible. As for *cfr* gene in Italian swine MRS, this is the first time that *optrA* is recovered in staphylococci from Italian pigs. Moreover, this resistant determinant was never detected in *S. cohnii* and *S. pasteuri* from livestock samples worldwide. Before this discovery, in Italy *optrA* gene has been only found in enterococci isolated from human clinical samples, swine and marine environmental samples [355,356,357]

Although two phenotypic linezolid-resistant staphylococci (G1PAS15 and P1SAS14) did not result positive to the PCRs (*cfr* and *optrA*), we cannot rule out that their resistance could be due to other genetic mechanisms, like the recently discovered *poxtA* gene*,* or a chromosomic point mutation at 23S rRNA [285,358].

Further tests like WGS would be necessary to uncover if *cfr* and *optrA* genes are located on mobile genetic elements and if they are near other AMR and virulence genes. In addition, it would be important to test *in vitro* transmissibility of *cfr* and *optrA*, to understand if both these genes can be transmitted to other bacterial species through HGT.

Contrary to MRS, resistance to gentamicin (aminoglycoside) was rare in ESBL-producing *E. coli.* However, they were frequently resistant to the CIA enrofloxacin, that according to Chervet et al. (2018) and Prendergast et al. (2022) is commonly recovered among ESBL-producing *E. coli* [359,360]. WGS confirmed the phenotypic resistance; in fact, we detected different quinolones associated resistant genes like *qnrS*1 and Aac(6')-Ib-cr, present sometimes in the same strain (see Chapter 7). Furthermore, they were all resistant to tetracycline and doxycycline, with the exception of G1PAE9, and this can be associated to the direct antibiotic selective pressure for the intensive farms. Precisely, we did recover an elevated frequency of doxycycline usage particularly in farm P and T, and WGS confirmed the presence of *tet* genes (A, B and M) in resistant strains (see Chapter 7). This is

accordance with a recent study conducted on nine European countries; indeed, this survey confirmed that the quantity of tetracycline resistance genes in faecal bacteria is associated to the amounts of tetracyclines administered to the animals [361]. These resistant determinants were also detected in ESBL-producing *E. coli* sampled in the organic farm. As said before, no selective pressure is exerted on these bacteria; however, a maternal bacterial transmission from already colonised sows can be the explanation of all these phenotypic resistances recovered not just in *E. coli*, but also in MRS [362].
Resistance to sulphonamides/trimethoprim was disseminated in all farms and all the strains were resistant to fluoroquinolones and tetracyclines too. These types of resistances were found in ESBL-producing *E. coli* sampled from livestock in a recent European study; indeed, the Authors hypothesised that multidrug resistance can support the co-selection of ESBL-associated genes, when all the resistant determinants are transmitted through the same plasmid [363]. Luckily, all selected *E. coli* were meropenem susceptible; this is in line with former European studies, where resistance to carbapenems has been rarely found in swine faecal samples [364]. In Italy only an *E. coli* encoding the carbapenemase $bla_{\text{NDM-4}}$ was recently reported [365].
All these findings indicate that MDR bacterial strains are commonly present in intensive swine farming; however, they can also be isolated in organic pigsties. Antibiotic selective pressure may be the most relevant cause of the various resistances observed in this survey; on the other hand, it resulted insufficient to explain what we found, where no antibiotic was used in rearing animals. Also, due to the limited number of tested strains and farms, we cannot state a direct association between AMU and antibiotic resistance detected in this study. Considering that a global high antibiotic consumption was recorded in all intensive farms, restrictions and a continuous monitoring of AMU are needed. Moreover, antibiotics were mainly administered orally to carry out group treatments. Taking into account all these factors, strategies to counteract the antibiotics' use should focus firstly on using always first-choice antibiotics, preferring parenteral route to the oral. Other preventive measures should be then implemented, like buying pigs from one supplier with elevated sanitary parameters, vaccinations (especially against the two most important respiratory pathogens, *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae* [366]), using boots sanitation system among diverse farm areas and disposable protective equipment for external visitors. This would contribute to decrease farm AMU and AMR, enhancing biosecurity and counteracting stressors that can reduce animal innate immune system opening the doors to preventable infectious diseases.

8.6. Acknowledgements

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Chapter 9 – Concluding remarks

Through the experimental studies conducted in the Northern Italy (Piedmont and Lombardy) livestock farms, it was possible to demonstrate the simultaneous presence of two different resistant bacterial groups, extended-spectrum β-lactamase (ESBL)-producing *E. coli* and methicillin-resistant staphylococci (MRS), that were never detected in combination in Italian poultry (**Chapter 5**) and swine farms (**Chapter 6** and **8**). Our results were revelant not only because of the aforementioned novel finding (contemporary presence of ESBL-producing *E. coli* and MRS), but also because they were carried out in different productive stages, including the farm environment. Taking into account different animal productive phases, it was possible to understand that animals, namely chicks (**Chapter 5**) and post-weaning piglets (**Chapter 4, 6** and **8**) are already coloniesed with MRS since the first days (chicks) or weeks (piglets) of life. This bacterial colonisation can be due to environmental contamination, especially for the staphylococcal species *S. sciuri* and *S. lentus*, that were recovered often in the nearby farm environment (**Chapter 4** and **5**), or can be linked to piglet to piglet transmission and a maternal colonisation, especially for the swine productive chain (**Chapter 4** and **8**). In fact, the highest percentages of MRS were recovered in the youngest animals, weaning (**Chapter 6**) and post-weaning piglets (**Chapter 4**), and in sows (**Chapter 4** and **6**). Furthermore, some swine MRS possessed a mutated *mecA* gene, that in some specifical cases (e.g. *S. xylosus* with G737A nucleotide mutation) was never detected in livestock and human medicine (**Chapter 4**). We recovered other mutations, sometimes in combination, like T675A and G682A, which were sequenced in *mecA* genes amplified from *S. aureus* and other staphylococcal species (*S. sciuri* and *S. haemolyticus*) from two different productive phases of an intensive swine farm.

Looking to ESBL-producing *E. coli* in poultry sector, animals were carriers during the first days of life, with an important decrease in the next weeks of the productive cycle. This event can be associated to parental colonisation and *in ovo* administration of third- generation cephalosporins (**Chapter 5**). The same early carrier status was detected in swine sector, where the highest prevalence of ESBL-producing *E. coli* was observed during the weaning and post-weaning stages of piglets (**Chapter 6** and **8**). For this important resistant bacterial species, we appreciated the potential pathogenicity, using the next generation molecular technique of Whole genome sequencing (WGS) (**Chapter 7**). Through WGS it was possible to unravel in samples from healthy animals, the presence of enterotoxins' associated genes (*astA*, *ltcA* and *stb*) and various virulence genes linked to other *E. coli* human and animal pathotypes. Moreover, we found a possible association of ESBL-producing *E. coli* and MRS prevalence recovered in swine sector, with antibiotic usage at farm level (**Chapter 8**). In fact, we detected higher prevalence for both bacterial groups in animal sectors, such as post-weaning, where antibiotics are more often administered. Although these results were only observed in 4 swine farms, a wider group of farms will be useful to understand if a direct link between antibiotic usage and resistant bacteria can be established. Surprisingly, we recovered oxazolidinone associated resistant genes, *cfr* and *optrA*, from different staphylococcal species, in which these genes were never reported in Italy. These results were relevant not only because these genes confer resistance to last resort human antibiotics, such as oxazolidinones, but also because these strains were multidrug resistant, and they were sampled from healthy animals (**Chapter 8**). Further studies are necessary to understand if the aforementioned genes are cromosomally encoded or are located on plasmids, to understand if they can be transmitted among diverse staphylococcal species. Also, it will be important to test farmers not only for these specific resistant staphylococci but also for ESBL-producing *E. coli*, to comprehend if the same bacterial strains are present in animals and humans at strict contact with them. Exposure assessment of farm workers to resistant bacteria was also an

important part of the experimental studies. In **Chapter 5**, we found which are the most at risk farm practices for MRS and ESBL-producing *E. coli* exposition (carcasses removal and litter removal) during daily work in poultry farm; while in **Chapter 6**, we unraveled the most dangerous farm practices detected during the exposure assessment in swine sector. The study revealed that removal of dead pigs >25kg (gilts), fecundation of gilts, and piglets' tattooing and castration were the most at risk activities for ESBL-producing *E. coli* exposition; instead for MRS exposition, piglets' tattooing and castration resulted the riskiest for farmers. All these findings are really important and should be shared with farm workers in order to prevent transmission of above mentioned resistant bacteria and to promote the use of personal protective equipment especially during at risk farm practices.

Considering our experimental studies carried out in poultry and swine productive sectors, our results are extremely important in contributing to fill the gap about AMR at livestock level. This is true especially for MRS, such as *S. sciuri* or *S. xylosus*, which are often not considered during AMR surveillance in livestock productive chains, preferring just to monitor MRSA presence. These findings do not represent the end of research in livestock sector, but indicate the need to strenghten AMR surveillance in livestock productive chain, including farm management factors such as antibiotic usage, biosecurity and overall farm hygiene; in fact, all these components can leverage and promote the dissemination of resistant bacteria on farm, as was supported by our studies (**Chapter 4**, **5**, **6** and **8**). Future studies should focus on understanding if horizontal gene transfer can happen easily at farm level among different staphylococcal species (e.g. *S. aureus* and *S. sciuri*) that possess *mecA or* oxazolidinone resistance associated genes. In this way it will be perhaps necessary to include MRS and not only MRSA in the routine sourveillance in livestock sector. Moreover, WGS should be implemented in the official controls not only for the AMR part but also to enlight if important virulence genes are carried by bacterial strains sampled from healthy and asymptomatic animals.

Part III- References

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