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BSc in Biology

EXPLORING THE FOOD PRODUCTION CHAIN AS A SOURCE OF BACTERIA RESISTANT TO LAST-RESORT ANTIBIOTICS

MASTER IN MEDICAL MICROBIOLOGY
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Exploring the food production chain as a source of bacteria resistant to last-resort antibiotics

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ABSTRACT

Antibiotic-resistant bacteria spread through the food chain is a public health problem. However, the role of the meat production chain in antibiotic resistance dissemination is still unknown. Of concern is the emergence of resistance to antibiotics that are the last resort for human infection treatment.

Samples collected along the pig processing chain in 2016, from live pigs to slaughterhouse operators, were screened for Gram-negative bacteria and staphylococci resistant to linezolid, tigecycline and colistin. Gram-negative bacteria resistant to last-resort antibiotics and representative staphylococci were identified by 16S rRNA/ *tuf* sequencing and susceptibility to other antibiotics was also evaluated. The whole genome was sequenced, and bioinformatics tools were used to determine the content in antimicrobial resistance genes and characterize the bacterial genetic background. Cross-transmission along the pig production chain was evaluated by single nucleotide polymorphisms (SNPs) analysis.

Overall, 34% of Gram-negative isolates were tigecycline resistant and 13% were colistin-resistant, being mainly from the operators and live pigs. Genes *oqxA/oqxB*, encoding multidrug efflux pumps, were carried by 71% of tigecycline-resistant *Enterobacteriaceae*, but colistin-resistant determinants were not found. Resistance to these last-resort antibiotics was probably associated with the overexpression of the efflux pumps (tigecycline) and mutations in genes involved in lipid A biosynthesis (colistin). We detected no resistance to last-resort antibiotics among staphylococci, but resistance to beta-lactams, tetracyclines and macrolides was frequent. Although we did not identify transmission of Gram-negative bacteria resistant to last-resort antibiotics, closely related *S. hyicus* were found in equipment, operators, and pigs.

Results suggest that slaughterhouses are reservoirs for multidrug-resistant bacteria, including those resistant to last-resort antibiotics and confirmed the occurrence of bacterial transmission between different sampling sites. The fact that high rates of resistance to last-resort antibiotics were detected, highlights the importance and urgency of taking action on both surveillance and control of these bacteria in the veterinary setting.

Keywords: Antibiotics; *Enterobacteriaceae*; Resistant; Slaughterhouse; Staphylococci

RESUMO

A disseminação de bactérias resistentes a antibióticos na cadeia alimentar é um problema de saúde pública. No entanto, o papel da cadeia de produção de carne na disseminação de resistências a antibióticos ainda é desconhecido. A emergência de resistência aos antibióticos de última linha é preocupante, pois estes são o último recurso para o tratamento de algumas infeções em humanos.

Rastreou-se a presença de bactérias Gram-negativas e estafilococos resistentes ao linezolide, tigeciclina e colistina em amostras recolhidas em 2016 ao longo da cadeia de produção de suínos, desde o porco vivo até às mãos dos operários. Bactérias Gram-negativas resistentes a antibióticos de última linha e estafilococos representativos foram identificadas por sequenciação dos genes rRNA 16S/ *tuf* e a suscetibilidade a outros antibióticos também foi avaliada. O genoma total foi sequenciado e, através de ferramentas bioinformáticas, determinou-se o conteúdo de genes de resistência a antibióticos e a sua linhagem genética. A ocorrência de transmissão cruzada no matadouro foi avaliada por análise de *single nucleotide polymorphisms* (SNPs).

Verificámos que 34% dos isolados Gram-negativos eram resistentes à tigeciclina e 13% eram resistentes à colistina, maioritariamente provenientes dos operadores e dos porcos vivos. Os genes *oqxA/oqxB*, que codificam para bombas de efluxo, estavam presentes em 71% das *Enterobacteriaceae* resistentes à tigeciclina, mas não foram encontrados genes de resistência à colistina. As resistências a estes antibióticos estão provavelmente associadas com a sobre-expressão de bombas de efluxo (tigeciclina) e mutações em genes envolvidos na biossíntese do lípido A (colistina). Não foram identificados estafilococos resistentes a antibióticos de última linha, mas verificou-se uma elevada freqência de resistência a beta-lactâmicos, tetracicilinas, e macrolidos. Apesar de não termos detectado transmissão entre bactérias Gram-negativas, alguns isolados de *S. hyicus* recolhidos em equipamentos, operários e porcos eram filogeneticamente próximos.

Os resultados sugerem que os matadouros são reservatórios de bactérias multirresistentes, incluindo aquelas resistentes a antibióticos de última linha e confirmou a ocorrência de transmissão cruzada de bactérias resistentes entre diferentes locais de amostragem. O facto de resistências a antibióticos de última linha terem sido detetadas em elevadas frequências, destaca a importância e a urgência da tomada de medidas de vigilância e controlo destas bactérias no sector veterinário.

Palavras-chave: Antibióticos; *Enterobacteriaceae*; Matadouro; Resistente; Staphylococci

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ACRONYMS

ACB - *Acinetobacter calcoaceticus–Acinobacter baumannii* complex

AGP - Antibiotic Growth Promoter

AMEG - Advice Ad Hoc Expert Group

ATCC - American Type Culture Collection

BSAC - British Society for Antimicrobial Chemotherapy

CA-MRSA - Community-Associated Methicillin-resistant *Staphylococcus aureus*

CARD - Comprehensive Antibiotic Resistance Database

CDC - Centre for Disease Control and Prevention

CFU - Colony Forming Unit

CGE - Centre of Genomic Epidemiology

CLSI - Clinical Laboratory Standards Institute

CO - CHROMagar Orientation

CoNS - Coagulase-negative staphylococci

CoPS - Coagulase-positive staphylococci

CSA - CHROMagar Staph aureus

DNA - Deoxyribonucleic acid

dNTP - Deoxynucleotide triphosphate

ECDC - European Centre for Disease Prevention and Control

ECOFF - Epidemiological cut-off value

EE - Exudative Epidermitis

EF-Tu - Elongation factor Tu

EHEC - Enterohaemorrhagic *Escherichia coli*

EMA - European Medicines Agency

EPEC - Enteropathogenic *Escherichia coli*

ESBL - Extended-Spectrum Beta-Lactamases

ESVAC - European Surveillance of Veterinary Antimicrobial Consumption

EU - European Union

EUCAST - European Committee on Antimicrobial Susceptibility Testing

Exo - Exonuclease

FDA - Food and Drug Administration

ICNP - International Code of Nomenclature of Prokaryotes

ICU - Intensive Care Unit

ISO - International Organization for Standardization

LA-MRSA - Livestock-associated methicillin-resistant *Staphylococcus aureus*

L-Ara4N - 4-amino-4-deoxy-L-arabinose

LPS - Lipopolysaccharide

MALDI-TOF MS - Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry

mcr - mobilized colistin resistance

MDR - Multidrug-resistant

MHA - Mueller-Hilton Agar

MHB - Mueller-Hilton Broth

MIC - Minimum Inhibitory Concentration

MLST - Multilocus Sequence Typing

MRCoNS - Methicillin-resistant Coagulase-negative staphylococci

mRNA - messenger Ribonucleic acid

MRSA - Methicillin-Resistant *Staphylococcus aureus*

MSA - Mannitol Salt Agar

nBLAST - Nucleotide Basic Local Alignment Search Tool

NCBI - National Center for Biotechnology Information

NDH-2 - type II nicotinamide adenine dinucleotide quinone oxidoreductase

OM - Outer Membrane

OMV - Outer Membrane Vesicle

PacBio - Pacific Biosciences

PBP - Penicillin-Binding Protein

PCR - Polymerase Chain Reaction

pEtN - phosphoethanolamine

RNA - Ribonucleic acid

RNase - Ribonuclease A

rRNA - ribosomal Ribonucleic acid

rSAP - Shrimp Alkaline Phosphatase

SNP - Single-Nucleotide Polymorphism

SSTI - Skin and Soft Tissues Infection

ST - Sequence Type

TAE - Tris-acetate-EDTA

tECOOF - tentative Epidemiological cut-off value

tRNA - Aminoacyl-transfer Ribonucleic acid

TSA - Trypticase Soy Agar

TSB - Tryptic Soy Broth

TSS - Type Secretion System

UK - United Kingdom

USA - United States of America

UTI - Urinary tract infection

UV - ultraviolet

VAP - Ventilator-associated pneumonia

WGS - Whole-Genome Sequencing

WHO - World Health Organization

1. INTRODUCTION

1.1 The *Staphylococcus* genus

1.1.1 Description and taxonomy of *Staphylococcus* spp.

The genus *Staphylococcus* is classified in the broad *Bacillus-Lactobacillus-Streptococcus*-cluster of Gram-positive bacteria with a low guanine-cytosine content (G/C) in its chromosomal Deoxyribonucleic acid (DNA) (Scheifer & Bell, 2009; Becker et al., 2015). In 2009, the Bergey's Manual classified the *Staphylococcus* genus along with the genera *Jeotgalicoccus, Macrococcus,* and *Salinicoccus* in a new family designated *Staphylococcaceae* within the order *Bacillales* and class *Bacilli* (Scheifer & Bell, 2009). More recently, phylogenomic analyses proposed a novel genus *Mammaliicoccus* and the *Nosocomiicoccus* formal integration in the *Staphylococcaceae* family (Madhaiyan et al., 2020).

Staphylococci were first described by Rosenbach in 1884 (Scheifer & Bell, 2009). They are coccoid-shaped bacteria with a diameter of 0.5 to 1.5 µm (Scheifer & Bell, 2009; Becker et al., 2015) which, under the microscope, group together in grape-like structures (from the Greek "staphyle" which means a bunch of grapes) (Scheifer & Bell, 2009). Staphylococcal species have the typical cell wall structure of Gram-positive bacteria, containing teichoic acids and peptidoglycan (Noble, 2004; Scheifer & Bell, 2009). With few exceptions, staphylococci are facultative anaerobes but grow more vigorous in the presence of oxygen (Scheifer & Bell, 2009). Rare strains have been previously reported as catalase-negative but most staphylococci are catalase-positive (Över et al., 2000; Scheifer & Bell, 2009). All current *Staphylococcus* members are oxidase negative (Becker et al., 2015; Madhaiyan et al., 2020). They are non-capsulated or have limited capsule formation, non-flagellate, nonmotile and non-spore-forming (Ryan, 2018; Scheifer & Bell, 2009). They are capable of surviving in low water availability or dry environments and are tolerant to high salt concentrations (7.5-10% NaCl) (Scheifer & Bell, 2009; Somerville & Proctor, 2009; Becker et al., 2015) due to osmoprotectants production (Amin et al., 1995). These bacteria are able to grow in a wide pH range (4.8-9.4) and survive in temperatures of up to 60ºC for 30 minutes (Scheifer & Bell, 2009; Somerville & Proctor, 2009). Staphylococcal species are susceptible to lysostaphin, with rare exceptions (Kusuma et al., 2007; Savini et al., 2009). Staphylococcal species' DNA G+C ratio ranges from 30% to 40% (Scheifer & Bell, 2009; Becker et al., 2015)

The genus *Staphylococcus* is divided into two groups based on the ability to form clots in rabbit plasma (Becker et al., 2015; Von Darányi, 1925). The coagulase-positive staphylococci (CoPS), originally named "*S. aureus* group" include the most pathogenic species within the genus, and contain coagulase, an enzyme that can convert fibrinogen into fibrin (Becker et al., 2015; Savini, 2018). In

opposition, the coagulase-negative staphylococci (CoNS) lack coagulase and are considered less pathogenic and generally cause opportunistic infections in compromised hosts (Becker et al., 2015; Savini, 2018). In addition to *S. aureus*, ten other CoPS are described: *S. argenteus, S. cornubiensis, S. delphini, S. intermedius, S. lutrae, S. pseudointermedius, S. coagulans, S. schweitzeri, S. roterodami, S. singaporensis,* besides the coagulase variable species *S. agnetis*, *S. chromogenes* and *S. hyicus* (dos Santos et al., 2016; Becker et al., 2019; González-Martín et al., 2020; Schutte et al., 2021; Chew et al., 2021). The CoNS group includes the majority of the *Staphylococcu*s spp., comprising important resident bacteria of the human and animal skin (Kong & Segre, 2012; Becker, Heilmann, et al., 2014; Rodrigues Hoffmann, 2017).

A total of 62 recognized Staphylococcal species and 30 subspecies have been described so far in the *Staphylococcus* genus, according to the International Code of Nomenclature of Prokaryotes (ICNP) and available in the List of Prokaryotic names with Standing in Nomenclature (LPSN) database (https://lpsn.dsmz.de/ [accessed 18th November 2021]) (Parte et al., 2020).

1.1.2 Methods for *Staphylococcus* spp. identification

The identification of the most clinically significant staphylococcal species and subspecies in humans and animals is based on key phenotypic characteristics (Becker et al., 2015) including the following: colony morphology, Gram staining, oxidase, catalase and coagulase production, hemolysins, urease, nuclease activity, clumping factors, oxygen requirements, alkaline phosphatase, agglutination assays, and novobiocin and polymixin B susceptibility (Scheifer & Bell, 2009; Somerville & Proctor, 2009; Becker et al., 2015). Additionally, classical fermentation, degradation, and hydrolysis assays have been incorporated into commercial manual and automated biochemical identification systems that are used in clinical laboratories for the identification of bacteria (Scheifer & Bell, 2009; Becker et al., 2015). However, these phenotypic methods frequently failed to reliably provide accurate identification of staphylococci to the species level (Heikens et al., 2005).

Various molecular biology methods have been increasingly incorporated into microbiology laboratories, proving to be extremely accurate in the identification of *Staphylococcus* species. The first molecular techniques used for species identification in staphylococci were typically based on polymerase chain reaction (PCR), DNA hybridization or restriction (Marcos et al., 1999; Martineau et al., 2001). Examples of such methodologies include PCR-restriction fragment length polymorphism (Hookey et al., 1998; Hauschild & Stepanović, 2008), amplified fragment length polymorphism (Taponen et al., 2007), and DNA microarray technologies (Cui et al., 2005). Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS), used to analyse the protein composition of a bacterial cell, has emerged as a tool for species identification (Singhal et al., 2015) that is, nowadays, widely used in clinical laboratories (Feucherolles et al., 2019).

More recently almost all molecular identification methods are based on DNA or gene sequencing, such as the sequencing of partial 16S ribosomal ribonucleic acid (rRNA) gene (Kwok et al., 1999). In addition, the 16S-23S rRNA intergenic spacer region (Maes et al., 1997), and several gene targets, such as the heat shock protein 60 (*hsp*60) gene) (Kwok et al., 1999), *femA* gene (Vannuffel et al., 1999),

sodA gene (Poyart et al., 2001), the *tuf* gene, encoding for elongation factor Tu (EF-tu) (Martineau et al., 2001), the *rpoB* gene encoding the B subunit of RNA polymerase (Mollet et al., 1997; Drancourt & Raoult, 2002) and the *gap* gene (Yugueros et al., 2001) proved to be useful and precise methods for the staphylococcal identification. The *tuf* gene analysis has shown to have more discriminatory power when compared to the 16S rRNA sequencing, especially for the identification of close related CoNS (e.g. *S. capitis, S. caprae*, and *S. epidermidis*) (Hwang et al., 2011). Although discordant results were found when comparing different databases, *tuf* identification provided fewer discordant results and fewer cases of multiple probable identities (Hwang et al., 2011). More recently, it has also been suggested the use of heptaplex PCR assays comprising several gene markers including, among others, the *16S rRNA*, *tuf, mecA* and *cns* (only present on CoNS), allows staphylococci identification even in polymicrobial samples (Okolie et al., 2015). Nowadays, identification methods based in the analysis of the whole genome sequencing (WGS), such as average nucleotide identity (Lavecchia et al., 2021) and digital DNA-DNA hybridization methods (Švec et al., 2004) are becoming to be also widely used, having an increased accuracy, when compared to all other methods.

1.1.3 *Staphylococcus* as colonizers of humans and farm animals

Staphylococcus is one of the most commonly isolated genera in animals and humans (Bierowiec et al., 2019). Most of the staphylococci are part of the mammalian microbiome and are naturally present in their skin, nasal cavities, auditory canal, and mucosal membranes (Becker, Heilmann, et al., 2014; Haag et al., 2019).

The CoNS constitute a significant fraction of the skin and mucous membranes microbiota of both humans and animals (Schoenfelder et al., 2010; Becker, Heilmann, et al., 2014). In humans, staphylococcal species can be found on nearly all body parts, but usually different species present site or niche preferences (W. E. Kloos et al., 1998; Becker, Heilmann, et al., 2014). *S. epidermidis*, is the more common human-associated CoNS species with a wide distribution in all moist zones such as the axillae, inguinal and perineal areas, anterior nares, conjunctiva and toe webs (Kong & Segre, 2012; Becker, Heilmann, et al., 2014).

Other frequent human skin colonizers such as *S. haemolyticus* and *S. hominis* are preferentially isolated from the axillae and pubic areas, which are rich in apocrine glands (Becker, Heilmann, et al., 2014). *S. capitis* is usually recovered from the sebaceous glands on the forehead, scalp and human arms (W. E. Kloos & Schleifer, 1983; Becker, Heilmann, et al., 2014), whereas *S. warneri* is commonly recovered from human hands (Cimiotti et al., 2007). In healthy humans, *S. lugdunensis* has been reported as a highly frequent species in some body parts such as the inguinal and breast area (van der Mee-Marquet et al., 2003; Hellbacher et al., 2006), whilst *S. auricularis* is found mostly in the ear canal (W. E. Kloos & Schleifer, 1983). Furthermore, humans may be transiently colonized by species that normally live on companion animals and livestock, such as *M. sciuri* (Stepanovic et al., 2003; Madhaiyan et al., 2020)) or *S. intermedius* (Devriese et al., 1985; Nemeghaire et al., 2014).

S. aureus can colonize several sites on the human body (skin, rectum, vagina, gastrointestinal tract, the pharynx and axillae) (Becker, Heilmann, et al., 2014), but the anterior nares are the main

reservoir (Sakr et al., 2018). Nasal persistent colonization with *S. aureus* is observed in approximately 20% to 30% of the healthy adult population (Roghmann et al., 2011; Sakr et al., 2018), about 30% are transient carriers and 50% are non-carriers (Roghmann et al., 2011).

Staphylococci also constitute part of the normal microbial flora of animals, including farm production animals. In particular, *S. aureus* is also a common colonizer of pig nose (Oppliger et al., 2012; Linhares et al., 2015; Mroczkowska et al., 2017; Strube et al., 2018; Pirolo, Gioffrè, et al., 2019), ventral and dorsal skin regions (Strube et al., 2018), axillae, rectum and on the vagina (Linhares et al., 2015) and mammary glands of sows (Kemper & Preissler, 2011). According to the literature, up to 100% of healthy pigs and cows and 90% of chickens may be colonized by *S. aureus* (Nagase et al., 2002). In swine nasal exudates, the carriage of *S. aureus* range from 36% in Switzerland to 65% in samples collected from the Netherlands, France and Germany (Verstappen et al., 2017). *S. aureus* can also colonize other livestock animals such as turkeys (Ribeiro et al., 2018), goats and sheep (Merz et al., 2016; Z. Zhou et al., 2017). Moreover, CoNS species such as *S. equorum, S. cohnii*, *S. saprophyticus* and also *M. sciuri* are also frequently found in healthy pigs (Strube et al., 2018). Additionally, *S. rostri*, was isolated for the first time from the nasal cavity of healthy pigs (Riesen & Perreten, 2010) and lather from faecal samples of healthy dairy cows (Wuytack et al., 2019).

Several species including *S. aureus* (Pirolo, Visaggio, et al., 2019)*, S. saprophyticus*, or *S. haemolyticus* can colonize both humans and animals and be present in the surrounding environment (Roberts et al., 2018). The fact that the same *Staphylococcus* spp. can be shared by different animals and humans suggests that the staphylococci, during their evolution, often crossed the species barrier in both directions, multiple times (S. Park & Ronholm, 2021). This has particular epidemiological importance because these staphylococcal species can be the main traffickers of antibiotic resistance and virulence genes.

Humans exposed to production animals have a higher risk of being colonized with animal-borne bacteria, which are frequently resistant to antibiotics. In particular, swine workers were found to be six times more likely to carry multidrug-resistant (MDR) *S. aureus* (Wardyn et al., 2015) than other individuals. Moreover, individuals sharing the same households tend to carry genetically similar strains in their nares (L. G. Miller et al., 2012). In the case of household members of swine workers, they also have a more elevated carriage of methicillin-resistant *S. aureus* (MRSA) and livestock-associated MRSA (LA-MRSA) (Wardyn et al., 2015). In these cases, the hands and everyday objects are considered the main sources of transmission (Arinder et al., 2016). Although less frequent, *S. aureus* airborne transmission has already been reported (Kozajda et al., 2019).

1.1.4 *Staphylococcus* as contaminants – a global health perspective

Being staphylococci a frequent colonizer of production animals, they are also frequently found contaminating both unprocessed and processed meat. In unprocessed raw meat, the most frequent species were *S. saprophyticus, S. warneri and also M. sciuri,* while the species *S. hyicus* and *S. aureus* only represented 17% of the isolates (G. Y. Lee & Yang, 2021). On the other hand, processed meat like pork ham and pork luncheon meat were described to be also frequently contaminated with *S. equorum*

(Mroczkowska et al., 2017). The steps of slaughtering, meat processing, cleaning, and sanitizing, include the use of large quantities of water (Genné & Derden, 2008). The resulting wastewaters can though be potential sources of food-borne bacteria including staphylococci (Savin et al., 2020), that can then contaminate soils and plants.

1.1.5 *Staphylococcus* as pathogens of humans and animals

Generally, CoPS are considered more pathogenic than CoNS (González-Martín et al., 2020) with *S. aureus* being the most common etiological agent of infections worldwide (Solberg, 2009; David & Daum, 2010). Skin and soft tissue infections (SSTIs) are the most frequent *S. aureus* associated-infections (David & Daum, 2010). These infections include boils, furuncles, styes, impetigo, carbuncle and other superficial infections like post-operative wound infections of various sites, which, if left untreated, may evolve into an abscess, tissue necrosis and may spread to nearby tissues (Ramakrishnan et al., 2015). *S aureus* is also notorious for causing life-threatening serious systemic infections like bacteraemia, pneumonia, septicaemia, osteomyelitis, endocarditis, mastitis, meningitis and infections in the urogenital tract, central nervous system and various intra-abdominal organs (David & Daum, 2010; W. E. Kloos et al., 1998). Moreover, *S aureus* can be the cause of food poisoning due to the production of enterotoxins (Argudín et al., 2010).

CoNS are opportunistic pathogens frequently responsible for hospital-acquired infections due to the increasing use of medical devices (Costerton et al., 1999) particularly in immunocompromised individuals (Morfin-Otero et al., 2012). Among these, *S. epidermidis* (Gomes et al., 2014) and *S. haemolyticus* (Czekaj et al., 2015) are the most frequent cause of nosocomial infections. The CoNS pathogenicity is mainly explained by their adherence capacity and the ability to form biofilms (F. Oliveira & Cerca, 2013; Tremblay et al., 2013). These infections can be particularly difficult to treat and lead to complications, including the spreading of bacteria from the biofilms to the bloodstream, which can originate bacteraemia (Chang et al., 2018; Oliveira et al., 2018).*S. haemolyticus* has been implicated in native valve endocarditis, septicaemia, peritonitis, urinary tract infections (UTIs), wound, bone and joint infections (W. Kloos & Bannerman, 1999).

S. saprophyticus is an important opportunistic pathogen frequently causing uncomplicated UTIs, especially in sexually active young women (W. Kloos & Bannerman, 1999; Becker, Heilmann, et al., 2014). Two staphylococcal species, *S. lugdunensis* and *S. schleiferi* have been reported to cause more serious infections than other CoNS (Frank et al., 2008; Davis et al., 2013). *S. lugdunensis* is known to cause skin infections and invasive infections, such as endocarditis, osteomyelitis and sepsis in humans (Frank et al., 2008). *S. schleiferi* is also found associated with endocarditis, metastatic infection and endophthalmitis (Kumar et al., 2007; Tzamalis et al., 2013)

Several staphylococcal species are additionally responsible for significant bacterial infections in animals (Aarestrup & Schwarz, 2006; Coetzer & Tustin, 2004). MRSA infections have been reported in different food production animals, including ruminants, swine and poultry (Holmes & Zadoks, 2011; Laranjo et al., 2018; McNamee et al., 2000; Udoh et al., 2019). In chickens and turkeys, *S. aureus* infections may cause subdermal abscesses, septic arthritis, gangrenous dermatitis and osteomyelitis

(Daum et al., 1990; Gornatti-Churria et al., 2018). In cattle, sheep and goats, *S. aureus* is a common cause of dermatitis (Foster, 2012) and mastitis (Bergonier et al., 2014). Moreover, *S. aureus* may cause botryomycosis, a chronic granuloma of the muscle or connective tissue, namely in horses and pigs (Cooper & Valentine, 2016).

S. hyicus is the main etiological agent of exudative epidermitis (EE) (greasy pig disease) in pigs (L. Schwarz et al., 2021), but other species such *as S. aureus*, *S. chromogenes* and *M. sciuri* were also isolated from primary skin lesions in piglets (L. Schwarz et al., 2021). *S. pseudintermedius* was commonly associated with infection in dogs, cats and horses including pyoderma, otitis externa and other suppurative conditions including mastitis, endometritis, cystitis, osteomyelitis and wound infections (Quinn & Quinn, 2011; Sykes, 2014). Furthermore, the bacterial species *S. chromogenes* (Piccart et al., 2016), *S. epidermidis*, *S. haemolyticus*, *S. simulans* and *S. xylosus* are frequently isolated from intramammary infections especially in dairy heifers (Hosseinzadeh & Dastmalchi Saei, 2014; Valckenier et al., 2021), but CoNS were also detected among milk samples of dairy buffaloes, goats and sheep with subclinical mastitis (El-Jakee et al., 2013). *S. rostri* was also found to be the cause of intramammary infection in dairy water buffaloes (Locatelli et al., 2013).

1.1.6 Treatment of *Staphylococcus* infections in humans and animals

The treatment of staphylococcal infections in humans depends on the type, location and severity of infection but also the antimicrobial susceptibility of the isolate (Ki & Rotstein, 2008). Beta-lactams are considered the primary line of treatment in *S. aureus* non-invasive SSTIs (David & Daum, 2010). However, for invasive infections caused by MRSA, like abscesses and bacteraemia, vancomycin is usually the drug of choice (C. Liu et al., 2011). Although, vancomycin is considered the drug of choice to treat methicillin-resistant staphylococci, treatment failure of staphylococcal infections is relatively common (25%) and in 6.7% of cases results in patient death (Dubée et al., 2013). Especially in MRSA invasive infections, some studies indicated that vancomycin monotherapy treatment failure was 28% in epidural abscess, 27% in surgical wounds and 12% in bacteraemia (Dombrowski & Winston, 2008). Linezolid has been used as an alternative to vancomycin not only for SSTIs but also for treating pneumonia (C. Liu et al., 2011). Alternatively, clindamycin or trimethoprim-sulfamethoxazole (L. G. Miller et al., 2015) are also used to treat MRSA infections and showed to be effective. Patients with healthcare-associated CoNS infections are usually treated with vancomycin, daptomycin, teicoplanin, tigecycline and linezolid (David & Daum, 2010).

For skin infections on food animals usually, beta-lactams, particularly penicillin G injections, are the first-choice antimicrobials (Pyörälä, 2009; J. Park et al., 2013; Zimmerman et al., 2019). In the case of EE in piglets, the use of topical antibiotics mixed with antiseptics and/or mineral oils (procaine penicillin G + novobiocin + topical oil), isolated or in combination with injectable antibiotics (Park et al., 2013) is common. In pigs, in case of dermatitis with penicillin-resistant strains or *S. aureus* severe infections (septicemia, osteomyelitis, endocarditis), the more reliable treatment options are third-generation cephalosporins (e.g. ceftiofur), second-generation fluoroquinolones (e.g. enrofloxacin) and trimethoprim-sulfamethoxazole (Zimmerman et al., 2019). To treat mastitis in big ruminants caused by

CoNS or beta-lactamase positive staphylococci, cloxacillin, macrolides or fluoroquinolones can also be used (Pyörälä, 2009).

1.1.7 Pathogenicity in *Staphylococcus*

The virulence factors play an important role in staphylococcal infections and pathogenicity (Cheung et al., 2021). Most virulence determinants are associated with *S. aureus* infections (Oogai et al., 2011), but numerous virulence factors have also been detected in CoNS (Otto, 2004). The infection process implicates different phases that can be facilitated by the presence of these virulence determinants. First, the bacteria adhere to the extracellular matrix or directly to the host cells (Josse et al., 2017). Then, the bacteria start to multiply and invade the tissues (Josse et al., 2017). And finally, they evade the host's immune response leading to infection (de Jong et al., 2019). Although the same virulence factor may be involved in different stages of an infection, they can be grouped into i) adherence factors; ii) membrane and tissue-damaging toxins; iii) host evasion facilitators; iv) iron acquisition facilitators and v) biofilm formation promoters.

Among all virulence factors described in staphylococci, the ones that probably are more relevant in food-borne bacteria are associated with the production of toxins and biofilms. The cytotoxic activity of some of these toxins can be responsible for the lysis of the host cells (e.g. monocytes, neutrophils, platelets, erythrocytes and epithelial cells) and the promotion of immune evasion (D. Oliveira et al., 2018). Included in this group of pore-forming toxins are the α-hemolysin (or α-toxin), β-hemolysin (or β-toxin) and Panton-Valentine Leukocidin (D. Oliveira et al., 2018). Additionally, other *S. aureus* associated toxins are staphylococcal enterotoxins (e.g. SEA, SEB), exotoxins and exfoliative toxins (ETA, ETB, ETC, ETD) (Bukowski et al., 2010; D. Oliveira et al., 2018). In *S. hyicus*, the presence of exfoliative toxins (ExhA, ExhB, ExhC, ExhD, and SHETB), promote skin exfoliation and epidermidis detachment (Nishifuji et al., 2008), is positively related to higher pathogenicity in EE diseased pigs (Futagawa-Saito et al., 2007).

In the case of other CoNS species, the adherence capacity and ability to biofilm are more relevant virulence factors. This capacity allows the colonization and persistence in tissues and medical devices such as intravenous catheters (Costerton et al., 1999) or cardiac pacemakers (Dengler et al., 2019). The polysaccharide intercellular adhesins produced by *S. epidermidis,* and other species including *S. aureus*, is one of the main components of the biofilm (H. T. T. Nguyen et al., 2020). Cells encased in the polysaccharide matrix are highly protected against disinfectants, antibiotics and metals (Flemming et al., 2016). Some studies suggest that biofilms-associated bacteria are up to 1000-times more resistant to antibiotics than planktonic cells (Mah, 2012). Consequently, approximately 80% of bacterial biofilm infections evolve into a chronic state (Jamal et al., 2018).

1.2 Gram-negative bacteria

Gram-negative bacteria are characterized by the presence of a thin peptidoglycan layer (1.5–10 nm thick) (Mai-Prochnow et al., 2016). Unlike Gram-positive, the peptidoglycan of Gram-negative bacteria does not present teichoic acids (Shiraishi et al., 2016). Gram-negative bacteria are also more structurally complex, having an additional layer on their cellular envelope called the outer membrane

(OM) (Nikaido, 2009). Additionally, some species can have external structures such as capsules, flagella or fimbriae (pili) (P. R. Murray et al., 2016)

The OM has a different architecture from the inner membrane, being mainly composed of glycerophospholipids (e.g. phosphatidylethanolamine, phosphatidylglycerol and cardiolipin in the enteric bacteria) (Nikaido, 2009). Although the OM is also a lipid bilayer, it does not contain phospholipids in its outer leaf (Silhavy et al., 2010). The outer leaf of the OM is composed of glycolipids, mainly lipopolysaccharides (LPS) (Kamio & Nikaido, 1976). As a part of the LPS, there is a chain of repetitive oligosaccharides units designed O-antigen or distal polysaccharide (Raetz & Whitfield, 2002). Numerous proteins are present on the OM layer, including transmembrane proteins such as porins (e.g. OmpF, OmpC), and enzymes (Silhavy et al., 2010).

Between the outer and the inner membrane lies the periplasm. This region contains peptidoglycan and unique proteins (Nikaido, 2009). In enteric bacteria e.g. *E.coli*, the peptidoglycan is bound to the OM through lipoproteins known as murein or Braun lipoproteins (V. Braun, 1975; Fadl et al., 2005). Several functions have been associated with the periplasm such as iron and metal transport, nitrate reduction, protein oxidation and secretion, among others (P. R. Murray et al., 2016; S. Miller & Salama, 2018). Finally, the most internal layer of the cell envelope is the inner membrane - a phospholipid bilayer (Silhavy et al., 2010).

Among the group of Gram-negative bacteria, our study will focus on genera that are part of the *Enterobacteriaceae* and *Moraxellaceae* families.

1.2.1 *Enterobacteriaceae* family

1.2.1.1 Description and taxonomy of *Enterobacteriaceae*

The family *Enterobacteriaceae* is part of the class Gammaproteobacteria, and order *Enterobacteriales* (Brenner & Framer, 2005; Adeolu et al., 2016). The *Enterobacteriaceae* includes a diverse array of Gram-negative bacilli. They are rod-shaped bacilli typically 1-5 µm in length (Brenner & Framer, 2005), possess a cell wall with LPS, are non-spore-forming and non-halophilic, facultative anaerobes (Brenner & Framer, 2005; Adeolu et al., 2016). Furthermore, *Enterobacteriaceae* members ferment sugars such as glucose, may or may not ferment lactose, reduce nitrate to nitrite, and produce catalase, but do not produce oxidase (Brenner & Framer, 2005; Adeolu et al., 2016). Most are motile by peritrichous flagella, have an optimal growth temperature between 22-35ºC (Brenner & Framer, 2005).

The order *Enterobacteriales* contains 60 genera and over 250 species with validly published names according to the ICNP (available from: https://lpsn.dsmz.de/ [accessed 18th November 2021]) and available at the LPSN (Adeolu et al., 2016; Parte et al., 2020). The family *Enterobacteriaceae* is one of the most taxonomically diverse bacterial families currently recognized (Adeolu et al., 2016, Parte *et al*., 2020). Presently, 33 valid taxa within the family *Enterobacteriaceae* are published under the ICNP (Parte et al., 2020). Most of the species/genera within the family can be assigned to seven different subfamilies which are designed as "*Escherichia* clade", "*Klebsiella* clade", "*Enterobacter* clade", "*Kosakonia* clade", "*Cronobacter* clade" and "*Cedecea* clade" and "*Enterobacteriaceae incertae sedis*

clade" (Alnajar & Gupta, 2017). In the *Escherichia* clade, besides *Escherichia* spp., other relevant genera such as: *Salmonella, Citrobacter* and *Shigella* are included (Alnajar & Gupta, 2017).

1.2.1.2 *Enterobacteriaceae* as colonizers of humans and animals and contaminants of food and environment

The family *Enterobacteriaceae* includes a diverse group of Gram-negative bacteria that are ubiquitous in the environment and the gastrointestinal tracts of humans and animals. *Enterobacteriaceae* can be found in a wide variety of niches such as: soil, faeces, water, plants, fruits, animals, meat and derivatives (Brenner & Framer, 2005)

The lower gastrointestinal of humans and warm-blooded animals is considered one of the main natural reservoirs (Ryan, 2018). In the gut microbiota of healthy humans, *E. coli* seems to be the dominant *Enterobacteriaceae* species (Martinson et al., 2019). Other resident isolates include *Citrobacter freundii* and *K. pneumonia*e, but in a lower proportion (Martinson et al., 2019). Moreover, *Klebsiella*, and *Proteus* spp. can transiently colonize the oropharynx and skin of healthy humans (Russo & Johnson, 2014).

Enterobacteriaceae are also frequently isolated from animals. In particular, the microbiota of healthy domestic pigs is very similar to the human microbiota, in terms of species composition and prevalence (Schierack et al., 2007). According to Schierack *et al.* 2007, in the microbiota of healthy domestic piglets, *E. coli, E. cloacae, C. freundii* and *K. pneumoniae* are the dominant *Enterobacteriaceae* species. Other species such as *Salmonella* spp., in particular *S. enterica* serotype Typhimurium, were mainly found in the intestinal tract of wild birds' (Tizard, 2004). This species can also be found frequently in domestic birds, swine, cattle, rodents, and companion animals (Percival & Williams, 2014).

Besides being commensals of the animal's intestine, *E. coli, Klebsiella* spp. and *Citrobacter* spp. frequently colonize animal carcasses, meat and the hands of slaughterhouse workers (Schwaiger et al., 2012; Gwida et al., 2014). Moreover, *Enterobacteriaceae* they can be found in raw (unpasteurized) dairy products and locally made dairy products (Garbaj et al., 2016) fresh beef, pork and chicken meat (Gwida et al., 2014; Jansen et al., 2018) and traditional fermented meat products (Talon et al., 2007). The finding of these *Enterobacteriaceae* in food and food-related sources are considered the main indicator of poor hygiene, inadequate storage temperatures or post-process contamination (Baylis et al., 2011).

Among *Enterobacteriaceae*, *E. coli* is considered the most important indicator organism of faecal contamination of water (Odonkor & Ampofo, 2013). But *Salmonella* spp. were also found in the polluted water and sewage (Percival & Williams, 2014; Munck et al., 2020). In particular, wastewater treatment plants are considered an important reservoir of *Enterobacteriaceae* mainly, *E. cloacae, K. pneumoniae, Raoultella planticola* carrying resistant genes (P. P. Amador et al., 2015). Furthermore, the wastewaters from animal slaughterhouses are also considered the main source of antibiotic-resistant *Enterobacteriaceae* (*K. pneumoniae, Enterobacter spp.* and *E. coli*), (Savin et al., 2020, 2021). Furthermore, contamination of farm soils may occur through the use of animal faeces, which frequently end up as manure or slurry in the soil for fertilization (P. Amador et al., 2019).

On the other hand, the hospital environment is also considered a relevant source of *Enterobacteriaceae*. *Klebsiella* spp., *Enterobacter* spp. and *E. coli* were frequently found in hospital toilet bowls and sinks (Decraene et al., 2018), and patients' beds (S. H. Kim et al., 2022) and even in cold tea machine dispensers (K. Ito et al., 2019).

1.2.1.3 *Enterobacteriaceae* as pathogens of humans and animals

1.2.1.3.1 *Enterobacteriaceae* – human infections and treatment

Enterobacteriaceae are members of the gut microbiome of humans and animals, where they reside without causing infection in their hosts. However, they may escape from the gastrointestinal tract and colonize new niches. This might occur through intestinal perforation (Kumar-M et al., 2019), colonization of the peri-urethra region (Najar et al., 2009), via faecal-oral route (Y. Nguyen & Sperandio, 2012) or the consumption of contaminated food (Solhan et al., 2011). *Enterobacteriaceae* can cause a wide variety of infections in humans including gastrointestinal infections, UTIs, bacteraemia, pneumonia, intra-abdominal infections, surgical site infections, meningitis and abscesses (Jenkins et al., 2017).

S. enterica, *E. coli* and *Shigella* spp. are the most relevant enteric pathogens that can cause intestinal infections. *S. enterica* serotype Enteritidis is mainly transmitted through contaminated chicken meat and undercooked eggs (Jackson et al., 2013; Gal-Mor et al., 2014). Enteric fever is caused by *S. enterica* serovar Typhi and less frequently by *S. enterica* serovar Paratyphi are transmitted through the faecal-oral route (Ray & Raha, 2021), especially in undeveloped countries (Dahiya et al., 2019).

Enteropathogenic E. coli (EPEC) and Enterohaemorrhagic *E.coli* (EHEC) are both considered food- and water-borne pathogens (Yen et al., 2016), that have been reported to cause intestinal diseases and which can be fatal, particularly in children living in developing countries (Afset et al., 2003). EHEC main reservoir is the bovine gastrointestinal tract (Sandhu & Gyles, 2002) and human infections are usually associated with the consumption of undercooked beef or raw dairy products (Moxley & Moxley, 2004; Vanitha et al., 2018). EHEC can cause haemorrhagic colitis and haemolytic uremic syndrome (Fitzpatrick, 1999). In addition, UTIs, including cystitis and acute pyelonephritis, are one of the most common human infections caused by *Enterobacteriaceae* (Jenkins et al., 2017; Bischoff et al., 2018), mainly Uropathogenic *E. coli* (Bischoff et al., 2018) and *K. pneumoniae* (Behzadi et al., 2010). In the hospital environment, *Enterobacter* spp., mainly *E. cloaceae*, are also relevant urinary pathogens (Kamińska et al., 2002; D. Ramirez & Giron, 2021). Regarding, respiratory tract infections, *K. pneumoniae, E. cloacae* and *E. coli* are among the most frequent carbapenem-resistant *Enterobacteriaceae* responsible for ventilator-associated pneumonia (VAP) (Gao et al., 2019; D. Ramirez & Giron, 2021)

Regarding the treatment of infections caused by *Enterobacteriaceae*, it differs depending on the bacteria, its antimicrobial resistance profile and the type of infection. In the case of enteric fever (typhoid or paratyphoid) caused by *S. enterica* serovar Typhi or serovar Paratyphi, ceftriaxone and cefixime are usually the first line of antibiotic choice (Dahiya et al., 2019). In the case of complicated EPEC infections with persistent diarrhoea, the recommended treatment for adults and children is trimethoprim/sulfamethoxazole or ciprofloxacin (DuPont, 2016). On the other hand, according to the

Guidelines on Urological Infections, in women with uncomplicated cystitis, fosfomycin is the first line of treatment (Bonkat et al., 2020). As an alternative, cephalosporins (e.g., cefadroxil) can also be prescribed (Bonkat et al., 2020). For uncomplicated pyelonephritis, ciprofloxacin or trimethoprim/sulfamethoxazole are recommended (Bonkat et al., 2020). To treat general severe infections caused by *Enterobacteriaceae*, carbapenems (e.g. meropenem, ertapenem) are usually considered the treatment of choice (Fritzenwanker et al., 2018). In the case of carbapenem-resistance strains, other antibiotics can also be considered such as trimethoprim/sulfamethoxazole, fosfomycin, aminoglycosides, colistin or tigecycline (Fritzenwanker et al., 2018).

Nevertheless, not all infections caused by *Enterobacteriaceae* require antibiotic treatment. For example, *Salmonella* gut infections are usually self-limited and antibiotic treatment is not considered beneficial to decrease the length of the illness or the symptoms (Sirinavin & Garner, 1999). Moreover, antibiotic therapy for EHEC causing haemorrhagic colitis is usually not recommended, because of the increased probability of the development of haemolytic uremic syndrome (Tarr et al., 2005) which is characterized by anaemia, thrombocytopenia and acute renal failure (Fitzpatrick, 1999).

1.2.1.3.2 *Enterobacteriaceae* – animal infections and treatment

Although salmonellosis is uncommon in cats and dogs (Callegari et al., 2014), *Salmonella* can cause gastrointestinal symptoms associated with fever, malaise and abdominal pain (Marks et al., 2011). In dairy cattle, *Salmonella* spp. infections may manifest through enteric diseases, septicaemia, reproductive diseases and pneumonia (Holschbach & Peek, 2018). In pigs, enterocolitis and septicaemia are the most frequent clinical manifestations caused by *S. enterica* serovar Choleraesuis (Uzzau et al., 2000; Zimmerman et al., 2019).

E. coli is also considered potentially pathogenic to animals, including livestock. They have been associated with infections such as colibacillosis, respiratory tract infections, pericarditis, and septicaemia in poultry (Sadeyen et al., 2014). In fact, the avian pathogenic *E. coli* is responsible for a high rate of morbidity and mortality among chickens and turkeys and consequent economic losses (Sadeyen et al., 2014). A similar scenario occurs in pigs. In particular, ETEC is the more relevant pathotype responsible for neonatal diarrhoea and post-weaning diarrhoea in piglets (Luppi, 2017; Zimmerman et al., 2019). The mortality rate of colibacillosis caused by ETEC in pigs is high and can reach 70% (Luppi, 2017). Other relevant *E. coli* infections in swine include edema disease, polyserositis, coliform mastitis and UTIs (Zimmerman et al., 2019). *Escherichia* along with *Enterobacter*, *Citrobacter* and *Klebsiella* are also frequently isolated from sows with mastitis (Zimmerman et al., 2019). In addition, *K. pneumoniae* can be responsible for mastitis in ruminants (Quinn & Quinn, 2011) and severe haemorrhagic enteritis in rabbits (Coletti et al., 2001).

Amoxicillin-clavulanic acid, fluoroquinolones, cephalosporins or trimethoprim are some of the antibiotics of choice to treat infected pigs with colibacillosis (Fairbrother & Nadeau, 2019). Colistin is also frequently administered to treat and prevent colibacillosis in pigs (Kempf et al., 2013; Zimmerman et al., 2019) and poultry (Kempf et al., 2013). Some studies have indicated that mastitis treatment is limited (Schukken et al., 2011), however, some have shown that ceftiofur reduced the risk of cattle death or culling (Erskine et al., 2002).

1.2.1.4 Pathogenicity in *Enterobacteriaceae*

Virulence factors in *Enterobacteriaceae* are associated with mobility, cell attachment, invasion, and immune-host evasion (OECD, 2016). Adhesins, like type 1 Fimbriae (Berne et al., 2015) are ubiquitous among *Enterobacteriaceae* and are responsible for the adhesion to mannose-containing receptors in the host cells and extracellular matrix (Struve et al., 2008). After the adhesion to the host, fimbriae may also promote the invasion of the host tissue via specific receptors (Mazariego-Espinosa et al., 2010). In *E. coli* UTIs, type 1 fimbriae have been proven to be critical for colonization and disease (C. M. Müller et al., 2009; Melican et al., 2011). *K. pneumoniae* has a type I fimbriae gene cluster similar to that of *E. coli*, with a very analogous structure that resembles that of *E. coli* (72 to 84% amino acid identity) (Struve et al., 2008). It has been demonstrated that the type I fimbriae was also essential to the ability of *K. pneumoniae* to cause UTIs, however, according to the tested mouse models, it does not play a role in gastrointestinal or lung colonization (Struve et al., 2008). Fimbriae are not the only adhesins produced by *Enterobacteriaceae*, a variety of OM proteins also serve as adhesins such as the invasin-intimin family of proteins (Heinz et al., 2016). *Enterobacteriaceae* are also able to produce toxins, which induce the lysis of host cells (Krzymińska et al., 2009; Engelsöy et al., 2019). Stx, particularly present on *E. coli* serotype O157:H7 (H. Singh et al., 2019), is highly cytotoxic, particularly on the kidneys and intestinal epithelium (Chan & Ng, 2016).

Other virulence factors that are very important in *Enterobacteriaceae* are the type III and IV secretion systems (T3SS and T4SS) (Backert & Meyer, 2006; Portaliou et al., 2016). These systems not only export proteins through the inner and outer bacterial membranes but also inject them through the host cell membrane (Backert & Meyer, 2006; Portaliou et al., 2016). Additionally, *Enterobacteriaceae* such as ETEC (Noroozi et al., 2018) and Shiga toxin *E. coli* O157:H7 (S.-H. Kim et al., 2010) can produce outer membrane vesicles (OMVs) that can transport virulence factors.

1.2.2 *Moraxellaceae* family

1.2.2.1 Description and taxonomy of *Moraxellaceae*

The *Moraxellaceae* are a family of organisms that belong to the phylum Proteobacteria, the class Gammaproteobacteria, and the order Moraxellales (Teixeira & Merquior, 2014; H. Liao et al., 2020). The *Moraxellaceae* family has fifteen genera, including the pathogenic genus *Moraxella* and *Acinetobacter* (Rossau et al., 1991). Microorganisms from this family are non-fermentative, Gram-negative rods, coccobacilli, or diplococci and often become more coccoid as the culture age (Whitman et al., 2015). The cells are non-motile in liquid media, but surface-bound motility may be observed (Whitman et al., 2015). They contain capsules and fimbriae and are chemo-organotrophic, aerobic, mesophilic or psychrophilic (Álvarez-Pérez et al., 2013; Whitman et al., 2015). They are usually catalase-positive, do not produce indole and can be either oxidase-positive or negative depending on the genus (e.g *Moraxella*: oxidase-positive; *Acinetobacter*: oxidase-negative) (Teixeira & Merquior, 2014).

The *Acinetobacter* genus, first described by Brisou and Prévot in 1954, is one of the genera within the Moraxellaceae family that contains important pathogenic species for humans (Rossau et al., 1991; Whitman et al., 2015; Parte et al., 2020). Currently, 73 valid species are included in this genus, according to the LPSN (https://lpsn.dsmz.de/ accessed on 26 November 2021) (Parte et al., 2020). One

of the most relevant phylogroups is the *Acinetobacter calcoaceticus–A. baumannii* (ACB) complex, which currently includes six valid species: *A. calcoaceticus, A. baumannii, A. pittii, A. nosocomialis*, *A. seifertii* and *A. dijkshoorniae* (Vijayakumar et al., 2019). The other group is the haemolytic clade characterized by containing a great proportion of species with strong-haemolytic activity on agar such as: *A. haemolyticus, A. beijerinckii, A. colistiniresistens*, and *A. proteolyticus* (Nemec et al., 2016, 2017). Several other phylogroups and species, mainly from environmental sources (e.g. freshwater, animals or plants), have been described recently (Álvarez-Pérez et al., 2013; Nemec et al., 2021). However, despite all the advances in nucleotide sequencing methods, the genus *Acinetobacter* still suffers from an ambiguous taxonomic position and confusing nomenclature of some species (Touchon et al., 2014).

The genus *Acinetobacter* can be presently defined as Gram-negative coccobacilli (0.9–1.6 × 1.5–2.5μm) (Whitman et al., 2015), being smaller and more spherical when compared with *Enterobacteriaceae*. In a Gram-staining microscope preparation, the bacterial cells usually appear in pairs or variable size chains (Whitman et al., 2015). *Acinetobacter* spp. are strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive, and oxidase-negative with a GC content of 38%-47% (Peleg et al., 2008; Whitman et al., 2015). Most of the strains are also unable to reduce nitrate (Whitman et al., 2015). The optimal temperature is between 33-35ºC (Whitman et al., 2015) and some of the species, mainly environmental ones, are not able to grow at 37ºC (Álvarez-Pérez et al., 2013; Whitman et al., 2015).

1.2.2.2 *Acinetobacter* as colonizers of humans and animals and contaminants of food and environment

Acinetobacter spp. can be found as human colonizers, particularly in the skin and intestine (Seifert et al., 1997; Dijkshoorn et al., 2005). Members of the genus *Acinetobacter* are considered one of the most frequent and probably the only resident group of Gram-negative bacteria found in human skin (Cosseau et al., 2016), particularly in moist regions such as hands, the groin, toe webs, the forehead, and the ears (Seifert et al., 1997). Furthermore, this study performed in Germany, revealed a high carriage rate of *Acinetobacter* spp. on human skin and mucous membranes among inpatients and healthy controls (75% and 43% respectively) (Seifert et al., 1997). In a similar study, a carriage rate of 40% was found on the human skin of healthy humans in Europe (Berlau et al., 1999). In Hong Kong, the skin and mucous membranes carriage rates were noticeably high in medical personnel and patients (Chu et al., 1999).

The faecal carriage rate of *Acinetobacter* spp. was 24.6% among community individuals from the Netherlands (Dijkshoorn et al., 2005). *A. baumannii*, the most clinically relevant *Acinetobacter* spp., was found only rarely on human skin and faeces (Dijkshoorn et al., 2005). However, the colonization rate of MDR *A. baumannii* in faecal samples from hospitalized patients in an intensive care unit (ICU) in a Spanish hospital was 41% (Corbella et al., 1996). Moreover, a high colonization rate of *A. baumannii* was also observed in the respiratory tract of the patients attending the ICU (Corbella et al., 1996). Additionally, *A. baumannii*. isolates were found in the hospital environment on frequently touched surfaces including patients' beds, intravenous pumps and ventilator faces (Montefour et al., 2008).

In animals, data regarding *Acinetobacter* colonization is still scarce. The few studies available reported that *Acinetobacter* spp. can be isolated from a wide range of animals such as mammals, birds and fishes (Askari et al., 2019). *Acinetobacter* spp. including *A. lwoffii*, *A. baumannii, A. junii, A. calcoaceticus* and *A. pittii* were collected from canine healthy skin (Mitchell et al., 2018). Nasal samples from rabbits also show that they are an important reservoir for MDR *A. baumannii* in Thailand (Pagdepanichkit & Chuanchuen, 2020). Food-producing animals, such as cattle, harboured these bacteria predominantly in the nose with a carriage rate of 21.1% in dairy cows and 2.4% in calves (Klotz et al., 2019). *A. baumannii* was also found in swine manure on a Croatian pig farm (Hrenovic et al., 2019) and wildlife (white stork nestlings) and on chickens and geese (Wilharm et al., 2017).

As part of animal microbiota, the *Acinetobacter* genus can contaminate food during production or processing. However, reports on the isolation of *Acinetobacter* spp. from food sources are uncommon (Adewoyin & Okoh, 2018). *A. baumannii* species have been identified in a variety of food items, such as raw vegetables, fruits, milk and other dairy products (Gennari & Lombardi, 1993; Carvalheira et al., 2017b, 2017a; Kačániová, 2019). Recently, *A. baumannii* was also found in raw meat of turkey and chicken (Ghaffoori Kanaan et al., 2020) and also in ovine, caprine and camel meats, beef and pork in an Iranian butcher (Askari et al., 2019). Other *Acinetobacter* species such as *A. lwoffii, A. johnsonii,* and *A. pittii* were found in raw vegetables and fruits (Carvalheira et al., 2017b), meat (Carvalheira et al., 2017a), raw milk and cheese (Kačániová, 2019) and fish (Gennari & Lombardi, 1993). In Portugal, *Acinetobacter* spp. (*A. guillouiae, A. baumannii*, *A. pittii, A. seifertii* and *A. nosocomialis*) were isolated from chicken, turkey, beef, and pork raw meat (Carvalheira et al., 2017a).

Besides being colonizers of humans and animals, *Acinetobacter* spp. has also been frequently isolated in the natural environment. Being mainly a hydrophilic organism, preferentially inhabiting aquatic environments including wastewaters, freshwater, and rhizosphere (Whitman et al., 2015; Maravić et al., 2016). Moreover, *Acinetobacter* spp. were also found in the ocean sediments and hydrocarbon-contaminated sites (e.g. oil-degrading from beach sands) (Kostka et al., 2011; Z. Zhao et al., 2011).

1.2.2.3 *Acinetobacter* as pathogens of humans and animals

1.2.2.3.1 *Acinetobacter* – human infections and treatment

Acinetobacter spp. is mainly a nosocomial pathogen, causing infections among patients at ICUs (Almasaudi, 2018), reaching mortality rates up to 43% (Falagas et al., 2006). Community-acquired infections caused by these bacteria are uncommon (Petrosillo et al., 2014). *Acinetobacter* spp., especially those from the ACB complex, are often MDR (Peleg et al., 2008) and have been implicated in several hospital-acquired infections including VAPs, bloodstream infections, catheter-associated UTIs and surgical site infections (Weiner et al., 2016), besides meningitis (Jiménez-Mejías et al., 1997) and endocarditis (Wisplinghoff et al., 2012; Ioannou et al., 2021). *A. baumannii* is the species most frequently involved in these infections, followed by *A. pittii* and *A. nosocomialis* (Peleg et al., 2008). However, identification methods in clinical laboratories are usually semi-automated, therefore, *A. pittii, A. nosocomialis* and *A. calcoaceticus* might have been misidentified as *A. baumannii* in some studies (Wisplinghoff et al., 2012).

Since nosocomial isolates are usually MDR, the antibiotics of choice to treat ACB pneumonia are usually polymyxin B or E (colistin) (Hartzell et al., 2007). Colistin-rifampicin combined therapy was also conducted in critically ill patients with pneumonia and bacteraemia (Motaouakkil et al., 2006) and treatment with tigecycline is also considered an acceptable alternative for VAP, with a success of 69.86% (Curcio et al., 2009). For post-neurosurgical meningitis caused by MDR *Acinetobacter* spp. treatments include polymyxins monotherapy or in combination with rifampicin (B.-N. Kim et al., 2009), parental monotherapy with carbapenems or ampicillin-sulbactam and combined therapy of aminoglycosides plus carbapenems (Rodriguez Guardado et al., 2008). Tigecycline is not recommended to treat meningitis due to poor penetration in the cerebrospinal fluid (Rodvold et al., 2006).

1.2.2.3.2 *Acinetobacter* – animal infections and treatment

Acinetobacter spp. are considered frequent and increasing animal nosocomial pathogens (Zordan, 2011). The companion animals, such as dogs and cats and horses, are the most frequently hospitalized animals and those in which *A. baumannii*-associated diseases are more frequently observed (Zordan, 2011; S. Müller et al., 2014) such as canine pyoderma, feline necrotizing fasciitis, UTIs (S. Müller et al., 2014), equine wound infections, septicaemia and bronchopneumonia (Wareth et al., 2019). In addition to pets, *A. baumannii* has also been isolated from other animals with different clinical signs, including rabbits, ferrets, snakes, rats, horses, and ducks (Ewers et al., 2017). A case of *A. baumannii* isolated from a lung sample of a pig with pneumonia and sepsis was reported in China (W.-J. Zhang et al., 2013).

Treatment of diseased animals is often supportive and is preferably based on antimicrobial susceptibility testing results (van der Kolk et al., 2019). It was reported the use of azithromycin and rifampin to threat bronchopneumonia in a horse caused by *A. baumannii* (Jokisalo et al., 2010) and amoxicillin-clavulanate for the treatment of a dog with a UTI caused by *A. ursingii* (Salavati et al., 2018)

1.2.2.4 Pathogenicity in *Acinetobacter*

In *Acinetobacter* spp. some of the most important virulence factors include the type I fimbriae (*fimH*) (Padmaja et al., 2020), the curli fimbriae (*csgA)* and the fibronectin receptor (*fnb*) (G. Braun & Vidotto, 2004), which are known to be involved in the attachment and invasion of host cells and contributing to biofilm formation (Pawar et al., 2005; C. Kim et al., 2012; Padmaja et al., 2020). Additionally, type II secretion systems (T2SS) were also found to be required for the transport of the most relevant effector proteins mediating virulence (lipases LipA and LipH and the protease CpaA)(Harding et al., 2016).

In particular, *A. baumannii* was found to produce an OM protein A (AbOmpA) which is simultaneously associated to biofilm formation, eukaryotic cell infection, antibiotic resistance and immunomodulation (J. S. Lee et al., 2010; Nie et al., 2020). Moreover, during bacterial growth *A. baumannii* was shown to secrete OMVs that contain virulence factors such as putative haemolysins, proteases, fimbrial proteins and efflux pumps (Jin et al., 2011) that may also contribute to disease.

1.3 Antibiotics use: a historical perspective

During the Nineteenth century, bacterial infections such as pneumonia, diarrhoea, typhus, and diphtheria were considered one of the major causes of mortality (Zaffiri et al., 2012; Blanton & Walker, 2015). The discovery of the first synthetic antibiotic known as arsphenamine, an arsenic-based compound used for syphilis treatment, is attributed to Paul Ehrich in 1907 (Bosch & Rosich, 2008; Valent et al., 2016). In 1928, Alexander Fleming discovered the first natural antibiotic (Fleming, 1929).

The discovery of penicillin represented one of the great developments in the history of medicine and the most important therapeutic invention for the control of diseases caused by bacteria and marked the beginning of an antibiotic revolution (Lewis, 2013). The first prescription of penicillin was made in the 1940s and mass production occurred between 1944 and 1945, by the end of World War II (Sengupta et al., 2013), and has saved many lives (Tan & Tatsumura, 2015). Penicillin was used to fight bacterial infections in soldiers and civilians such as staphylococcal wounds infections and pneumococcal infections (C. K. Murray et al., 2006; Herst, 2018)

Almost all the antibiotic drug classes used in clinical practice today were discovered during the golden era (from 1940s-1960s) (Lewis, 2013). Until the early 1960s, more than twenty new classes of antibiotics were commercialized worldwide (Coates et al., 2011). However, in the last decades, oxazolidinones (FDA, 2000), glycylcyclines (FDA, 2005; Greer, 2006) and diarylquinolines (Mahajan, 2013) were the only novel class of antibiotics that have been introduced into the clinical setting.

Unfortunately, soon after the introduction of antibiotics, bacteria resistant to antibiotics emerged and MDR developed (Kumarasamy et al., 2010), which constituted a significant clinical concern. According to the World Health Organization (WHO), in the European Union (EU) alone, 25 000 people die per year from infections caused by drug-resistant bacteria (WHO, 2015). In other countries the panorama is similar. One of the more recent reports by the Centre for Disease Control and Prevention (CDC) refers that more than 2.8 million antibiotic-resistant infections occur in the United States of America (USA) per year, and more than 35 000 people die as a result of those infections (CDC, 2019)

1.4 History and legislation of antibiotics in the veterinary setting

In the veterinary settings, antibiotics have been used either in the treatment of infections, as prophylactics or as growth promoters (Tang et al., 2019). Prontosil and other sulphonamides were the first antibiotics commercialized for use in the treatment of animals in 1938 (Kirchhelle, 2018). With the mass production of penicillin in the 40s (Lombardino, 2000; Aminov, 2010), this antibiotic started to be also an option in the treatment of animals, for example, to treat bovine mastitis (Bryan, 1947). Soon, it was discovered that the use of feed supplemented with fungi mycelium cloud hasten animal growth (Stokstad & Jukes, 1949). Sub-therapeutic doses of antibiotics were also used for a prophylactic effect, protecting animals from bacterial infections (Kirchhelle, 2018).

Several antibiotics like chlortetracycline, streptomycin and penicillin were associated with growth-promoting effects on swine (Luecke et al., 1951). Polymyxins and macrolides were also heavily used as growth promoters, prophylaxis and metaphylaxis in swine, chickens and cattle (EMA, 2010; Nordmann & Poirel, 2016). In most of the cases, these results were observed when the antibiotics were given in combination with vitamin B12 (Jukes, 1972). Thus, the use of antibiotic growth promoters (AGPs) together with vitamin B12 became very popular across the globe. Their use without veterinary prescription was allowed in 1951 in the USA and AGP quickly spread to European countries (Castanon, 2007). From the 1950s onwards, antibiotics began to be used routinely on farm animals to maintain their health and increment productivity (Lekagul et al., 2019). By 1958, it was estimated that up to half of pigs in England were fed with antibiotics and that almost all piglets had access to food suplemented with tetracyclines (Kirchhelle, 2018). A few years later, in 1966, West Germany also reported that 80% of the mixed feed designed for piglets, veal calves and poultry contained antibiotics (Kirchhelle, 2016).

Concerns regarding meat and milk contamination with antibiotics and the rising antimicrobial resistance lead to the implementation of monitoring programs (Kirchhelle, 2018) and limitations on antibiotic usage. In 1970, a European Council Directive established that only the antibiotics listed in the Directive such as bacitracin zinc, tetracyclines, penicillin G, and macrolides could be incorporated in animal feeds (European Parliament and the Council of the European Union, 1970). However, by 1980, China started to routinely use antibiotics, including colistin, as AGPs (Kirchhelle, 2018; Schoenmakers, 2020).

Sweden was the pioneer country in the implementation of legislation against the use of AGPs. In 1986, in Sweden, AGPs were completely banned (Wierup, 2001), an action that was followed by Denmark's voluntary ban in 1999 (Aarestrup et al., 2001). Also, in 1999 the European council banned five popular AGPs: bacitracin zinc, spiramycin, virginiamycin and tylosin phosphate (European Parliament and the Council of the European Union, 1998, p. 98). Only on 1st January 2006, all AGPs were prohibited in the EU and United Kingdom (UK) (European Parliament and the Council of the European Union, 2003). The legislation was applied to all antibiotics, whether they were used in human medicine or not (European Parliament and the Council of the European Union, 2003). Furthermore, the use of some antibiotics has been restricted or reserved for human use only, to preserve their efficacy (EMA, 2019). For example, oxazolidinones (e.g. linezolid), monobactams (e.g. aztreonam), streptogramins (e.g. pristinamycin, virginiamycin), glycylcyclines (e.g. tigecycline), carbapenems and other antibiotics listed into category A (“avoid”) of Advice Ad Hoc Expert Group (AMEG) were prohibited for veterinary use (EMA, 2019). However, they might be used in non-food animals (e.g. dogs, cats) in exceptional circumstances, conforming to the prescribing “cascade” (EMA, 2019).

On 28th January 2022, additional legislation was implemented in the EU. This recent regulation defined that antimicrobials shall not be “applied routinely or to compensate poor hygiene, inadequate husbandry or lack of care or to compensate for poor farm management” (European Parliament and the Council of the European Union, 2018). Also, antibiotics should not be used for prophylaxis or metaphylaxis, unless when the risk of infection and spread in the group of animals is high and the consequences are likely to be severe (European Parliament and the Council of the European Union,

2018). Additionally, the import from third countries of food-production animals produced with AGPs was also prohibited (European Parliament and the Council of the European Union, 2018). New goals were set for the coming years, including reports on antimicrobial data collection per species starting from 2024 for pigs, poultry and veal calves and by 2027 for all food-producing animals (European Parliament and the Council of the European Union, 2018).

In non-EU countries, the restrictive regulations regarding AGPs took longer to be implemented. In China, the discovery of the *mcr-1* led to the colistin ban as a feed additive in late-2016 (Walsh & Wu, 2016) and to the launch of a national pilot program to decrease unnecessary antimicrobial use (Tian et al., 2021). On 10 July 2019, the Ministry of Agriculture and Rural Areas of China stipulated that from 1 January 2020, all AGPs feed additives, except traditional Chinese medicines, should be withdrawn (Tian et al., 2021). Besides that, 2017-2019 data from 129 countries reported that 14% of the countries located in the Americas, Asia, the Far East and Oceania still use antibiotics for growth-promoting purposes (Gochez et al., 2021). In Africa, eight countries out of thirty-nine used AGPs (Gochez et al., 2021). America (n=20/35 countries), Asia, the Far East and Oceania (n=12/31 countries) had the highest proportions of countries using AGPs and Europe had the lowest (n=2/48 countries) (Gochez et al., 2021). According to the same report, the number of countries which do not have a regulatory framework is substantial-high in Africa (75%) when compared with the remaining regions: 60% (n=12/20) in America and 25% (n=3/123) in Asia, Far East and Oceania (Gochez et al., 2021).

Although the regulatory authority of drugs in the USA, the Food and Drug Administration (FDA), prohibited the use of important medical antibiotics for growth promoters purposes since 2017, the use of non-medically relevant antibiotics is still allowed (FDA, 2013). Furthermore, antibiotics remain licensed for the treatment and prevention of diseases caused by poor husbandry (e.g. post-weaning diarrhoea in pigs which is exacerbated by early weaning) (Nunan, 2020). The current list of medically important antimicrobial drugs was published in 2003 and includes, among others: aminoglycosides, lincosamides, macrolides, penicillins, streptogramins, sulfonamides, tetracyclines, oxazolidones (e.g. linezolid) and polymyxin B (FDA, 2003).

1.5 Current antibiotic consumption in food-producing animals

In 2009, the European Medicines Agency (EMA) launched the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project (EMA, 2012). This allowed the reporting of data on the use of antimicrobial agents in animals from the EU, including data for a specific country (EMA, 2012). In 2018, a total of thirty-one countries integrated into the project, including non-EU countries: Iceland, Norway, the UK and Switzerland (EMA, 2020). Like in Europe, the FDA also published the "Annual Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals" from 2009 onwards (FDA, 2014).

According to recent data, tetracyclines and beta-lactams are the most sold classes destined for food-production animals according to the EMA and FDA (EMA, 2021b; FDA, 2021). The 2019-2020 EMA report showed that penicillin (31.1%), tetracyclines (26.7%) and sulphonamides (9.9%) were the most sold antibiotics, accounting for 67.7% of the total sales (EMA, 2021b). In the case of the USA,

tetracyclines alone accounted for 66% of the sales, followed by penicillin (13%), macrolides (7%), sulphonamides (5%) and aminoglycosides (5%) (FDA, 2021). The sales of high priority antibiotics e.g., third and fourth-generation cephalosporins, fluoroquinolones, other quinolones and polymyxins, according to the last EMA report, accounted for 0.2%, 2.6%, 0.2% and 2.8%, respectively, of the total sales (EMA, 2021b). In Portugal, sales of third- and fourth-generation cephalosporins remained relatively stable and polymyxins and fluoroquinolones sales have fluctuated over the years (EMA, 2021a). Currently, in Portugal, polymyxins (including colistin) represent more than 5% of the antibiotic sales in 2020, with an increment of 37.4% when compared to 2019 (EMA, 2021a). Portugal is currently one of the European countries with more sales of polymyxins for veterinary purposes (EMA, 2021a, 2021b).

EMA reported that pigs and cattle represent 32 and 31% respectively, of the overall antibiotic consumption in the EU, followed by poultry (15%), and sheep along with goats (13%) (EMA, 2021b). The FDA reported that antibiotics were sold in equal proportion to pigs and cattle (41% each), 12% were destined for turkeys and 2% were for chickens (FDA, 2021). Since 2016, the FDA provided estimates of sales and distribution in the major food-producing species which include cattle, swine, chickens, and turkeys (FDA, 2017). The EMA reports do not provide this information. The ESVAC is currently preparing a system for the collection of standardised data on consumption by animal species (EMA, 2018). According to the FDA, 87% of lincosamides, 49% of tetracyclines and 42% of macrolides sold were intended for use in swine (FDA, 2021). Studies in some European countries like Denmark, Netherlands, Spain, and France, reported that tetracyclines are the most used class of antibiotics in pigs (Lekagul et al., 2019) and aminopenicillin, trimethoprim-sulphonamides, tylosin and colistin are also commonly applied (Lekagul et al., 2019). These antibiotics are used in all stages of pig production from nursery pigs to finishing, however, they are particularly used in post-weaning periods (Lekagul et al., 2019).

1.6 Antibiotics and antibiotic targets

Antibiotics are antimicrobial agents that have antibacterial activity (Walsh & Wu, 2016). Antibiotics can kill (bactericidal) or inhibit bacteria growth (bacteriostatic) and are primarily used for therapeutic purposes (Barker, 1998; Russell, 2004; Walsh & Wu, 2016). According to the range of microorganisms against which antibiotics are active, antibiotics can be divided into the broad-spectrum and narrow spectrum. The broad-spectrum antibiotics can act against a large range of Gram-positive and Gram-negative bacteria, while the narrow spectrum is active only against a limited variety of bacteria (Walsh & Wu, 2016). The biggest advantage of using broad-spectrum antibiotics is to be able to use them empirically in the treatment and prevention of infections (e.g. post-surgery, organ transplants and chemotherapy), even without knowing the infection-causing agent (Walsh & Wu, 2016; Melander et al., 2018). However, they have the great disadvantage of, besides acting against the causative agent of infection, also inhibiting commensal bacteria (Walsh & Wu, 2016; Melander et al., 2018). This frequently leads to microbiota disruption, selection of resistant bacteria and, eventually, permanent immunological and metabolic disorders (Walsh & Wu, 2016; Melander et al., 2018). Therefore narrow-spectrum

antibiotics are usually preferred, being the first line of treatment for children and whenever is possible (Gerber et al., 2017).

Different antibiotics act on different functions of bacteria, which are usually essential functions. These include inhibitors of cell wall synthesis; disruption of the cell membrane; inhibitors of protein synthesis; inhibitors of nucleic acid synthesis; and antimetabolites.

1.6.1 Inhibitors of the cell wall

The cell wall of bacteria is composed of peptidoglycan, which consists of glycan strands cross-linked by short peptides. The glycan strands consist of alternating N-acetylglucosamine and N-acetylmuramic acid residues linked by β(1,4) glycosidic bonds (P. R. Murray et al., 2016). These cross-links are catalysed by a set of enzymes known as penicillin-binding proteins (PBPs) (P. R. Murray et al., 2016; Kapoor et al., 2017). Since the peptidoglycan layer is a vital component present in bacteria, numerous antibiotics act by interfering with its biosynthesis (P. R. Murray et al., 2016). The most clinically relevant antibiotics in this category comprise glycopeptides (e.g. vancomycin), β-lactams (Walsh & Wu, 2016) and phosphonic acids (e.g. fosfomycin) (Epand et al., 2016).

The β-lactam class include penicillins, cephalosporins, cephamycins, carbapenems and monobactams (P. R. Murray et al., 2016). All antibiotics from this class have a β-lactam ring in their chemical structure (P. R. Murray et al., 2016; Walsh & Wu, 2016). The target of the β-lactam antibiotics are the PBPs to which they bind covalently, interrupting the last step of the peptidoglycan synthesis and resulting in lysis and cell death (P. R. Murray et al., 2016, p. 20116; Walsh & Wu, 2016). β-lactamase inhibitors, such as clavulanic acid, can be used together with β-lactam antibiotics (e.g. penicillin-clavulanic acid or amoxicillin-clavulanic acid) to overcome β lactamase enzymes' produced by bacteria (Bhattacharjee, 2016b)

The glycopeptide vancomycin disrupts the cell wall peptidoglycan (P. R. Murray et al., 2016) by interacting with the d-alanine-d-alanine termini of the pentapeptide side chains, hindering the continuation of cell wall biosynthesis (P. R. Murray et al., 2016; Walsh & Wu, 2016). Vancomycin is only active against Gram-positive bacteria because is a molecule too large to transpose the OM of Gram-negative bacteria and reach the peptidoglycan layer (P. R. Murray et al., 2016).

1.6.2 Disruptors of the cell membrane

The cytoplasmic cell membrane and the OM of Gram-negative bacteria constitute other possible targets of antimicrobial agents. One of the main differences between the cytoplasmic cell membrane and the OM is the presence of LPS (Nikaido, 2009). Polymyxins, which include polymyxin B and E (colistin) and lipopeptides (daptomycin) disrupt membranes in a “detergent-like” manner having, therefore, a bactericidal action (P. R. Murray et al., 2016; van Bambeke et al., 2017). Polymyxins target lipid A, the hydrophobic domain of LPS, and are only active against Gram-negative bacteria (P. R. Murray et al., 2016). Lipopeptides target the cell membrane, being only active against Gram-positive bacteria, because the antibiotic cannot penetrate through the OM of Gram-negative bacteria (van Bambeke et al., 2017).

The polymyxins colistin and polymyxin B have a very similar chemical structure, consisting of cyclic lipopeptide compounds with 10 amino acids that only differ in a single amino acid residue at position 6 (colistin: d-leucine; polymyxin B: d-phenylalanine) (Ledger et al., 2022). They act primarily by electrostatic interaction of the polymyxin L-α,γ-diaminobutyric acid side-chain with the lipid A's phosphate groups, resulting in divalent cations (Ca^{2+} and Mg^{2+}) displacement (Z. Li & Velkov, 2019; Segovia et al., 2021; Ledger et al., 2022) and LPS layer destabilization, which allow the entrance of the polymyxin hydrophobic acyl chain at the N-terminus through the bacterial membrane (Z. Li & Velkov, 2019; Segovia et al., 2021). In a second step, the polymyxin molecule penetrates the inner membrane and inhibits the respiratory enzyme known as type II Nicotinamide adenine dinucleotide quinone oxidoreductase (NDH-2) (Z. Li & Velkov, 2019). Therefore, polymyxins also involve the inhibition of bacterial respiration (Z. Li & Velkov, 2019).

Lipopeptides (e.g. daptomycin) consist of linear or cyclic peptides, with either positive or negative charge, to which a fatty acid moiety is covalently attached to its N-terminus (Straus & Hancock, 2006). In particular, daptomycin is a cyclic lipopeptide that binds to the cytoplasmic cell membrane, resulting in depolarization and disruption (P. R. Murray et al., 2016). There are solid evidence that daptomycin targets the phosphatidylglycerol of the bacterial membrane, however, it is still not clear the mechanism by which this results in cell death (Ledger et al., 2022).

1.6.3 Inhibitors of protein synthesis

The second larger group of antibiotics are those that interfere with the protein synthesis of bacteria (P. R. Murray et al., 2016). The anabolism of proteins requires a complex of ribonucleoproteins – the 70S bacterial ribosomes, composed of two unequal size subunits (50S and 30S), Ribonucleic acid (RNA) molecules and associated co-factors (Kapoor et al., 2017). The larger subunit contains the 23S and 5S rRNA molecules and more than 30 proteins (Ghosh & Joseph, 2005). The smaller subunit is composed of 16S rRNA and 21 proteins (Ghosh & Joseph, 2005). For the translation to initiate, the small subunit needs to bind to the messenger RNA (mRNA) and the aminoacyl-transfer RNA (tRNA) anticodon (Laursen et al., 2005). The tRNA binds to three sites: aminoacyl (A), peptidyl (P), and exit (E) sites of the ribosome (Schmeing et al., 2003). Aminoglycosides, tetracyclines and glycylcyclines interfere with the minor 30S subunit, although they do it using different approaches (Kavčič et al., 2020).

Antibiotics can interrupt the translation steps by binding to different sites including the 70S bacterial ribosome, one of the two ribosomal subunits or interfering directly with the binding site of the tRNA (P. R. Murray et al., 2016; Bhattacharjee, 2016b; Kapoor et al., 2017). Aminoglycosides, tetracyclines and glycylcyclines interfere with the minor 30S subunit, although they do it using different approaches (Bhattacharjee, 2016b). Other antibiotics such as clindamycin, chloramphenicol, macrolides and oxazolidinones prevent the initiation of protein synthesis at the 50S (Bhattacharjee, 2016b).

Oxazolidinones (e.g linezolid) are synthetic molecules that inhibit protein synthesis at an earlier step (van Bambeke et al., 2017). Linezolid bounds to the 23S rRNA of the 50S ribosomal subunit (Bhattacharjee, 2016b), in particular to the A site pocket at the peptidyltransferase centre of the ribosome and seems to interfere with the placement of the tRNA (D. N. Wilson et al., 2008; van Bambeke

et al., 2017). Linezolid shows activity against staphylococci, streptococci, and enterococci (including strains resistant to penicillins, vancomycin, and aminoglycosides) (P. R. Murray et al., 2016; van Bambeke et al., 2017), but is not effective against Gram-negative bacteria due to active efflux in most species (van Bambeke et al., 2017).

Glycylcyclines (e.g. tigecycline) have the same central carbocyclic skeleton present in the tetracycline's chemical structure (Pankey, 2005). However, glycylcyclines have an additional substitution of the N-alkyl-glycylamido group on the D ring at the ninth position, which facilitates the broader spectrum of activity and capability to overcome most tetracycline resistance mechanisms (Pankey, 2005). Tigecycline binds to the 30S ribosomal subunit (Bhattacharjee, 2016b), to the pocket formed by the 16S rRNA helices 31 and 34, which overlap with the primary tetracycline binding site (Schedlbauer et al., 2015). Thus, tigecycline blocks the entry of tRNA molecules into the A site of the ribosome, preventing peptide translation (Schedlbauer et al., 2015). Tigecycline has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria (Pankey, 2005).

1.6.4 Inhibitors of the nucleic acid synthesis

Quinolones are a big antibiotic family that have as a target the topoisomerase type II (DNA gyrase) and the topoisomerase type IV, essential for DNA replication recombination, and repair (P. R. Murray et al., 2016). DNA gyrase acts before the replication fork, removing positive supercoils to allow the replication and transcription to carry on (Sissi & Palumbo, 2010). Topoisomerase type IV acts after the replication fork, unlinking precatenanes that could prevent cell division (Sissi & Palumbo, 2010). There are currently four generations of quinolones, with increasing order of effectiveness (P. R. Murray et al., 2016; Bhattacharjee, 2016b).

The chemical structure of quinolones is heterocycles with a bicyclic core (Fàbrega et al., 2009). Quinolones have been shown to have interactions with both subunits of the enzyme: GyrA and GyrB for gyrase and ParC and ParE for topoisomerase type IV (Bush et al., 2020). In crystal structures models, the antibiotics intercalated between DNA bases at the DNA-cleavage site (Bush et al., 2020). The formation of a gyrase-DNA complex blocks access to the DNA and RNA polymerases, preventing both replication and transcription (Bush et al., 2020). Furthermore, the formation of this complex causes DNA breaks, which is pointed out as the mechanism that leads to bacterial death (C.-R. Chen et al., 1996).

The first generation of quinolones includes nalidixic acid and oxolinic which inhibit DNA gyrase activity (Sugino et al., 1977). The second generation includes norfloxacin, ciprofloxacin and ofloxacin, which display considerably improved activity against gyrase and greater penetration into Gram-positive bacteria (Aldred et al., 2014). The most significant change in the quinolone skeleton was the introduction of fluorine at position C6 and a major ring substituent (piperazine or methyl-piperazine) at C7 (Aldred et al., 2014). Because of the fluorine addition, these quinolones are often called "fluoroquinolones" (Aldred et al., 2014).

In the third generation, new extended-spectrum fluoroquinolones with improved activity against Gram-positive bacteria and anaerobes were developed (Fàbrega et al., 2009; Perry et al., 1999), such as levofloxacin (Scoper, 2008). Fourth-generation fluoroquinolones, e.g. moxifloxacin and gatifloxacin,

have increased potency against Gram-positive bacteria while maintaining the broad spectrum of activity against Gram-negative bacteria (Scoper, 2008). Substitution of a methoxy group at position eight of the quinolone ring results in the simultaneous inhibition of both DNA gyrase and topoisomerase IV in Gram-positive bacteria, increasing its potency and decreasing the emergence of resistant mechanisms (Scoper, 2008).

1.6.5 Antimetabolites

The metabolite synthesis such as nucleotides or amino acids can, additionally, be potential targets for antibiotics. Antibiotics that have a similar structure to a certain metabolite are known as antimetabolites (Bhattacharjee, 2016b). They bind to the active site of an enzyme that catalyzes a reaction function, acting as a competitive inhibitor (Bhattacharjee, 2016b). Although the term antimetabolite can apply to any metabolic step of a biochemical pathway, it is frequently used to refer to nucleic acids anabolism(Bhattacharjee, 2016b). The most relevant nucleic acid antimetabolites are trimethoprim and sulfamethoxazole (P. R. Murray et al., 2016).

Trimethoprim is a dihydrofolate reductase inhibitor, its blocks the reduction step of dihydrofolate to tetrahydrofolate, a folic acid derivate (Gleckman et al., 1981). Tetrahydrofolate is a cofactor needed for fundamental reactions such as the synthesis of nitrogenous bases of the nucleic acids (DNA and RNA) and thus for bacterial replication and amino acid synthesis (P. R. Murray et al., 2016). Sulfamethoxazole, a sulphonamide drug, is an analogue of para-aminobenzoic acid and inhibits the synthesis of the dihydrofolic acid from its precursors (Masters et al., 2003). These two antimetabolites are usually combined to produce a synergism in two different steps of the synthesis of folic acids (P. R. Murray et al., 2016; Bhattacharjee, 2016a). Trimethoprim-sulfamethoxazole is effective against a wide range of Gram-positive and Gram-negative bacteria (P. R. Murray et al., 2016; Bhattacharjee, 2016a).

1.7 Resistance to last-resort antibiotics

The use of antibiotics frequently leads to the emergence of bacteria that are resistant to antibiotics. Actually, for most antibiotics, resistance has emerged shortly after their introduction into clinical practice and has spread afterwards, leading to the emergence of high resistance prevalence and MDR strains.

In 2017, the WHO introduced a classification of antibiotics according to their essentiality in human medicine and impact on antimicrobial resistance as a tool to guide antibiotic prescription at local, national and global levels. Antibiotics were classified into three groups, Access, Watch and Reserve (“AWaRe”), to emphasize the importance of their appropriate use and reduction of antibiotic resistance (WHO, 2021). In the reserve group, also known as “last-resort antibiotics”, are included the ones that should be used to treat severe or life-threatening infections due to MDR and when alternative treatments are not suitable or have failed (WHO, 2021). According to the most recent AWaRe classification from 2021, in the reserve group are included colistin, tigecycline, linezolid, aztreonam, 3rd and 5th generation cephalosporins, among others (WHO, 2021). Most of these “last-resort” antibiotics, except for colistin, are also classified as category A (“Avoid”), according to the EMA (EMA, 2019). In Europe, all antibiotics integrated into category A are not authorized to use in veterinary medicine (EMA, 2019). In the Watch

group are included critical important antibiotics for human medicine such as fluoroquinolones (e.g. ciprofloxacin), aminoglycosides, macrolides (e.g. erythromycin), and glycopeptides (vancomycin) (WHO, 2021). The access group includes the first and second line of antibiotic empiric treatment: first-generation cephalosporins, sulfamethoxazole/trimethoprim, penicillin, and tetracyclines, are some of the included antibiotics (WHO, 2021).

In our study, we have focused on resistance to last-resort antibiotics, in *Staphylococcus* spp., *Acinetobacter* spp. and *Enterobacteriaceae*, including resistance to colistin, tigecycline and linezolid. Due to its epidemiological importance and broad use in animals and humans, we have also analysed to some extent resistance to β-lactams.

1.7.1 Linezolid Resistance

Resistance to linezolid emerged in 2001 in an *S. aureus* clinical isolate from a patient undergoing peritoneal dialysis with recurrent peritonitis, shortly after the introduction of linezolid in clinical practice (Tsiodras et al., 2001). Resistance to linezolid has been associated with mutations in the 50S ribosomal subunit, particularly in the 23S rRNA, which corresponds to the binding site of this antibiotic (Bhattacharjee, 2016b). In clinical staphylococcal isolates, one of the most common mechanisms is associated with the G2576T mutation on the V domain of the 23S rRNA (Tsiodras et al., 2001; Hong et al., 2007; Mazzariol et al., 2012). Additionally, resistance to linezolid has also been associated with mutations in the L3 and L4 ribosomal proteins, positioned in the 50S subunit (Long & Vester, 2012).

Another alternative resistance mechanism is associated with the *cfr* gene that encodes a methyltransferase enzyme involved in the methylation of carbon-8 on the A2503 nucleotide of the 23S rRNA (Giessing et al., 2009). The *cfr* gene was first discovered in the year 2000 present on a 6.5 kilobase (kb) plasmid pSCFS1 from *M. sciuri,* which was isolated from a calf and was associated with chloramphenicol and florfenicol resistance (S. Schwarz et al., 2000). That same plasmid carried additional antimicrobial-resistant genes conferring resistance to macrolide-lincosamide-streptogramin B, spectinomycin, and lincosamides (S. Schwarz et al., 2000). Some hospital studies had reported linezolid-resistant strains of *Staphylococcus* spp. carrying the *cfr* gene alone or in conjunction with 23S rRNA G2576T mutations (Quiles-Melero et al., 2013).

Recently, a novel transporter that coffers resistance to oxazolidinone, encoded by the *optrA* gene, has been identified on *M. sciuri* strains of pig origin (Fan et al., 2016; D. Li et al., 2016). In contrast to the *cfr* gene, the *optrA* only confers resistance to phenicols and oxazolidinones (Michalik et al., 2021). The *poxtA* gene, which encodes for the PotxA protein with 32% amino acid identity with OptrA, has been identified in MRSA from clinical origin (Antonelli et al., 2018). Besides conferring resistance to phenicols and oxazolidinones, it also confers resistance to tetracycline (Antonelli et al., 2018). Furthermore, in 2021, an *S. aureus* strain harbouring the *optrA* and *poxtA* genes has been found in patients with laryngological infections in Poland (Michalik et al., 2021).

According to European data collected between 2014 and 2018, the percentage of *S. aureus* linezolid-resistance isolated from patients with bloodstream infections was 0.28% (Markwart et al., 2021). A global review also reported a reduced frequency of linezolid-resistant among MRSA and CoNS

(0.1% and 0.3%, respectively) (Shariati et al., 2020). These isolates were obtained from several human samples including skin infections, bronchopulmonary infections, pneumonia and bacteraemia (Shariati et al., 2020). Most of the reported studies were published in the USA, and also in other countries including Spain, Italy, France, Germany, Brazil, and China, among others (Shariati et al., 2020). However, In the last decade, outbreaks of hospital-acquired infections due to oxazolidinone-resistant CoNS were reported (Balandin et al., 2016). Higher percentages of linezolid resistance were reported in *S. aureus* isolates (2.3%) from pig carcasses (Kang et al., 2020) and in CoNS (1.3%) isolated from healthy slaughter poultry in Portugal (Silva et al., 2022).

1.7.2 Tigecycline Resistance

Tigecycline approval for clinical use occurred in 2005 by the FDA and in 2006 by the EMA (Bassetti et al., 2013). Even so, a Portuguese study reported enterococcal isolates with reduced susceptibility to tigecycline from food animals, meat and human origins, even before the introduction of tigecycline in clinical practice (Freitas et al., 2011). Tigecycline acts in bacteria in a way similar to tetracyclines (Rose & Rybak, 2006). After entering in bacteria cell by active or passive diffusion, the antibiotic reversibly binds to the 30S ribosomal subunit, blocking the entry of the tRNA into the ribosome site (Rose & Rybak, 2006).

Resistance to tigecycline is related to several mechanisms in both Gram-positive and Gram-negative bacteria. Resistance can occur through overexpression and accumulation of mutations on *tet* genes such as *tet*(A), *tet*(K), *tet* (L) and *tet* (M) leading to reduced tigecycline susceptibility (Fiedler et al., 2016; Linkevicius et al., 2016; He et al., 2019). The *tet*(A), *tet*(K) and *tet*(L) encode for efflux pumps, whereas *tet*(M) encodes for a ribosomal protection protein (Linkevicius et al., 2016). Another mechanism of tigecycline resistance is associated with the mobile tetracycline-resistant *tet*(X) gene (Yang et al., 2004). This gene codifies a flavin-dependent monooxygenase that has been shown to inactivate all known tetracyclines and also tigecycline (Yang et al., 2004). Tet(X) enzyme accept the tigecycline as a substrate and modify it into 11a-hydroxytigecycline, a product with a weak ability to inhibit protein translation (Moore et al., 2005). Gene variants *tet*(X3) and *tet*(X4) have been detected in *E. coli* and *A. baumanni* isolates from pigs with tigecycline minimum inhibitory concentration (MIC) values ≥32 mg/l (He et al., 2019). In another study, several *tet*(X) variants were identified in *Acinetobacter* spp. from pig farms, migratory birds, and clinical samples in China (C. Chen et al., 2020). All the tested *tet*(X)-positive isolates exhibited resistance to tetracycline and tigecycline (C. Chen et al., 2020). Furthermore, it has been shown that both *tet*(X3) and *tet*(X4) compromise tigecycline action (He et al., 2019).

In *S. aureus*, tigecycline resistance was associated with mutations in MepRAB efflux system (R. Fang et al., 2020). In Gram-negative bacteria, overexpression of resistance-nodulation division efflux pumps, encoded in the bacterial chromosome, such as AdeABC (Haeili et al., 2021), AdeFGH (Coyne et al., 2010), AdeIJK (Damier-Piolle et al., 2008), MexAB-OprM and MexXY (Dean et al., 2003), AcrAB (Y. Park et al., 2020) and OqxAB (H. B. Kim et al., 2009; Perez et al., 2013, p. 20) can also contribute to the emergence of tigecycline resistance strains. The *oqxA* and *oqxB* genes were first reported to be carried on a pOLA52 plasmid in *E.coli* from swine manure (L. H. Hansen et al., 2007).

Although the *oqxAB* operon was originally reported as plasmid-borne, it was also found in the bacterial chromosome (J. Li et al., 2019) and has been reported among several *Enterobacteriaceae* such as *E. coli* (H. B. Kim et al., 2009; J. Zhao et al., 2010), *K. pneumoniae* (H. B. Kim et al., 2009; Perez et al., 2013; Zhong et al., 2014) and *E. cloacae* (H. B. Kim et al., 2009). When overexpressed, the OqxAB multidrug efflux pump can confer resistance not only to antibiotics (quinoxalines, quinolones, tigecycline, nitrofurantoin and chloramphenicol) but also to detergents and disinfectants (benzalkonium chloride, triclosan and SDS) (J. Li et al., 2019). The OqxAB can be functionally independent of other efflux pumps however, a higher MIC (≥16mg/L) was associated with the presence of both AcrAB-TolC and OqxAB (Zhong et al., 2014). It has been proved that the overexpression of both efflux pumps and, consequently, the decrease in tigecycline susceptibility, is dependent on transcriptional regulators such as RarA, an AraC-type transcriptional activator (Veleba et al., 2012; Q. Xu et al., 2021), RamA, an integral transcriptional regulator (Q. Xu et al., 2021), and OqxR, an GntR-type transcriptional repressor (Q. Xu et al., 2021).

A recent systematic review indicated that the frequency of tigecycline resistance in clinical samples is low among *S. aureus* (0.1%), and that the higher frequencies are found in CoNS (1.6%) (Shariati et al., 2020). In a study with bacterial isolates collected from cats and dogs from 2003 to 2016, antimicrobial susceptibility showed that more than 20% of *Klebsiella* spp. isolates were non-susceptible to tigecycline (Sato et al., 2018), but all the *E. coli* isolates collected from the same clinical specimens were susceptible (Sato et al., 2018). Few studies have been focused on the prevalence of tigecycline resistance among animals for food consumption. However, carriage of antimicrobial-resistant determinants like *tet*(X) can reach 50% in pigs and 44% in chickens in certain Chinese provinces (Sun et al., 2020).

1.7.3 Colistin Resistance

Colistin is a 60-years-old antibiotic, however, its clinical use was suspended in the 1970s because of reports of significant renal (Brown et al., 1970, p. 197; Koch-Weser, 1970) and neurological toxicity (Koch-Weser, 1970, p.). Only in 1990s, the colistin therapy recovers interest and it was salvaged for the treatment of human infections caused by MDR Gram-negative bacteria (Bialvaei & Samadi Kafil, 2015). Almost ten years later, in 1999, was reported in the Czech Republic the first clinical colistin-resistant *A. baumannii* isolated from inpatients' blood samples (Hejnar et al., 1999). By 2004 the first reported colistin-resistant *K. pneumoniae* was isolated in Athens from hospitalized patients at the ICUs and, since then, colistin-resistant *Enterobacteriaceae* has spread all over the world (Antoniadou et al., 2007).

The mechanisms of colistin-resistance reported in *A. baumannii* and *Enterobacteriaceae* were associated with two-component systems PmrAB (Adams et al., 2009; Jayol et al., 2014) and PhoPQ (Jayol et al., 2015). Briefly, the PmrAB and PhoQ systems activate the *pmrCAB* and *pmrHFlJKLM* operons, respectively (Gunn, 2008; Chin et al., 2015). The activation of these operons mediates the addition of a cationic phosphoethanolamine (pEtN) and/or 4- amino-4-deoxy-L-arabinose (L-Ara4N) to the LPS (Jayol et al., 2015). Thus, the cationic charge of the LPS is increased and, consequently, the affinity to colistin is reduced, preventing the initial binding (Aghapour et al., 2019). Both systems can be induced by an environmental stimulus such as low pH (5.5), macrophage phagosomes (Chin et al.,

2015; Gunn, 2008), iron increment or magnesium decrement (Gunn, 2008). PmrAB system modulation can also be regulated by CrrAB regulatory system (Wright et al., 2015). It was reported that mutations in the *crrB* gene can induce colistin resistance in *K. pneumoniae* (Wright et al., 2015).

In 2015, a plasmid-borne colistin-resistant gene, designated mobilized colistin resistance (*mcr*), was first described in *E. coli* isolated from humans and livestock in China (Y.-Y. Liu et al., 2016). Since then, several *mcr* variants have been described worldwide. The MCR-1, codified by the *mcr-1* gene, is a pEtN transferase that modifies the pEtN moiety of lipid A, conferring colistin resistance (Hu et al., 2016). Until now, ten *mcr* variants have been reported (*mcr-1* to *mcr-10*) (C. Wang et al., 2020) in *Enterobacteriaceae* and other Gram-negative bacteria from human and animal origins including *K. pneumoniae* (F. J. Chen et al., 2021) *S. enterica* (Borowiak et al., 2019)*, E. hormaechei* (Khodor et al., 2021)*.* and *A. baumannii* (Martins-Sorenson et al., 2020)*.*

Alternative resistance mechanisms include the inactivation of the lipid A biosynthesis, through mutations in *lpxA, lpxC, and lpxD* genes, which are part of the lipid A biosynthesis pathway (Moffatt et al., 2010). Mutations in these genes can result in the complete loss of the LPS and can occur spontaneously (Moffatt et al., 2010). The EmrAB pump systems in *A. baumannii* can also contribute to colistin resistance (Lin et al., 2017). Furthermore, MgrB, a small regulatory transmembrane protein, also can contribute to colistin resistance (Cannatelli et al., 2013). Its production is induced by the PhoQ/PhoP system and has been demonstrated in *E. coli* and *S. enterica* (Lippa & Goulian, 2009) and the disruption of the *mgrB* gene was shown to be associated with polymyxin B resistance in *K. pneumoniae* (Aires et al., 2016)*.*

Some species such as *Proteus*, *Serratia, Morganella and Providencia spp.*, previously classified as *Enterobacteriaceae* (Adeolu et al., 2016), are intrinsically resistant to colistin (Samonis et al., 2014). The mechanism of intrinsic resistance is linked to the expression of *arnBCADTEF* operon and the *eptB* gene that add, respectively, pEtN and L-Ara4N cationic groups to the LPS (Aghapour et al., 2019).

Data on polymyxin resistance from the European Centre for Disease Prevention and Control (ECDC) in 2013 suggested that 7.1% of the human carbapenem-resistant isolates were also polymyxin resistant (ECDC, 2014). Only five countries reported polymyxin data, most of them corresponding to *E. coli* isolates (ECDC, 2014). Difficulties in the interpretation of the susceptibility testing methods used for colistin resistance detection may explain these low numbers (European Food Safety Authority et al., 2021). In China, between 2013 and 2014 the frequency of colistin resistance in *E. coli* from pigs was 24.1% (Huang et al., 2017) and *mcr*-1-harboring *E. coli* isolates in pigs was 45% in 2016 (Shen et al., 2020). But in a more recent study, performed between 2018 and 2019 in Chinese pig farms, a lower frequency was reported, 5.56% for colistin-resistant *E. coli* and 6.23% for *mcr*-positive *E. coli* (Peng et al., 2021).

1.8 Methicillin-resistance in staphylococci

The first reports of MRSA occurred in the UK in 1961 in human infection, three years after methicillin introduction into clinical practice (Jevons, 1961). Methicillin resistance is usually associated with the presence of *mecA* gene, which codifies for an additional PBP known as PBP2a or PBP2′, with

low affinity for virtually all β-lactams (Hartman & Tomasz, 1984; Katayama et al., 2000). The acquisition of *mecA* by different genetic backgrounds of *S. aureus* originated in what is known as the MRSA pandemics which included the dissemination of different MRSA clonal types in hospitals worldwide (Deurenberg et al., 2007). Following the MRSA pandemics, MRSA emerged also as a cause of infections in healthy persons in the community, the so-called community-associated *S. aureus* (CA-MRSA) (CDC, 1999).

In 2005 the first LA-MRSA was reported in swine, suggesting that animals might also be a potential source of this pathogenic bacteria (Voss et al., 2005). A few years later, an MRSA strain designated *S. aureus* LGA251 was isolated from a bulk tank of milk in southwest England (García-Álvarez et al., 2011). Genome sequencing revealed that the strain belonged to ST425 and carried a *mecA* form that was different from *mecA* designated as *mecC* (former $mecA_{LGA251}$) (García-Álvarez et al., 2011; Becker, Ballhausen, et al., 2014). The *mecC* was additionally found in other genetic backgrounds, mainly associated with isolates of animal origin (García-Álvarez et al., 2011), but the extent of its dissemination is limited when compared to *mecA*.

Clinical isolates of MRSA are frequently MDR, showing additional resistance to fluoroquinolones, macrolides, lincosamides, rifampicin and tetracycline (Abdolmaleki et al., 2019). Between countries, there is a wide variation in MRSA prevalence in human infection, even across Europe. The latest ECDC report showed in Scandinavia, only 1-5% of the notified *S. aureus* isolates were MRSA strains (ECDC, 2019). However, in Southern European countries including Portugal, Italy and Greece MRSA rates reach up to 25-50% (ECDC, 2019).

Similar to what happens with human isolates, MRSA prevalence in pigs varied substantially between countries (EFSA, 2009). According to the European Food Safety Authority, in 2008 the higher prevalences of MRSA in breeding pigs were found in Spain (46%), Germany (43.5%), Belgium (40%) and Italy (34.9%), the majority belonging to the sequence type (ST) 398 (EFSA, 2009). According to the same report, the prevalence of MRSA in Portuguese pigs was 14.7% (EFSA, 2009). However, a recent study performed on a Portuguese farm reported that 99% of the staphylococci were MRSA (Conceição et al., 2017).

The ST398 is currently the most widely spread LA-MRSA (Pirolo, Gioffrè, et al., 2019), is found frequently in pigs (Sahibzada et al., 2017; Bouchami et al., 2020) and other production animals, such as dairy cattle and veal calves (J. E. Hansen et al., 2019). A recent study by Bouchami *et al.* 2020 showed that MRSA ST398 could be additionally transmitted along the pig processing chain to humans in close contact with pigs (Bouchami et al., 2020). Although MRSA ST398 has been also found to cause infections in humans, these are uncommon and frequently associated with humans exposed to animals or animal husbandry (Pirolo, Gioffrè, et al., 2019).

Besides being frequently found in *S. aureus*, *mecA* was also found associated with CoNS. Methicillin-resistant CoNS (MRCoNS) were found to be highly prevalent in hospitals worldwide in colonization and infection (Petinaki et al., 2001; Seng et al., 2017). In Greece, 91% of *S. haemolyticus*, 73% of *S. epidermidis* and 48% of S. *hominis* isolate collected from ICUs and surgical wards in hospitals

carried the *mecA* gene (Petinaki et al., 2001). Furthermore, MRCoNS have been also reported in veterinary settings. A frequency of 19.6%, 20.3% and 51% of MRCoNS isolates were collected in 2020 in Portugal, from commercial chickens, homebred chickens and quails, respectively (Silva et al., 2022). Furthermore, MRCoNS prevalence in intensive and organic swine farms' in Italy was reported to reach 64.6% (Bonvegna et al., 2021).

1.9 Detection of antibiotic-resistant bacteria

Nowadays detection of antimicrobial resistance can be assessed by either phenotypic and/or genotypic methods.

1.9.1 Phenotypic methods for detecting antimicrobial resistance

The most commonly used methods to determine phenotypic antibiotic susceptibility are those based on agar diffusion methods and dilution methods (Wiegand et al., 2008). The disk diffusion (Kirby–Bauer test) (Bauer et al., 1966) is an example of an agar diffusion method (EUCAST, 1998). It consists of the spreading of a pure bacterial suspension, in a well-defined growth medium and standardized turbidity, in agar plates on the top of which antibiotic disks with a specific concentration are placed (Khan et al., 2019). Inhibition zones (halo) correspond to the region around the antibiotic disk free of bacterial growth (EUCAST, 1998), after incubation at a specified temperature (Khan et al., 2019), according to the bacterial species.

Two types of dilution methods are defined: macrodilution and microdilution (EUCAST, 1998) which can be performed in liquid media (broth) or solid media (agar) (Khan et al., 2019). The macrodilution in broth consists in growing bacteria in a medium containing serial dilutions of the antibiotic and then observing the presence of turbidity as a surrogate for bacterial growth detection (Khan et al., 2019). Additionally, the dilutions can be plated in agar plates to determine the number of colony forming units (CFU/mL) (Boukouvalas et al., 2019). The microdilution follows the same principle of the macrodilution method, but on a smaller scale. The antibiotics susceptibility testing is performed on microwells plates (Khan et al., 2019). After the incubation period, the growth and MIC can be assessed through specialized optical instruments (Khan et al., 2019).

These methods allow defining the MICs by agar or liquid dilution methods (EUCAST, 1998). This parameter defines as the lowest concentration of an antimicrobial agent (usually express in mg/L) that prevents the growth of a microorganism within defined conditions (EUCAST, 1998; Wiegand et al., 2008). With the development of the Epsilometer testing (Etest) (Picard, 1990), which consists of plastic strips with a pre-defined gradient of antibiotic concentrations, it became possible to determine the MIC also through diffusion in agar (Sader & Pignatari, 1994). Elliptical inhibition zones appear around the strips, after the incubation period, and the point of intersection between the inhibition zone and the strip edge is considered the MIC (Sanchez & Jones, 1992; Sader & Pignatari, 1994).

Breakpoints for phenotypic antimicrobial susceptibility testing (disk diffusion and dilution methods) have been defined by specific committees and are part of regulatory processes for the approval of each drug. The use of such breakpoints assumes that well-defined protocols are used, in which growth conditions (e.g. inoculum concentration, temperature), growth medium, and antibiotic concentrations

are standard and quality control procedures are followed. Several factors are taken into consideration for the definition of these breakpoints, such as dosages, pharmacokinetics and pharmacodynamics dynamics (Mouton et al., 2012), resistance mechanisms, MIC distributions, and epidemiological cut-off values (ECOFFs) (EUCAST, 2019). Based on the MIC breakpoints it is possible to determine if a bacterial species is susceptible or resistant to a certain antimicrobial. Some of the most popular MIC guidelines are those provided by the Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST). Both committees provide MIC and breakpoints values that represent guidance to clinicians and impact decisions regarding the most appropriate treatment.

In clinical laboratories, more automatized methods are used to detect phenotypic antibiotic resistance, such as the VITEK (Shetty et al., 1998). Additionally, some studies have been performed that suggest that MALDI-TOF MS, a protein ionization profiling method traditionally used for bacterial species identification, can be additionally applied to detecting resistance determinants (Lau et al., 2014; Rahi et al., 2016).

1.9.2 Whole-genome sequencing (WGS)-based methods for detection of antimicrobial resistance

The high throughput technologies that allow the sequencing of the whole genome of multiple bacteria simultaneously, including Illumina, IonTorrent (Quail et al., 2012) and Nanopore Technologies (Y. Wang et al., 2021), have provided an opportunity to detect antimicrobial resistance in a fast and comprehensive way in clinical laboratories. Pacific Biosciences (PacBio) technology has been used mainly to obtain completely closed genomes and is not used in high-throughput (Goodwin et al., 2016).

WGS technologies can be divided into long reads (Nanopore, PacBio) and short reads (Illumina and Ion Torrent) sequencing (Goodwin et al., 2016), depending on the length of the sequencing reads produced. Also, the different technologies have different error rates in base-calling, Illumina is still the most accurate (0.1% base-calling errors) (Glenn, 2011) when compared to PacBio single-molecule real-time sequencing (<1%) (Wenger et al., 2019), Ion Torrent (1.5%) (Song et al., 2017) and Nanopore (<5% error) (Jain et al., 2018). Long read sequencing technologies, like PacBio and Nanopore, have the great advantage of providing a more correct structural genomic analysis (Quail et al., 2012).

The short reads sequences obtained by Illumina are usually subjected to steps of multiplexing, adapter trimming, and quality check and then assembled into contigs using either a *de novo* or mapping strategy (Chaitankar et al., 2016). Usually, it is obtained a large number of contigs (Smits, 2019), more than 100 contigs are obtained for bacteria of approximately 2 Gb. To obtain draft genomes, contigs can be then aligned against a reference closed genome (Illumina, 2010). In opposition to Illumina, Nanopore technologies allow obtaining extreme long reads, without a complex preparation of mate-pair libraries (Kono & Arakawa, 2019).

The detection of antibiotic resistance determinants from WGS data is usually done by submitting the obtained raw reads or the assembled contigs into web-based databases containing reference sequences for antibiotic resistance genes and mutations, like ResFINDER (Bortolaia et al., 2020) and

the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013). The sequencing contig/reads are aligned against the gene reference database and considered to be present/absent according to defined nucleotide identity and gene coverage. Although these databases are frequently curated and updated, they usually contain antibiotic resistance genes only for the most common human pathogens, which makes the detection of antibiotic resistance genes in non-pathogenic species a challenge. Moreover, the majority of the databases developed to contain data regarding acquired resistance only, not including the resistance associated with the emergence of mutations, which limits considerably the detection range.

Nevertheless, WGS-based sequencing methods have been considered reliable for the detection of antibiotic resistance in human pathogens (Ellington et al., 2017). And, usually, a good correlation between the carriage of antibiotic genes and the phenotypic data has been reported (Gordon et al., 2014). However, there are certain mechanisms of resistance that are particularly difficult to detect, like those based on multiple mutations, multiple mechanisms or gene expression variation.

1.10 Detection of bacterial transmission events by WGS

Given the high resolution provided by WGS, this technology has been also applied for the detection of transmission of bacteria. This is usually done using either a single nucleotide analysis of the core genome Single-Nucleotide Polymorphism (SNP) analysis (Gilchrist et al., 2015; Lawal et al., 2021) or a core genome Multilocus Sequence Typing (MLST) (Leopold et al., 2014; Mellmann et al., 2017). SNPs analysis implies the alignment of draft genomes against a reference closed genome and the identification of the SNPs that are core. These SNPs are then concatenated for each strain, a new alignment is performed, resulting in a production of an SNPs distance matrix, that is used to construct a phylogenetic tree. Isolates are then considered to belong to the same chain of transmission if they differ in a certain number of SNPs (usually a low number). This cut-off has to be defined and optimized for each bacterial species, commonly by using isolate collections that are representative of the population, multiple typing methods, and epidemiological data (Coll et al., 2020). However, still, there is not a standardized way of defining these cut-off values.

The cgMLST is an extension of MLST in which all core genes defined for the species are considered in the analysis (Leopold et al., 2014). In this analysis, the sequence contigs obtained are submitted and aligned with the core genes database containing all the gene alleles described for the species. To each gene, an allele number is attributed and for each isolate, a gene profile with a code containing a number for each core gene is obtained. The allelic profiles of the different isolates are compared and the number of allelic differences is identified. The cut-off for defining an isolate as belonging to the same chain of transmission is usually defined for each species when the methodology is developed for the species and is called cluster type distance. However, the cgMLST scheme is only defined for a limited number of human pathogens.

1.11 Objectives

In this present study, we aimed to evaluate the frequency of resistance to antibiotics in the pig meat processing chain, including last-resort antibiotics, among *Staphylococcus* spp. and Gram-negative

bacteria. Furthermore, we intended to assess the extent of dissemination of antibiotic-resistant bacteria and antibiotic resistance genes in the pig production chain and identify the most frequent antibiotic-resistant mechanisms among these bacteria.

2. MATERIAL & METHODS

2.1 Sample collection and sampling strategy

A total of 24 samples from a slaughterhouse in the metropolitan area of Lisbon (Portugal) were analyzed (see Annexe 1). The samples were collected in 2016 at two-time points (summer and winter) and included samples from the live pig (ear and rectum), processed pig (fresh meat), hands of the operators and surfaces in direct contact with meat. Sampling was done on two consecutive days.

On the first day, samples from the ear (n=5) and rectum (n=3) of live pigs were collected at the slaughterhouse entrance, after electrical stunning but before the bleeding. On the following day, the samples from the pork, cutting operator hands and equipment surfaces were collected. All samples from the meat pieces were obtained from the previously sampled pigs and included the streaky pork (belly bone) (n=4) and the pig shoulder bone (n=2). The sampling of the operator's hands involved in the pork cutting was performed before and after cleaning (n=5). The slaughterhouse cutting room surfaces in contact with the meat, including cutting tables and conveyors from deboning line (n=5), were also performed before and after cleaning.

The samples from pig ears, pork, workers' hands, conveyors and cutting tables were obtained within a defined area (500–1000 cm^2) using 10×10 cm sterile cotton gauze humidified with physiologic NaCl concentration (0.9%) according to the guidelines of the International Organization for Standardization (ISO) 18593:2004 (ISO, 2004). The samples from the pig rectum were collected with a sterile swab. All samples were then enriched in peptone water solution for 24h at 37ºC and conserved in Tryptic Soy Broth (TSB, Bacto™, BBL, Becton Dickinson, Sparks, MD, USA) with 15% of glycerol at -72ºC.

2.1.1 Bacterial isolation and selection

2.1.1.1 Isolation of *Staphylococcus* spp.

Enriched samples (n=24, different origins) were serially diluted (10^{-1} to 10^{-4}) in TSB and 100 µl of each of these dilutions was inoculated on the surface of a selective chromogenic media-CHROMagar™ Staph aureus (CSA, CHROMagar™, Paris, France) (Gaillot et al., 2000; Carricajo et al., 2001) and spread using sterile glass beads. The plates were incubated at 37ºC for 20h. The dilution, showing countable and isolated CFUs (30-300 colonies per plate) and with the highest diversity in colony morphology were selected for further study.

Ten colonies with a morphology characteristic of staphylococci in the CSA medium were picked from the selected dilution and streaked into Mannitol Salt Agar (MSA, Difco™, BBL, Becton Dickinson, Sparks, MD, USA) medium and then incubated at 37ºC for 24-48h. The selected colonies had the

following features in CSA medium: a regular shape, moderate size, pink to mauve, cream to turquoise/light blue. All isolates that grew in MSA were considered putative staphylococci and were then grown into Trypticase Soy Agar (TSA, Difco™, BBL, Becton Dickinson, Sparks, MD, USA) at 37ºC for 24h and conserved in TSB with 15% glycerol at –72°C. The strain *S. aureus* American Type Culture Collection (ATCC) 29213 was used as a control for the CSA media performance.

2.1.1.2 Isolation of Gram-negative bacteria

Ten enriched samples recovered from different origins were serially diluted (10^{-1} to 10^{-3}) and 100 µl of each dilution was spread on CHROMagar Orientation (CO, CHROMagar™, Paris, France) (Merlino et al., 1996; A. K. Singh & Bhunia, 2016) using sterile glass beads and grown for 20h at 37ºC. The dilution, showing countable (30-300 colonies per plate) and isolated colonies with the highest diversity in colony morphology were selected for further study. Up to ten colonies with the morphology characteristic of Gram-negative bacteria were picked from the selected dilution and streaked in TSA medium at 37ºC for 24h. The selected colonies had the following features in CO medium: dark pink to reddish, turquoise blue, metallic blue, brown halo, cream or translucent; and moderate size. Isolates considered as putative Gram-negative were then conserved in TSB with 15% glycerol at –72°C.

The following controls of medium performance were used: *S. saprophyticus* ATCC 15305; *E. coli* ATCC 25922; *Enterococcus faecium* ATCC 6057; *E. faecalis* ATCC 29212; *S. aureus* ATCC 25923; *S. epidermidis* ATCC 12228; *K. pneumoniae* (OND 218 and OND 228A) and *Pseudomonas aeruginosa* SIPD4.

2.2 Species identification

2.2.1 Staphylococci identification by *tuf* sequecing

Staphylococcus species identification was done by amplification and sequencing of an internal fragment of the *tuf* gene (Martineau et al., 2001). A 320-bp *tuf* DNA fragment was amplified by PCR using the *Staphylococcus*-specific *tuf* primers: TStaG422 5'-GGC CGT GTT GAA CGT GGT CAA ATC A-3' and TstaG765 5'-TIA CCA TTT CAG TAC CTT CTG GTA A-3' (Invitrogen, ThermoFisher Scientific, Wilmington, DE, USA).

The amplification was performed in a thermocycler (MiniAmp™ Plus Thermal Cycler, Thermo Fisher Scientific, USA) and PCR conditions were optimized as follows: a total reaction mix of 50 µl containing 1X PCR Buffer I with 15 mM $MgCl_2$ (Applied Biosystems, Roche, Branchburg, New Jersey, USA), 160 µM deoxynucleotide triphosphate (dNTP) mixture (BIORON, Ludwigsmanfen, Germany), 0.075 U/µl Amplitaq® DNA Polymerase (Applied Biosystems, Roche, Branchburg, New Jersey, USA), 0.4 mM from each of primer (Invitrogen, ThermoFisher Scientific, Wilmington, DE, USA), 5 µl of diluted DNA (50-100 ng/µl) and MilliQ water.

The PCR optimized program was the following: 96ºC for 3 min, followed by 35 (or 40) cycles at 95ºC for 30 sec, 55ºC (or 53ºC) for 1 min, and 72ºC for 30 sec, and a final extension step of 3 min at 72ºC. The type strain *S. saprophyticus* ATCC 15305 was used as a positive control for the *tuf* PCR and a reaction without a DNA template was run as a negative control.

Before sequencing, enzymatic cleanup of the PCR products was performed using Exonuclease I (Exo I, NEB #M0293) and Shrimp Alkaline Phosphatase (rSAP, NEB #M0371) (New England BioLabs, Beverly, USA). Briefly, 30 µl of each PCR product was mixed with Exo I (5 U/µl) and rSAP (1U/µl) under the following program conditions: 30 min at 37ºC and 15 min at 80ºC.

All the PCR products were visualized on a 1% agarose gel (SeaKem LE Agarose, Lonza, Rockland, ME, USA) in Tris Acetate EDTA (TAE) 1x, stained with 0.003% v/v Green Safe (GreenSafe Premium, NZYTech – Genes & Enzymes, Lisbon, Portugal). The amplicons were observed under ultraviolet (UV) light and photographed using GelDoc-EZ apparatus (Gel Doc™ EZ Imager, Bio-Rad Laboratories, Hercules, USA). Purified amplicons were then sequenced with the primers TstaG422 and TstaG765 (5 pmol/µl each) using Sanger sequencing (GATC, Eurofins Genomics, Ebersberg, Germany).

To identify the species, the nucleotide sequences obtained were analysed using Sequence Analysis Software DNASTAR Lasergene (version 7.0.0) (SeqMan package, DNASTAR, Madison, WI, USA) and submitted to the National Center for Biotechnology Information (NCBI) database (Nucleotide Basic Local Alignment Search Tool (nBLAST), available at https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on November 2021). Identity in the nucleotide sequence of ≥97% was considered the cut-off for species identification.

2.2.2 Gram-negative identification by 16S sequencing

Identification of Gram-negative bacterial species was performed by amplification and sequencing of the 16S rRNA gene. Universal 16S rRNA bacterial primers 27f 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492r 5′-TACGGYTACCTTGTTACGACTT-3′ (Invitrogen, ThermoFisher Scientific, Wilmington, DE, USA) (Edwards et al., 1989; Weisburg et al., 1991) were used to amplify this gene, generating a fragment size of 1500 bp (Ghoreishi & Etemadifar, 2017). The PCR reaction mixture of 50 µl volume consisted of 1x Colorless GoTaq™ Flexi Buffer(Promega, WI, USA); 1.5 mM of $MgCl_2$ (Applied Biosystems, Roche, Branchburg, New Jersey, USA), 16 µM of the dNTPs mixture (BIORON, Ludwigshafen, Germany), 0.2 µM of 27f primer (20pmol/µl); 0.24 µM of 1422r primer (Invitrogen, ThermoFisher Scientific); 0.075 U/µl of GoTaq® DNA Polymerase (Promega, WI, USA), and 5 µl of diluted DNA (50-100 ng/µl) and MilliQ water.

The PCR was run with the following conditions: 5 min at 95ºC, 30 cycles at 95ºC for 30s, 55ºC for 30 s, and 72 ºC for 1 min; followed by 10 min at 72 ºC (MiniAmp™ Plus Thermal Cycler). Amplicons were visualized on a 1% agarose in TAE 1x after staining with 0.003% v/v Green Safe. The amplicons (SeaKem LE Agarose, Lonza, Rockland, ME, USA) were visualized and photographed under ultraviolet illumination using the GelDoc-EZ apparatus (Gel Doc™ EZ Imager, Bio-Rad Laboratories). *S. aureus* subsp. *aureus* COL (MRSA) strain was used as a positive control for the *16S* PCR and a reaction without the DNA template as a negative control.

PCR products were purified using EXO/SAP clean up enzymes (New England BioLabs) as described above (See section 2.3.1.). Products were then sequenced using forward and reverse primers (5 pmol/µl) and sequences were analysed with the DNASTAR package version 7.0.0 and online with

the nBLAST program. Identity in the nucleotide sequence of ≥97% was considered the cut-off for species identification.

2.3 Antimicrobial susceptibility testing to last-resort antibiotics

Susceptibility testing of *Staphylococcus* spp. and Gram-negative isolates to last-resort antibiotics – linezolid, tigecycline and colistin - was done following the EUCAST breakpoint guidelines (EUCAST, 2020a)

2.3.1 Antimicrobial susceptibility testing to linezolid

To screen for linezolid-resistant staphylococci, all the presumptive *Staphylococcus* spp. isolates (n=161) were grown on Mueller-Hilton II agar plates (MHA II, BBL™, Becton Dickinson, Sparks, MD, USA) containing 5 mg/L of linezolid, a concentration above the resistant breakpoint defined by EUCAST (MIC= 4 mg/L, EUCAST, 2020a). For this purpose, one or several colonies of *Staphylococcus* spp. isolates from TSA agar plates were suspended in saline solution (NaCl 0.85%), adjusted to a turbidity of 0.5 McFarland ($1x10^8$ CFU/mL) and spotted (approximately 5 μL per drop) into freshly prepared MHA II agar plates supplemented with linezolid with a concentration of 5 mg/L. All the plates were dried for 5 min before being incubated at 37ºC for 18±2h. After the incubation, if visible growth was detected in the spot sites, the isolates were considered linezolid resistant. Staphylococci that were considered resistant by the method described previously were additionally tested for linezolid (LIN, 30μg) susceptibility by the disk diffusion method.

MHA II agar plates without antibiotics served as a growth control and *S. epidermidis* ATCC 12228 was used as a negative control. Furthermore, four linezolid-resistant *S. epidermidis* (MIC > 256 mg/L) were used as positive controls: LZD9, LZD10 (*cfr* positive) and LZD3, LZD15 (*cfr* negative).

2.3.2 Antimicrobial susceptibility testing to tigecycline

To select for tigecycline resistant isolates, all putative staphylococci (n=161) and Gram-negative bacterial isolates (n=121), were grown overnight at 37ºC. The bacterial suspensions were adjusted to 0.5 McFarland standard and were manually spotted (5 μl drop) over the surface of MHA II agar plates supplemented with the following tigecycline concentrations: 1.125 mg/L, 1.25 mg/L and 1.5 mg/L. Those concentrations were above the MICs defined by EUCAST for different bacterial species: *Staphylococcus* spp. and Enterobacterales (*E. coli* and *C. koseri*) with MIC=0.5 mg/L (EUCAST, 2020a).

S. aureus ATCC 29223 (MIC= 0.25 mg/L) was included as negative control. All the plates were allowed to dry for approximately 5 min and then incubated at 37ºC for 18±2h. If a visible growth in spots inoculation site was observed, isolates were considered tigecycline-resistant.

2.3.3 Antimicrobial susceptibility testing to colistin

To select for colistin-resistant Gram-negative isolates (n=121), bacterial suspensions at the final concentration of 5×10^5 CFU/mL were inoculated in 96-microtiter plates (BrandTech Ref. 781660, BRANDplates® pureGrade™ S, Wertheim, Germany) containing Muller Hilton Broth (MHB, Difco, BBL, Becton Dickinson, Sparks, MD, USA) supplemented with colistin at a concentration of 5 mg/L, above the EUCAST MIC defined for Enterobacterales and *Acinetobacter* spp. (MIC>2mg/L) (EUCAST, 2020a),

and incubated for 18±2h at 37ºC without shaking. The following controls were used: MHB wells without antibiotics as growth control; the *E. coli* ATCC 25922 was used as a colistin-susceptible control; and *E. coli* OND 396 (*mcr-1* positive, MIC >16 mg/L), *Hafnia alvei* F9Z2.18 (MIC >16 mg/L) and *Serratia marcescens* F43.11A (MIC=16 mg/L) were used as colistin-resistant control strains. The isolates were considered resistant if visible growth (turbidity and/or pellet bottom) was observed and were considered colistin-susceptible when no visible growth was detected.

The MIC was determined for the previous colistin-resistant isolates (n=15) in MHB by microdilution method according to the EUCAST guidelines (EUCAST, 2020b) and the ISO 20776-1 (ISO, 2006). Briefly, bacterial suspensions at a final concentration of 5×10^5 CFU/mL were inoculated in serial two-fold dilutions ranging from 0 to 16 mg/L of colistin concentrations in microtiter plates and incubated for 18±2h at 37ºC without shaking. Assays were performed in triplicate. *Escherichia coli* ATCC 25922 was used as the susceptible control strain and *H. alvei* F9Z2.18 as the colistin-resistant control strain.

2.4 Antimicrobial susceptibility testing to non-last resort antibiotics

Antimicrobial susceptibility testing to non-last resort antibiotics was performed for presumptive *Staphylococcus* spp. from different sampling sites and with different colony characteristics (n=51) and for all colistin and/or tigecycline resistant *Enterobacteriaceae* isolates (n=15) and *A.pittii* (n=1). Antimicrobial susceptibility was determined by the disk diffusion method on MHA II plates using 0.5 McFarland bacterial inoculum. Moreover, for some bacterial species and antibiotics, susceptibility was performed using the antibiotic gradient strips (Etests), as recommended by EUCAST (EUCAST, 2020a).

2.4.1 Antimicrobial susceptibility testing in staphylococci

For *Staphylococcus* spp., a total of fifteen antibiotics (Oxoid, Basingstoke, UK) were tested by disk diffusion method: penicillin (P, 10UI), oxacillin (OXA, 1µg), cefoxitin (CXI, 30µg), erythromycin (ERY, 15µg), clindamycin (CLI, 2µg), gentamicin (GEN, 10µg), fosfomycin (FOS, 50µg), teicoplanin (TEI, 30µg), tetracycline (TET, 30µg), chloramphenicol (CHL, 30µg), quinupristin/dalfopristin (QUD, 15µg), rifampicin (RIF, 5µg), fusidic acid (FUS, 10µg), ciprofloxacin (CIP, 5µg), and trimethoprim/sulfamethoxazole (TRS, 1.25/23.75µg). Briefly, a 0.5 McFarland bacterial inoculum was prepared and spread into an MHII plate, and the antibiotic disk was placed on the top of the bacterial layer and incubated at 37ºC for 18±h.

Resistance data were interpreted mostly according to the guidelines of EUCAST (EUCAST, 2020a). However, the interpretation criteria were exceptionally made according to the British Society for Antimicrobial Chemotherapy (BSAC) (BSAC, 2015) (for fosfomycin) and CLSI (CLSI, 2015) (for penicillin and teicoplanin) when EUCAST breakpoints were not available for the disk antimicrobial concentrations used.

The MICs were determined by E-test (AB bioMérieux, Solna, Sweden) for oxacillin and cefoxitin for presumable staphylococci (n=51) and quinupristin-dalfopristin-for the resistant and intermediate isolates, as classified by disk diffusion (n=8). E-tests were performed and interpreted as recommended by EUCAST (EUCAST, 2020a). Briefly, a 0.5 McFarland bacterial inoculum was prepared and spread into an MHII plate, and the antibiotic strips were placed on the top of the bacterial layer and incubated

at 37ºC for 18±h. The strain *S. aureus* ATCC 259213 was used as quality control for antimicrobial susceptibility testing.

To further confirm resistance to beta-lactams in staphylococci (n=51), the *mecA* and *mecC* genes were amplified by PCR as previously described (McClure et al., 2006; C. Kim et al., 2012) using primers: *mecA*-1 (5′ GTAGAAATGACTGAACGTCCGATAA-3′) and *mecA*-2 (5′ CCAATTCCACATTGT TTCGGTCTAA-3′) for *mecA* gene and *mecA*LGAF3 (5'ACACCTTTTAGGTTATGTGG-3') and *mecA*LGAR2 5'TTTACTAGTATCTCGCCTTGG-3' for *mecC* gene. The following controls were included in amplification reactions: *S. aureus* COL strain (*mecA*-positive) and *S. aureus* subsp. *aureus* LGA251 (*mecC*-positive).

Isolates that presented resistance to three or more antibiotic categories were classified as having a MDR profile as defined by Magiorakos *et al.* 2012 (Magiorakos et al., 2012).

2.4.2 Antimicrobial susceptibility testing in Gram-negative bacteria

The *Enterobacteriaceae* isolates were tested against a panel of eleven antimicrobial agents tested by disk diffusion method (described above) including amoxicillin-clavulanate (AMC, 30 µg), piperacillin-tazobactam (PIT, 36µg), ticarcillin (TIC, 75 µg), temocillin (TEM, 30 µg), cefepime (CEP, 30 µg), ceftazidime-avibactam (CTV, 14 µg), imipenem (IMI, 10 µg), meropenem (MER, 10 µg), ciprofloxacin (CIP, 5 µg), gentamicin (GEN, 10 µg) and trimethoprim-sulfamethoxazole (TRS, 25µg). *E. coli* ATCC 25922 was included for quality control of antimicrobial susceptibility testing. One *A. pittii* isolate was tested against eight of the eleven antibiotics, except for amoxicillin-clavulanate, piperacillin-tazobactam and ceftazidime-avibactam. Isolates were considered MDR if they showed acquired resistance to at least one agent from three different categories as defined by Magiorakos *et al.* 2012 (Magiorakos et al., 2012).

2.5 DNA extraction for long-read sequencing

The genomic DNA was extracted from overnight TSB cultures for 25 *Staphylococcus* spp. and Gram-negative (n=57) bacterial isolates using the DNeasy Blood & Tissue kit (Qiagen, GmbH, Hilden, Germany), according to the manufacturer instructions (QIAGEN, 2020). Briefly, for staphylococci, cells were lysed with lysostaphin (10 mg/ml) (Sigma-Aldrich Co.), lysozyme (10mg/ml) (Sigma-Aldrich Co.) and RNase A (10 mg/ml) (Ribonuclease A from bovine pancreas, Sigma-Aldrich Co.) and resuspended in LP1 buffer (20 mM Tris HCl, 20mM Sodium EDTA, 1.2% Triton x100; pH=8) (Sigma-Aldrich Co.). Lysis of Gram-negative bacteria was achieved by resuspending the bacterial cultures in the ATL buffer.

To ensure the use of high-quality DNA for WGS, the concentration and purity of the extracted DNA were determined using a Qubit double-stranded DNA high sensitivity assay kit (Qubit 2.0 Fluorometer, Invitrogen, Carlsbad, CA) and a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), respectively. The integrity of the genomic DNA was checked by visualization on a 0.8% agarose gel in TAEX 1x electrophoresis stained with 0.003% v/v Green Safe (GreenSafe Premium, NZYTech – Genes & Enzymes, Lisbon, Portugal). The genomic DNA was then observed under UV light and photographed using GelDoc-EZ apparatus (Gel DocTM EZ Imager, Bio-Rad Laboratories, Hercules, USA).

2.5.1 Library preparation and long-read sequencing

Long-read sequence data was obtained for Gram-negative bacteria showing resistance to last-resort antibiotics (n=15) and representative *Staphylococcus* spp. isolates (n=9). The representative *Staphylococcus* spp. selected were from different sample origins and had a MDR profile. Long read sequencing was performed with a Nanopore MinION system (MinION Mk1C, Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's instructions. DNA libraries were prepared using the ligation sequencing 1-12 kit (SQK-LSK109) and the native barcoding expansion kit (NB09 of EXP-NBD104), and sequencing libraries were subsequently loaded into a MinION flow cell (R9.4). Data was acquired and basecalling was performed using the graphical user interface MinKNOW v19.12.5. and Guppy basecaller v2.3.7. The demultiplexing of barcodes and quality control of the reads were achieved using EPI2ME platform. All quality reads were extracted after 48 h of the sequencing run for downstream analysis.

2.5.2 *de novo* assembly of sequencing reads

The *de novo* assembly of the MinION reads was performed using the Flye assembler version 2.9 (https://github.com/fenderglass/Flye , accessed on 20 August 2021) (Kolmogorov et al., 2020) and the genome error correction, implemented in the Flye pipeline. The generated contigs were used to confirm the species identification of the 24 representative isolates using Speciator tool in PathogenWatch (https://pathogen.watch, accessed on September 2021 (CGPS, 2020).

2.6 Detection of antibiotic resistance genes from WGS data

The assembled contigs were analyzed for the presence of acquired antimicrobial resistance determinants using ABRIcate version 1.0.0 pipeline (https://github.com/tseemann/abricate, accessed in September 2021) (Zankari et al., 2012; L. Chen et al., 2016; Seemann, 2021). Genes with a threshold of 70% identity and a minimum coverage length of 90% were considered present. ABRIcate screening was done using the following databases: ResFinder v. 4.1 at the Centre of Genomic Epidemiology (CGE) (https://cge.cbs.dtu.dk/services/ResFinder/ , accessed on September 2021) (Camacho et al., 2009; Zankari et al., 2017; Bortolaia et al., 2020) and CARD (http://arpcard.mcmaster.ca/ , accessed on September 2021) (Alcock et al., 2019).

2.7 Molecular characterization of genetic backgrounds by *in silico* multilocus sequence typing (MLST)

The *in silio* MLST was determined by using MLST v. 2.0 (https://cge.cbs.dtu.dk/services/MLST/, accessed on February 2021) (Larsen et al., 2012). The sequences were also queried against the MLST database on the website http://pubmlst.org (Jolley et al., 2018) and on the Pathogenwatch (CGPS, 2020) to infer the MLST. The MLST of the available species were defined according to the exact or closest matches.

2.8 Single-nucleotide polymorphism(SNP)-based phylogenetic analysis

For SNP-based phylogenetic analysis, the genomes were submitted to CSI Phylogeny (v. 1.4. online server available at CGE (https://cge.cbs.dtu.dk/services/CSIPhylogeny/, accessed on September 2021) (Kaas et al., 2014) with the following default setting: minimum depth at SNP positions 10, relative

depth at SNP positions 10, the minimum distance between SNPs (prune) disabled, minimum SNP quality 30, minimum read mapping quality 25 and minimum Z-score of 1.96.

The SNPs were called against *S. hyicus* strain ATCC 11249 (GenBank accession number CP008747) and *E. coli* strain AH04 (GenBank acession number NZ_CP081706.1), concatenated, and multiple-aligned. Maximum likelihood trees were generated based on concatenated SNP alignment using CSI Phylogeny 1.4 server (RAxML) and visualized using Microreact v. 197.0.0 (Argimón et al., 2016). Strains were considered to belong to the same direct chain of transmission if they had less than 20 SNPs difference as in previous studies for *S. aureus* (Goyal et al., 2019) and the Enterobacterales order (Hassan et al., 2021).

3. RESULTS

3.1 Prevalence of last-resort resistant bacteria collected from the pig production chain

To understand if the pig production chain could be a reservoir of *Staphylococcus* and Gram-negative bacteria resistant to last-resort antibiotics, we first grew dilutions of twenty-four samples collected over the entire pig production chain (live pigs, meat, surfaces and workers) in selective media. Up to ten isolated colonies were picked from each sample (total, n=282 isolates: 161 putative staphylococci and 121 putative Gram-negative bacteria) and screened for resistance by growth in media supplemented with linezolid, tigecycline and colistin in concentrations above the MIC breakpoints defined for these groups of species (see Materials and Methods).

3.1.1 Prevalence of resistance to last-resort antibiotics in Gram-negative bacteria

This approach allowed isolating a total of 53 isolates (44%; 53/121) that were resistant to at least one last-resort antibiotic all of which were putative Gram-negative bacteria. These bacteria (n=53) were further characterized at the molecular level (see sections below).

3.1.1.1 Species identification of Gram-negative bacteria

To identify at the species level the putative Gram-negative bacteria that were resistant to last-resort antibiotics (n=53 isolates), an internal fragment of the 16S rRNA gene was sequenced and compared with web-based databases. This methodology allowed identifying at species level 45 Gram-negative isolates. Overall, eight different species were identified (see Figure 3.1) that belonged to *Enterobacteriaceae* (n=38), *Morganellaceae* (n=4) and *Moraxellaceae* (n=3) families. *K. pneumoniae* was the most predominant species (n=13/45 isolates, 29%), followed by *E. coli* (n=11/45, 24%), *E.hormaechei* (n=8/45, 18%), *P. mirabilis* (n=4/45, 9%), *E. kobei* (n=3/45, 7%), *A. pittii* (n=3/45, 7%), *E. ludwigii* (n=2/45, 4%) and *E. asburiae* (n=1/45, 2%).

Eight of the remaining twelve isolates were Gram-positive bacteria, including *Enterococcus casseliflavus* (n=4), *E.faecalis* (n=2), *S. borealis* (n=1) *and S. simulans* (n=1). Moreover, for four isolates, the Sanger sequencing data obtained had bad quality and the species could not be determined. Since our objective was to select Gram-negative bacteria with acquired resistance mechanisms, *P. mirabilis* isolates (n=4), which are intrinsic resistant to both tigecycline and colistin (EUCAST, 2020c), and Gram-positive isolates (n=8) were not further analysed.

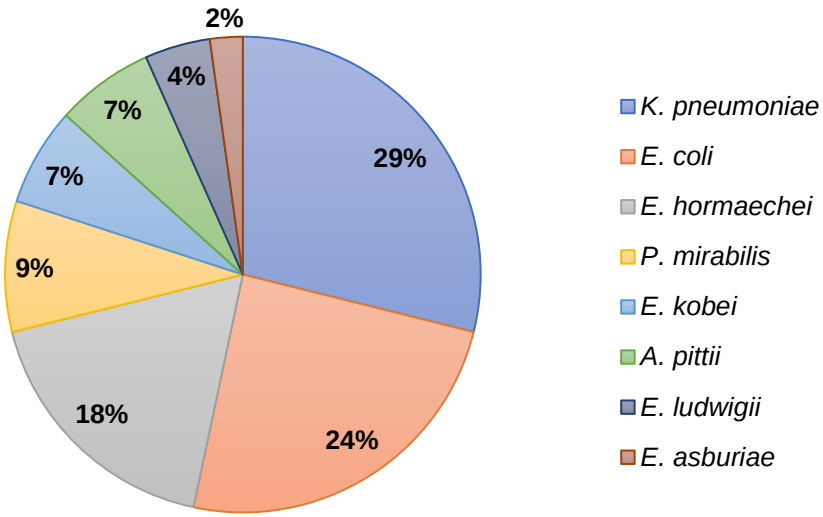


Figure 3.1. Distribution of Gram-negative bacterial species (n=45) identified in the pig production chain by 16S rRNA gene sequencing.

3.1.1.2 Susceptibility to last-resort antibiotics of Gram-negative bacteria

Overall, among the Gram-negative isolates identified at the species level, almost all of them were resistant to tigecycline (n=41/45, 91%) and 36% (n=16/45) were resistant to colistin (MIC= 8 to >16 mg/L), of which 9% (n=4/45) correspond to *P. mirabilis* isolates intrinsically resistant to both tigecycline and colistin (EUCAST, 2020c). The highest colistin-resistant level was found in *E. asburiae* (n=1) and *E. kobei* (n=1) (MIC >16 mg/L). Regarding tigecycline, the highest resistance levels were found in *E. coli* (n=2), *K. pneumoniae* (n=6) and *E. hormaechei* (n=2) (MIC >1.5 mg/L). In addition, it was found that as many as 27% (n=12/45) of the isolates showed resistance to both tigecycline and colistin (see Figure 3.2).

All three *A. pittii* isolates were obtained from operators' hands' samples (see Annexe 2) and have grown at a tigecycline concentration of 1.125 mg/L. However, no tigecycline breakpoints are currently defined for *Acinetobacter* spp. (EUCAST, 2020a), even in the more recent EUCAST guidelines (EUCAST, 2022a). Even so, according to the EUCAST program of antimicrobial wild type distributions for *A. pittii* isolates it was established a tentative ECOFF (TECOFF) of 0.5 mg/L (available on https://mic.eucast.org/search/ accessed on 18th March 2022) (EUCAST, 2022b). Using the TECOFF as the MIC breakpoint, our isolates were considered tigecycline-resistant.

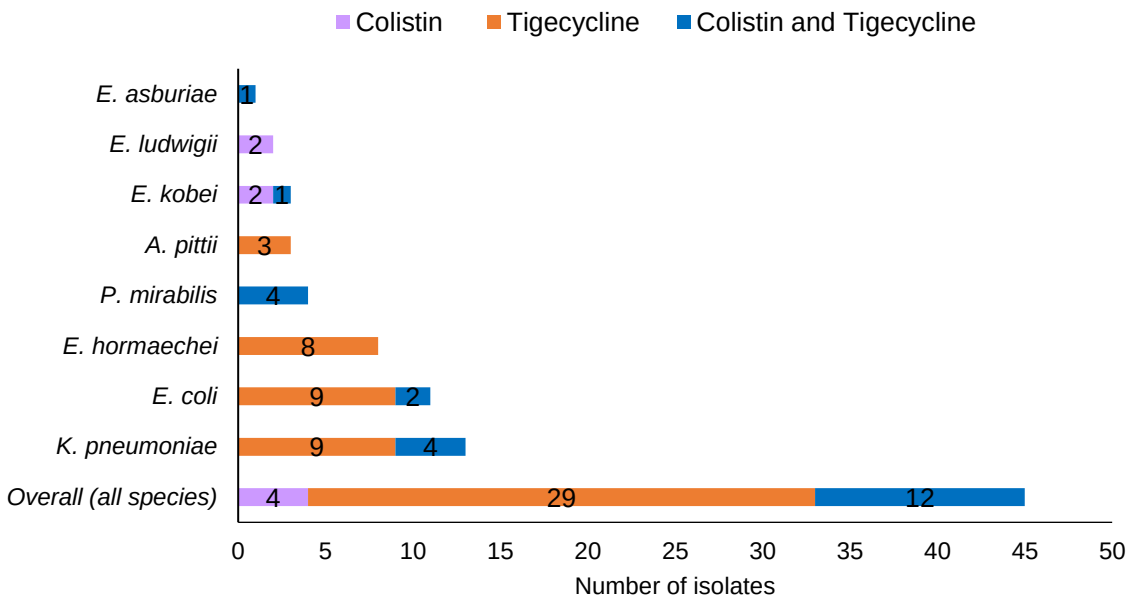


Figure 3.2**.** Prevalence of phenotypic resistance to tigecycline and colistin for Gram-negative bacteria. Numbers within bars correspond to the partial numbers of isolates.

The highest prevalence of resistance to tigecycline was found in workers' hands (37%, n=15/41) and live pigs (34%, n=14/41), followed by meat (17%, n=7/41) and equipment (12%, n=5/41) (see Figure 3.3 and Annexe 2). Live pigs' samples included both pig ear (n=11 tigecycline-resistant isolates) and rectum (n=3 tigecycline-resistant isolates). A similar distribution of resistant isolates was observed for colistin (Figure 3.3 and Annexe 2), wherein isolates from the hands of workers (38%, n=6/16) and live pigs (25%, n=4/16) showed the highest resistance rates. All colistin-resistant isolates from live pigs were obtained from pig rectum samples (100%, n=4/4).

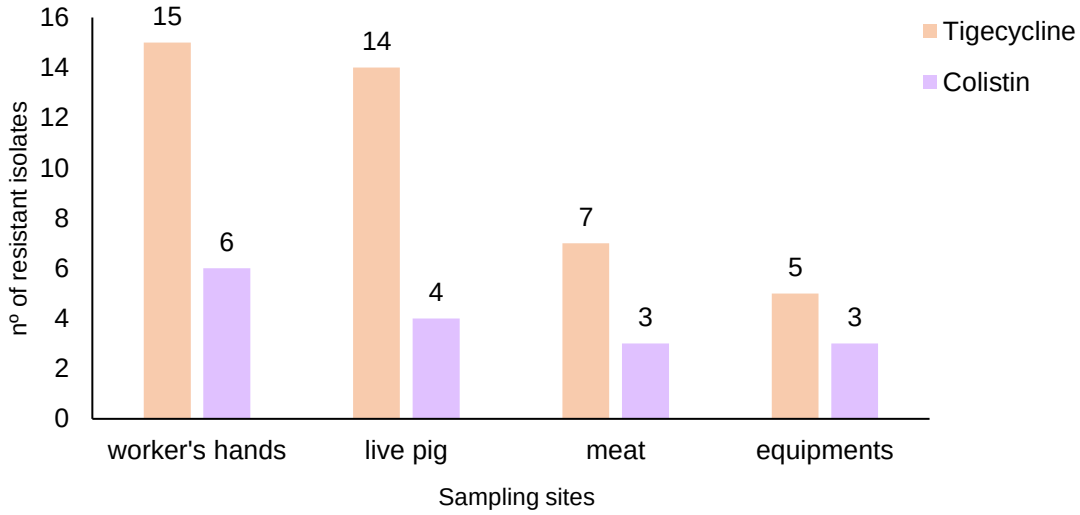


Figure 3.3**.** Distribution of the tigecycline and colistin-resistant isolates (n=45) among different sampling sites.

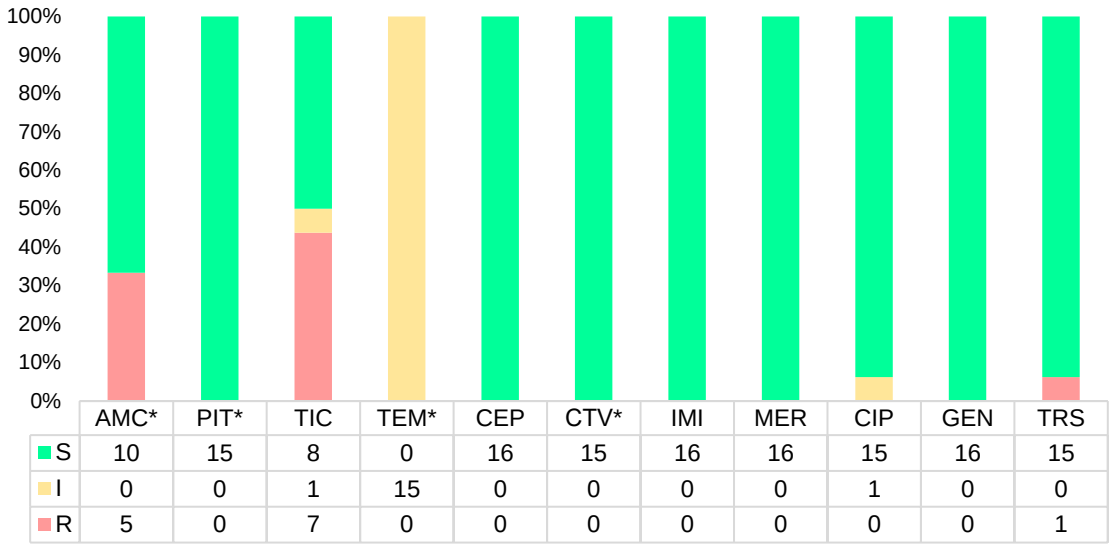
It was also observed that in a single sample, several isolates were found to be resistant to last-resort antibiotics. In particular, up to seven colonies from the same sample were found to be tigecycline resistant in a single sample from dirty operator hands (sample ZP27) (see Annexe 2). Those isolates correspond to *K. pneumonia* (n=3), *E. coli* (n=2) and *P. mirabilis* (n=2).

3.1.1.3 Susceptibility patterns to non-last resort antibiotics of Gram-negative bacteria

To characterize the antimicrobial resistance profile of the Gram-negative bacteria resistant to last-resort antibiotics isolated in this study (n=45), we additionally tested susceptibility to a panel of non-last resort antibiotics in a representative collection. Fifteen *Enterobacteriaceae* (*K. pneumoniae* (n=6), *E. coli* (n=4), *E. kobei* (n=3) and *E. hormaechei* (n=2)) were tested for eleven different antimicrobials and *A. pittii* (n=1) was tested against a panel of eight antibiotics (see Materials and Methods). *Proteus* spp. isolates were not included in this analysis.

The antibiotic susceptibility testing revealed that the *Enterobacteriaceae* isolates resistant to tigecycline and/or colistin were additionally resistant to ticarcillin (47%, n=7/15), amoxicillin/clavulanic acid (33%, 5/15) and trimethoprim-sulfamethoxazole (7%, 1/15) (see Figure 3.4). Moreover, all the fifteen *Enterobacteriaceae* isolates showed intermediate resistance to temocillin (100%, n=15/15) and one *E. coli* isolate from meat (ZP16 P2) (Annexe 2) showed intermediate resistance to ticarcillin (7%, n=1/15, each) (see Figure 3.4). Furthermore, none of the tested isolates was resistant to carbapenems (imipenem, meropenem), piperacillin-tazobactam, cefepime, ceftazidime-avibactam, and gentamicin. The *A. pittii* isolate was intermediate to ciprofloxacin, being susceptible to all of the remaining tested antibiotics. According to the MDR definition used, only one *Enterobacteriaceae* isolate - *E. asburiae* from meat (ZP16 B1.1) (Annexe 2), can be considered MDR, being resistant to three of the defined categories: penicillins + β-lactamase inhibitor (amoxicillin-clavulanic acid), polymixin (colistin) and glycylcycline (tigecycline) (Magiorakos et al., 2012).

Furthermore, the remaining four *Enterobacter* spp. isolates, although not considered MDR, were all amoxicillin-clavulanic acid-resistant and resistant to one of the two last-resort antibiotics. *E. kobei* isolates (n=2) were amoxicillin-clavulanic acid and colistin-resistant and *E. hormaechei* (n=2) were amoxicillin-clavulanic acid and tigecycline-resistant (see Annexe 2. It was also found that all *K. pneumoniae* isolates (n=6) were non-susceptible to tigecycline, temocillin and ticarcillin (Annexe 2). In addition, one *E. coli* isolate from pig rectum (ZA2 W3) was non-susceptible to four antibiotics including tigecycline, temocillin, ticarcillin and trimethoprim-sulfamethoxazole (Annexe 2). Since antipseudomonal penicillins without β-lactamase inhibitors (e.g. ticarcillin) is not a defined antimicrobial category for MDR according to Magiorakos *et al.* 2012, this isolate was not considered MDR.



	AMC*	PIT*	TIC	TEM*	CEP	CTV*	IMI	MER	CIP	GEN	TRS
S	10	15	8	0	16	15	16	16	15	16	15
I	0	0	1	15	0	0	0	0	1	0	0
R	5	0	7	0	0	0	0	0	0	0	1

Figure 3.4**.** Antibiotic susceptibility to a panel of 11 antibiotics of Gram-negative isolates from the pig production chain (n=16). S: susceptible; R: resistant; I: susceptible with increased exposure; AMC: amoxicillin-clavulanic acid; PIT: piperacillin-tazobactam; TIC: ticarcillin; TEM: temocillin; CEP: cefepime; CTV: ceftazidime-avibactam; IMI: imipenem; MER: meropenem; CIP: ciprofloxacin; GEN: gentamicin; TRS: trimethoprim-sulfamethoxazole. Asterisk indicates that the antimicrobial susceptibility testing was not performed on these four antibiotics for *A. pittii.*

3.1.1.4 Distribution of resistance determinants and SNP-based phylogenic analysis of Gram-negative bacteria

To characterize the content in antibiotic resistance genes of the Gram-negative bacteria resistant to last-resort antibiotics, we selected a total of fifteen Gram-negative (n=14 *Enterobacteriaceae* and n=1 *A. pittii*) bacterial isolates for WGS that were previously selected for antimicrobial susceptibility testing (Figure 3.4). WGS was not performed for one *K. pneumoniae* isolate (ZP18 B5) showing tigecycline and ticarcillin resistance profile, as it did not meet the necessary DNA quantity and purity required for the sequencing. To do the mass screening of the isolates for the presence of antibiotic-resistant genes, Abricate and ResFinder/CARD databases were used for antimicrobial resistance search using the identity of 70% and a gene coverage of 90%.

All isolates (100%, n=15/15), including *A. pittii*, carried *form*(A) gene (see Figure 3.5), which, according to the CGE (available at https://cge.cbs.dtu.dk/services/ResFinder/gene_overview.php accessed on 20th March 2022), is involved in disinfectants resistance. Furthermore, the *mdf*(A) gene (which codifies for a multidrug transporter) (Edgar & Bibi, 1997) was carried by all *Enterobacteriaceae* (100%, n=14/14). The genes *sitABC*, involved in the metal transport and resistance to hydrogen peroxide (Sabri et al., 2008), and *fosA,* conferring resistance to fosfomycin (R. Ito et al., 2017), were detected each in 71% (n=10/14) of the *Enterobacteriaceae* isolates. Tetracycline resistance genes (*tet*(A), *tet*(C) and *tet*(34),) (Roberts, 2005) were detected in four *Enterobacteriaceae* isolates (29%, n=4/14) and the *tet*(39) gene in the *A. pittii* isolate. The *oqxA* and *oqxB* genes, previously reported to be associated with tigecycline resistance (J. Li et al., 2019), were detected in half of the *Enterobacteriaceae* (50%, n=7/14 each).

Trimethoprim resistant dihydrofolate reductase *dfrA*1 and *dfrA*5 genes (Alcock et al., 2019; Ambrose & Hall, 2021) were both detected in an *E. coli* isolate (7%, n=1/14) and chloramphenicol

resistance gene (*floR*) (Doublet et al., 2005) was detected in a single *K. pneumoniae* isolate (7%, n=1/14). Several *blaSHV* variants were detected in 36 % of the *Enterobacteriaceae* (n=5/14), including an Extended-Spectrum Beta-lactamase (ESBL) *bla*SHV-42 (Mulvey et al., 2004; Alcock et al., 2019) carried by one *K.pneumoniae* isolate (7%, n=1/14). Another ESBL, *bla*TEM-1B (Salverda et al., 2010; Alcock et al., 2019), was also detected (7%, n=1/14). Furthermore, AmpC β-lactamases were frequently found, including several *bla*ACT variants (36%; n=5/14), the *bla*CFE-1 gene (21%; n=3/14) (Nakano et al., 2004) and *bla*CMY-155 (Alcock et al., 2019) (7%; n=1/14). The *A. pittii* isolate carried two genes codifying for β-lactamases: *bla*ADC-25, a cephalosporinase-encoding gene (Zong et al., 2008), and *bla*OXA-421 (Kamolvit et al., 2015), which were not present in the *Enterobacteriaceae.*

Tigecycline resistance was associated with the presence of *oqxA*/*oqxB* in *E. asburiae* (100%, n=1/1), *E. kobei* (100%, n=2/2) and *K. pneumoniae* (80%, n=4/5). However, we could not identify any genes associated with colistin resistance in the isolates analysed in this study by WGS (n=4). Notably, the tigecycline-resistant isolates co-carried *oqxA/oqxB* genes together with other antibiotic resistance determinants, such as *fos*(A), *tet*(A), *tet*(C), *tet*(34), *floR*, *blaSHV*; *blaTEM, blaACT,* besides *form*(A), *sitABC* and *mdf*(A). Similarly, analysis of the WGS data from colistin-resistant isolates revealed the presence of *fos*A, *tet*(A), *tet*(C), *tet*(34), *bla*SHV, *bla*ACT*, oqxA, oqxB, sitABC, form*(A) *and mdf*(A) genes. Furthermore, all *Enterobacteriaceae isolates* (100%, n=14/14) also harboured both *acrA* and *acrB*, which overexpression have been previously associated with tigecycline resistance (Zhong et al., 2014). The single *A. pittii* sequenced carried *adeF, adeG* and *adeH* genes encoding for the AdeFGH pump, which overexpression has also been associated with diminished susceptibility to tigecycline in *A. baumannii* (Coyne et al., 2010).

Phenotypic resistance was in accordance with the genotypic resistance in all isolates resistant to beta-lactams (100%, n=15/15). However, no correlation was found between the presence of the resistance genes and the resistance phenotype in seven cases, namely for isolates resistant to ciprofloxacin (n=1), tigecycline (n=2) and colistin (n=4). In these cases, we could not identify any genetic determinants that could explain the phenotypic resistance observed.

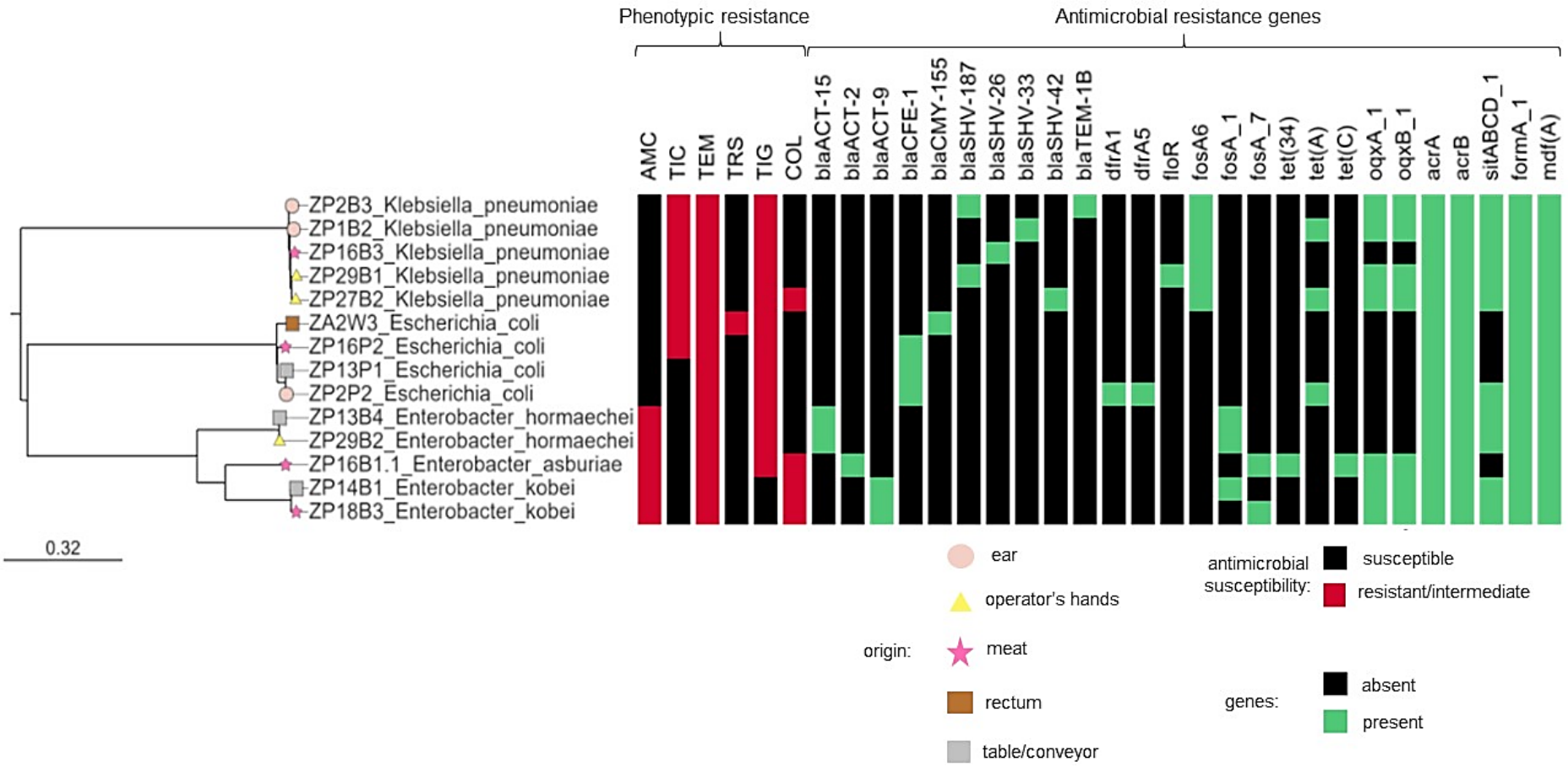


Figure 3.5- Maximum likelihood tree of *Enterobacteriaceae* isolates (n=14) constructed from the comparison of core SNPs identified through CSIPhylogeny v1.4 analysis and distribution of antimicrobial resistance phenotypes and genes SNPs tree visualization was performed with Microreact server v. 197.0.0. Red and Green boxes indicate the phenotypic resistance to antibiotics and the presence of acquired antimicrobial resistance genes, respectively. Black boxes indicate the susceptible phenotype and the absence of genetic determinants. AMC: amoxicillin-clavulanic acid; TIC: ticarcillin; TEM: temocillin; TRS: trimethoprim-sulfamethoxazole; TIG: tigecycline; COL: colistin

3.1.1.5 Assessment of cross-transmission of Gram-negative bacteria in the pig production chain

To understand if Gram-negative could be being transmitted along the pig production chain, previously sequenced isolates were compared by a core SNP analysis using the isolates sequencing data and the reference strains *E. coli* strain AH04, *K. pneumoniae* B31 (accession number CP035929), and *E. kobei* ENHKU01 (accession number CP003737.1) and *E. hormaechei* CAV1311 (accession number GCA_001022015) available at the NCBI database (NCBI, 1988). The analysis was only performed for thirteen *Enterobacteriaceae*, because *E. asburiae* and *A. pittii* were single isolates, thus it was not possible to verify the occurrence of transmission events. The SNP analysis of the *Enterobacteriaceae* (n=13) revealed that all strains of each species (*K. pneumonia, E. coli and E. kobei and E. hormaechei*) were distantly related (884-66355 SNPs), suggesting the absence of transmission between different sampling sites (see Figure 3.6).

To further understand if the isolates collected from the food production chain belonged to previously identified clonal types, we identified the MLST type by using pubMLST and Pathogenwatch platforms (Jolley et al., 2018; CGPS, 2020). Results obtained showed that *K. pneumoniae* and *E. coli* isolates all had different and new STs, that contained three or more new gene alleles in their allelic profile (Table 3.1), suggesting they are unrelated to any clonal type previously identified in humans. In the remaining species, it was not possible to determine the MLST, since an MLST scheme was not available.

Isolate transmission was not detected in the analysed collection (see Figure 3.6). However, by observing the distribution of antibiotic resistance genes in the phylogenetic tree constructed based on the SNPs analysis, it is possible to infer that all the pairs of isolates (n=13 isolates) belonging to the four different species and recovered from different sampling sites shared *formA* and *mdf*(A) genes (Figure 3.5). Furthermore, nine isolates shared the *sitABCD* gene and seven isolates the *oqxA* and *oqxB* genes. These results suggest that the pig production chain might be an environment in which antibiotic resistance genes are frequently transmitted between different strains and species.

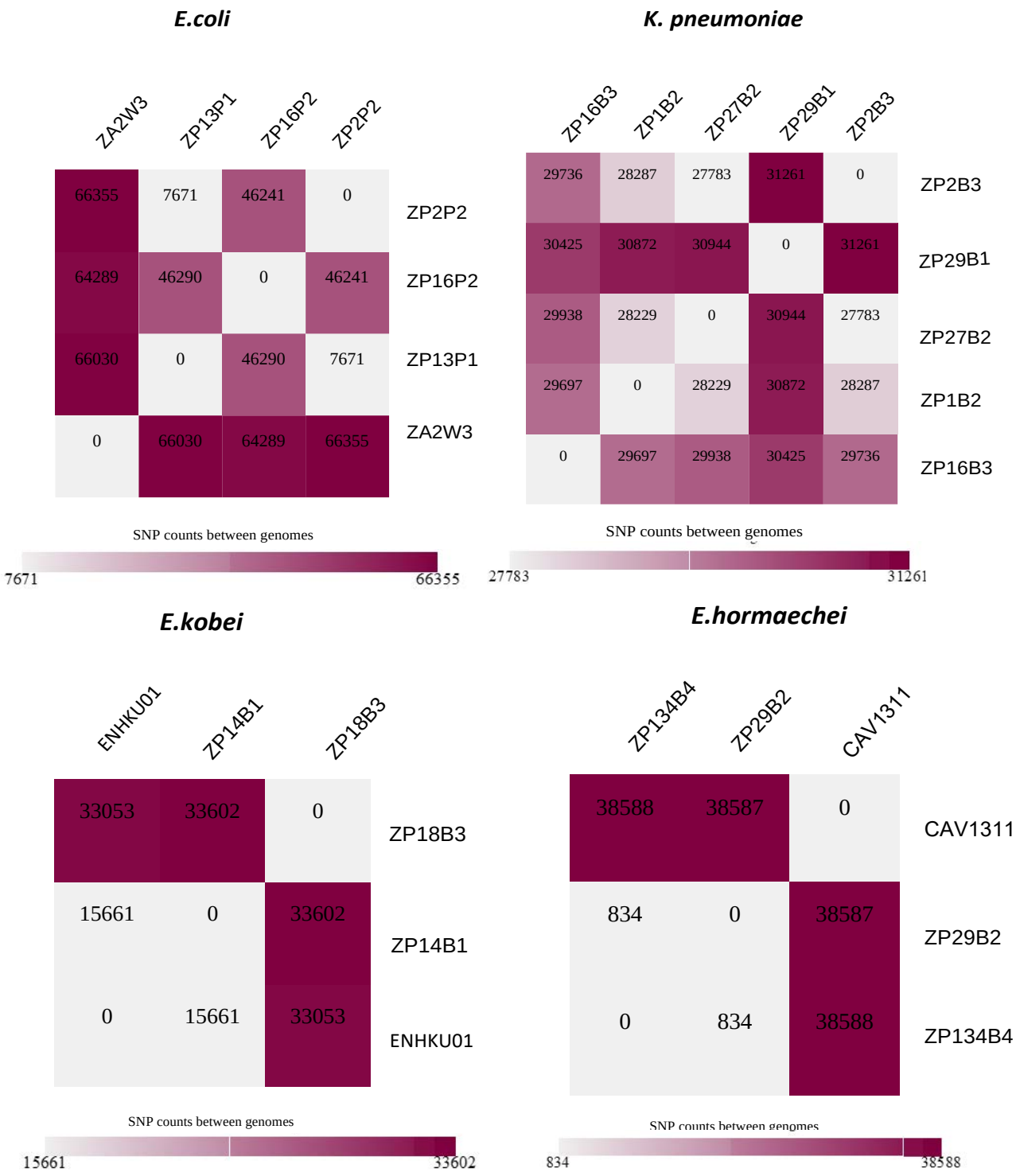


Figure 3.6. SNP matrix for *E. coli, K. pneumonia, E. kobei and E. hormoechei* isolates after core SNPs analysis using CSI phylogeny software. *E. coli* strain AH04, *K. pneumonia* B31, and *E. kobei* ENHKU01 and *E. hormaechei* CAV1311 were used as reference strains for mapping strains of each species.

Table 3.1- MLST and SNP results of Gram-negative bacteria.

Isolate ID	Source	Species ID	PubMLST Database								SNPs
			Locus [allele]							ST	
ZP1 B2	Pig ear	*K. pneumoniae*	*gapA* [new?]	*infB* [1]	*mdh* [new?]	*pgi* [144]	*phoE* [new?]	*rpoB* [new?]	*tonB* [19]	Not Attributed	28229-3126
ZP2 B3	Pig ear	*K. pneumoniae*	*gapA* [new?]	*infB* [1]	*mdh* [new?]	*pgi* [1]	*phoE* [3]	*rpoB* [new?]	*tonB* [31]	Not Attributed	
ZP29 B1	Clean hands	*K. pneumoniae*	*gapA* [214]	*infB* [1]	*mdh* [new?]	*pgi* [1]	*phoE* [387]	*rpoB* [new?]	*tonB* [12]	Not Attributed	
ZP27 B2	Dirty hands	*K. pneumoniae*	*gapA* [new?]	*infB* [1]	*mdh* [new?]	*pgi* [1]	*phoE* [new?]	*rpoB* [new?]	*tonB* [18]	Not Attributed	
ZP16 B3	Meat	*K. pneumoniae*	*gapA* [new?]	*infB* [3]	*mdh* [new?]	*pgi* [2]	*phoE* [6]	*rpoB* [new?]	*tonB* [4]	Not Attributed	
ZP2 P2	Pig ear	*E. coli*	*adk* [new?]	*fumC* [41]	*gyrB* [15]	*icd* [18]	*mdh* [new?]	*purA* [7]	*recA* [6]	Not Attributed	46241-66355
ZP16 P2	Meat	*E. coli*	*adk* [new?]	*fumC* [11]	*gyrB* [4]	*icd* [8]	*mdh* [923]	*purA* [18]	*recA* [2]	Not Attributed	
ZP13 P1	Clean conveyor	*E. coli*	*adk* [new?]	*fumC* [41]	*gyrB* [15]	*icd* [18]	*mdh* [new?]	*purA* [7]	*recA* [6]	Not Attributed	
ZA2 W3	Pig rectum	*E. coli*	*adk* [new?]	*fumC* [172]	*gyrB* [new?]	*icd* [158]	*mdh* [new?]	*purA* [new?]	*recA* [342]	Not Attributed	
ZP13 B4	Clean conveyor	*E. hormaechei*	No supported species							Not Attributed	884-38588
ZP29 B2	Clean hands	*E. hormaechei*									
ZP14 B1	Dirty cutting tables	*E. kobei*	Not supported species							Not Attributed	15661-33602
ZP18 B3	Meat	*E. kobei*									

3.1.1.6 Prevalence of resistance to last-resort antibiotics in *Staphylococcus* spp.

No resistance to last-resort antibiotics was found among the 161 putative staphylococci screened. However, to understand if staphylococci could constitute reservoirs of antibiotic-resistant genes, we further characterized at the molecular level a representative collection of the staphylococci. This collection was selected to include the highest diversity in colony morphology and isolates from all the steps in the swine processing chain (n=25 /161) (see sections below).

3.1.1.7 Species identification *of Staphylococcus* spp.

Staphylococcus spp. were identified by sequencing an internal fragment of the *tuf* gene and by comparison to web-based databases. This approach showed that the collection analysed was composed of six different species, including *S.hyicus* (n=7), *S. haemolyticus* (n=2), *S. rostri* (n=1), *S. simulans* (n=1), *S. pseudointermedius* (n=1) and *S. aureus* (n=1) (see Figure 3.7). In addition, non-staphylococci species were also identified, including *Vagococcus lutrae* (n=6) *M.caseolyticus* (n=5) and *M. sciuri* (n=1). The non-staphylococcal isolates were excluded from the subsequent analysis.

Most of the isolates identified as *Staphylococcus* were isolated from live pigs (46% n=6/13) half of them from pig ears (n=3) and the other half from pig rectum (n=3). The remaining isolates were obtained from equipment (23%, n=3/13), hands of workers (23%, n=3/13) and meat (8%; n=1/13).

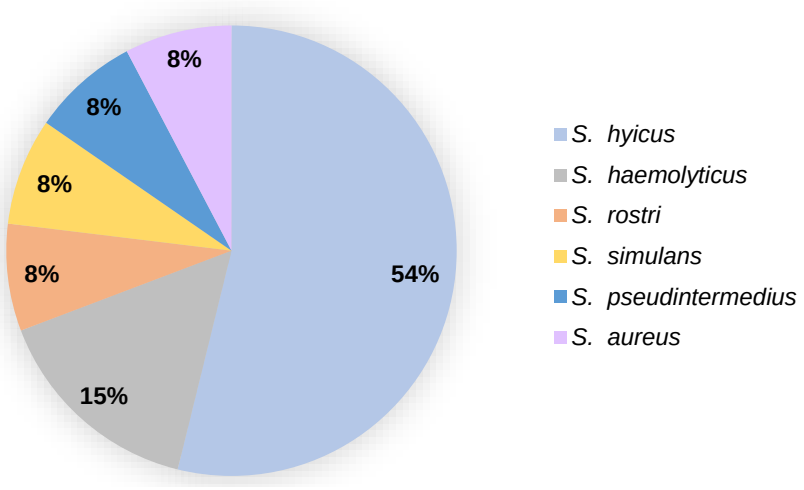


Figure 3.7. Distribution of staphylococcal species identified (n=13) in the pig production chain by *tuf* gene sequencing.

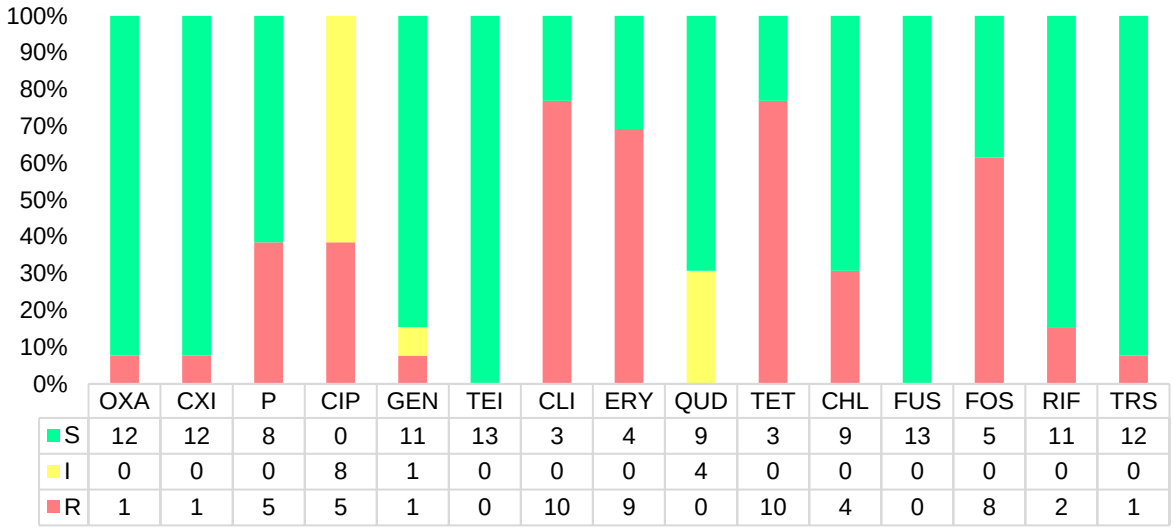
3.1.1.8 Susceptibility patterns of staphylococci to non-last resort antibiotics

To understand if staphylococci (n=13) from the pig production chain could constitute reservoirs of antibiotic resistance, it was determined the susceptibility to a panel of fifteen non-last resort antibiotics by disk diffusion. It was additionally determined the MIC for quinupristin-dalfopristin, oxacillin and cefoxitin and tested by PCR the presence of the beta-lactam resistance determinants *mecA* and *mecC* genes. Except for *S. rostri*, for all the remaining species (*S. hyicus, S. haemolyticus* and *S. simulans, S. aureus* and *S. pseudintermedius*) there is a EUCAST clinical breakpoint defined (EUCAST, 2020a). Since it is a species belonging to the CoNS group (Riesen & Perreten, 2010), the criteria used for *S. rostri* was the one considered for the other CoNS.

All thirteen *Staphylococcus* spp. isolates were resistant to at least one of the non-last resort antibiotics tested (Annexe 3). The great majority of the isolates showed high rates of resistance to tetracycline (n=10/13 isolates, 77%), clindamycin (n=10/13, 77%), erythromycin (n=9/13, 69%) and fosfomycin (n=8/13, 62%), followed by penicillin (n=5/13, 38%), chloramphenicol (n=4/13, 31%) and ciprofloxacin (n=5/13, 38%) (see Figure 3.8). Low frequencies of resistance were found for rifampin (n=2/13, 15%) and oxacillin, cefoxitin, gentamycin and trimethoprim-sulfamethoxazole (n=1/13, 8% each). Although there were no isolates resistant to quinupristin-dalfopristin according to the disk diffusion method, 31% (n=4/13) were considered intermediate (MIC=0.5-3 mg/L). More than half of the isolates (62%, n=8/13) were also intermediate to ciprofloxacin, according to the 2020 EUCAST criteria (EUCAST, 2020a). None of the isolates was resistant to teicoplanin or fusidic acid.

Following the antimicrobial categories and agents used to define MDR in *S. aureus* (Magiorakos et al., 2012), as many as eleven isolates (85%, n=11/13) were MDR. These include *S. hyicus* (n=5), *S. haemolyticus* (n=2), *S. pseudintermedius* (n=1), *S. aureus* (n=1), *S. simulans* (n=1) and *S. rostri* (n=1). *Staphylococcus* spp. MDR were recovered from all sampling sites: live pig (pig ear(n=3) and pig rectum (n=2)), workers' hands (n=3), equipment (n=2) and meat (n=1). The non-MDR isolates correspond to

two *S. hyicus* isolates recovered from pig rectum (ZA1 C2) and equipment (ZP14 Cd), both intermediate to ciprofloxacin and resistant to fosfomycin (see Annexe 3).



	OXA	CXI	P	CIP	GEN	TEI	CLI	ERY	QUD	TET	CHL	FUS	FOS	RIF	TRS
S	12	12	8	0	11	13	3	4	9	3	9	13	5	11	12
I	0	0	0	8	1	0	0	0	4	0	0	0	0	0	0
R	1	1	5	5	1	0	10	9	0	10	4	0	8	2	1

Figure 3.8. Antibiotic susceptibility pattern of *Staphylococcus spp*. (n=13) isolates from the pig production chain. S: susceptible; R: resistant; I: intermediate; OXA: oxacillin; CXI: cefoxitin; P: penicillin; CIP: ciprofloxacin; GEN: gentamycin; TEI: teicoplanin; CLI: clindamycin; ERY: erythromycin; QUD: quinupristin-dalfopristin; TET: tetracycline; CHL: chloramphenicol; FUS: fusidic-acid; FOS: fosfomycin; RIF: rifampicin; TRS: trimethoprim-sulfamethoxazole (EUCAST, 2020a).

To confirm the intermediate phenotype to quinupristin-dalfopristin, obtained by the disk diffusion method, was determined the MIC by Etest (n=4) (see Table 3.2). The Etest results confirmed the intermediate phenotype in two out of the four isolates (n=2, MIC=2 mg/L) and one was classified as resistant (n=1, MIC=3 mg/L). The remaining isolate was susceptible (n=1, MIC=0.5 mg/L). Overall, considering the MIC results, one *S. hyicus* isolate was considered resistant to quinupristin/dalfopristin and two *S. hyicus* were intermediate resistance profiles to this antibiotic. The only susceptible isolates correspond to *S. aureus*.

Table 3.2 - Comparison of susceptibility to quinupristin/dalfopristin for *Staphylococcus* spp. (n=4) showing intermediate resistance profile by disk diffusion method

		QUD	
Isolate	**Species (*tuf*)**	**disk diffusion**	**MIC (mg/L)**
ZPA12 C9	*S. hyicus*	I	2 (I)
ZA1 C6	*S. hyicus*	I	2 (I)
ZP14 C5	*S. hyicus*	I	3 (R)
ZAP1 C10	*S. aureus*	I	0.5 (S)

R: resistant; I: intermediate; S: susceptible; QUD: quinupristin/dalfopristin

To confirm oxacillin/cefoxitin resistance, all isolates were further tested for the presence of *mecA* and *mecC* by PCR and the MIC to oxacillin was determined by E-test (Table 3.3). As expected, the oxacillin/cefoxitin resistant *S. aureus* isolate, as determined by the disk diffusion test, harboured the *mecA* gene. This *S. aureus* isolate was classified as susceptible according to the MIC for oxacillin (MIC= 0.75 mg/L) but was resistant to cefoxitin (MIC=8 mg/L). Since cefoxitin MIC is a poorer predictor of

methicillin resistance for species other than *S. aureus, S. lugdunensis* and *S. saprophyticus* (EUCAST, 2020a), no breakpoint values are defined for the remaining species. Therefore, It was only possible to interpret the cefoxitin MIC for the *S. aureus* isolate. Furthermore, *M. siucri*, although is a former member of the *Staphylococcus* genus (Madhaiyan et al., 2020), EUCAST still considers this species as belonging to *Staphylococcus* genus within CoNS group (EUCAST, 2020a, 2022a). The *M. siucri* isolate had an oxacillin MIC of 1.5 mg/L, being considered resistant and also harboured the *mecA* gene.

For six additional isolates, discrepant results were obtained for disk diffusion and the Etest, when the EUCAST criteria were applied (EUCAST, 2020a). In particular, the two *S. haemolyticus* isolates, previously characterized as susceptible to oxacillin/cefoxitin by the disk diffusion method, were considered resistant to oxacillin by the Etest (MIC=0.5 mg/L and MIC=0.38 mg/L) according to the EUCAST breakpoints. Additionally, there were four *S. hyicus* isolates, that were classified as susceptible by the disk diffusion method, that also showed resistance to oxacillin by Etest (MIC=0.38 mg/L). However, none of this *S. haemolyticus* and *S. hyicus* oxacillin resistant isolates harboured *mec*A or *mec*C.

Table 3.3. - Comparison of susceptibility to beta-lactams as determined by disk diffusion and Etest for 13 *Staphylococcus* spp. collected in the pig production chain.

				Disk diffusion		MIC (mg/L)	
Isolate	**Source**	**Species *(tuf)***	***mecA***	**OXA**	**CXI**	**OXA**	**CXI**
ZAP1 C10	Pig ear	*S. aureus*	+	R	R	0.75 (S)	8 (R)
ZP11 C7	Operators' hands	*S. haemolyticus*	-	S	S	0.5 (R)	ND
ZP11 C8	Operators' hands	*S. haemolyticus*	-	S	S	0.38 (R)	ND
ZP8 C5	Pig ear	*S. rostri*	-	S	S	0.125 (S)	ND
ZPA12 C9	Operator's hands	*S. hyicus*	-	S	S	0.38 (R)	ND
ZA1 C2	Pig rectum	*S. hyicus*	-	S	S	0.38 (R)	ND
ZA1 C6	Pig rectum	*S. hyicus*	-	S	S	0.19 (S)	ND
ZP14 C5	Equipment	*S. hyicus*	-	S	S	0.38 (R)	ND
ZP15 C5	Equipment	*S. hyicus*	-	S	S	0.9 (S)	ND
ZP1 C7	Pig ear	*S. hyicus*	-	S	S	0.125 (S)	ND
ZP14 Cd	Equipment	*S. hyicus*	-	S	S	0,38 (R)	ND
ZA2 C10	Pig rectum	*S. simulans*	-	S	S	0.19 (S)	ND
ZP17 C3	Meat	*S. pseudointermedius*	-	S	S	0.25 (S)	ND

+ presence; − absence; ND: no interpretation criteria of resistance are defined for these *Staphylococcus* spp. (EUCAST, 2020a); R: resistant; I: intermediate; S: susceptible; OXA: oxacillin; CXI: cefoxitin.

3.1.1.9 Content in antibiotic-resistant genes of *Staphylococcus* spp.

To understand which was the genetic content of the staphylococci in antibiotic resistance determinants, a group of nine isolates was selected for WGS by Nanopore technology. The obtained sequencing reads were assembled and screened for the presence of resistance genes present in ResFinder and CARD databases using the mass screening software Abricate.

The analysis of WGS data showed that staphylococcal isolates carried genes conferring resistance to at least seven antibiotic classes (Figure 3.9). In particular, it was found genes conferring resistance to beta-lactams (*blaZ*, 44%, n=4/9) (Olsen et al., 2006), macrolide-lincosamide-streptogramins determinants including several *erm* genes: *erm*(T) (33%, n=3/9), *erm*(B) (22%, n=2/9), *erm*(C) (22%, n=2/9), *erm*(A) (11%, n=1/9) and *erm*(45) (11%, n=1/9) (Dzyubak & Yap, 2016), lincosamides (*lnu*(B), 44%, n=4/9) (Bozdogan et al., 1999), pleuromutilin-lincosamide-streptogramin A (*lsa*(E) *44%,* n=4/9*; vga*(A)LC 22%, n=2/9; *vga*(A)V 11%, n=1/9 and *vga*(E) 11%, n=1/9) (Novotna & Janata, 2006; Schwendener & Perreten, 2011; Wendlandt et al., 2013), aminoglycosides (*ant*(9)-*Ia* 56%, n=5/9; *ant*(6)-*Ia* 44*%,* n=4/9; *aadD* 22%, n=2/9; *aph*(3')-*III* 11%, n=1/9; *str* gene 11%, n=1/9) (Ramirez & Tolmasky, 2010; Hauschild *et al*., 2008) and tetracyclines (*tet*(K) 11%, n=1/9; *tet*(L) 44%, n=4/9 and *tet*(M) 22%, n=2/9) (Roberts, 2005). Other antimicrobial resistant genes found, although in a lower proportion of the isolates, include chloramphenicol (*fexA* 33%, n=3/9; *cat*(pC221) 11%, n=1/9) (Trieu-Cuot et al., 1993; Kehrenberg & Schwarz, 2004)), trimethoprim (*dfrK* 11%, n=1/9) and fosfomycin (*fosD* 11%, n=1/9) (Nakaminami et al., 2008; Kadlec et al., 2012).

The isolates carrying the highest number of antibiotic resistance genes belong to the species *S. hyicus* (n=2) and *S. rostri* (n=1), transporting as many as ten different antibiotic resistance genes each (ZP14 C5, ZPA12 C9 and ZP8C5, respectively). In contrast, the species with isolates that had the lowest number of resistant genes were *S. haemolyticus* (n=3 genes, ZP11 C7) and *S. simulans* (n=2 genes, ZA2 C10). No antimicrobial determinants were detected in one of the *S*. *hyicus* isolates (ZP15 C5).

A comparison between the phenotypic antimicrobial susceptibility and genotypic data obtained showed that there were antibiotics for which there was a correlation between the phenotype and genotype. However, there were also cases in which the resistance phenotypes did not correlate with the genotypes identified. All chloramphenicol and penicillin phenotypically resistant isolates carried the respective resistant determinants. However, there was disagreement in some results obtained for tetracycline, macrolides, ciprofloxacin, rifampicin, fosfomycin, and trimethoprim-sulfamethoxazole. Some isolates resistant to tetracycline (n=2, ZA2C10 and ZP14 C5), macrolides (n=2, ZA2 C10 and ZP11C7), ciprofloxacin (all isolates, n=9), rifampicin (n=2, ZP17 C3, ZP15C5), trimethoprim-sulfamethoxazole (n=1, ZP15 C5) and fosfomycin (n=5, ZA2C10, ZP11C7, ZA1C6, ZP15C5 and ZP1C7) contained none of the antibiotic resistance genes screened, suggesting that resistance in these isolates might have emerged by mutations. On the other hand, a fosfomycin resistant isolate harboured the fosD gene (n=1, ZP17C3) and several isolates harboured aminoglycoside resistant determinants (n=4; ZP8 C5, ZA1 C6, ZPA12 C9, ZP1 C7), but did not show a resistant phenotype.

To evaluate the extent of dissemination of resistant genes in the staphylococcal population, a phylogenetic tree based on SNPs was constructed using the S. hyicus strain ATCC 11249 as a reference and investigated the distribution of antibiotic-resistant genes. It was found that some of the resistant determinants were shared by isolates of different species and different sampling sites.

This was the case of the *ant(6)-Ia, lnu(B), lsa(E),* and *tet(L)* genes that were found in both *S. rostri* (n=1) and *S. hyicus* (n=3); and tet(M) that was found in both *S. rostri* and *S. pseudintermedius.* Antibiotic resistance genes ant(6)-Ia_1, ant(6)-Ia_2, lnu(B) and erm(T) were found in isolates collected from four different sampling sites along the chain, namely the ear (ZP8 C5), rectum (ZA1 C6), equipment (ZP14 C5) and worker's hands (ZPA12 C9).

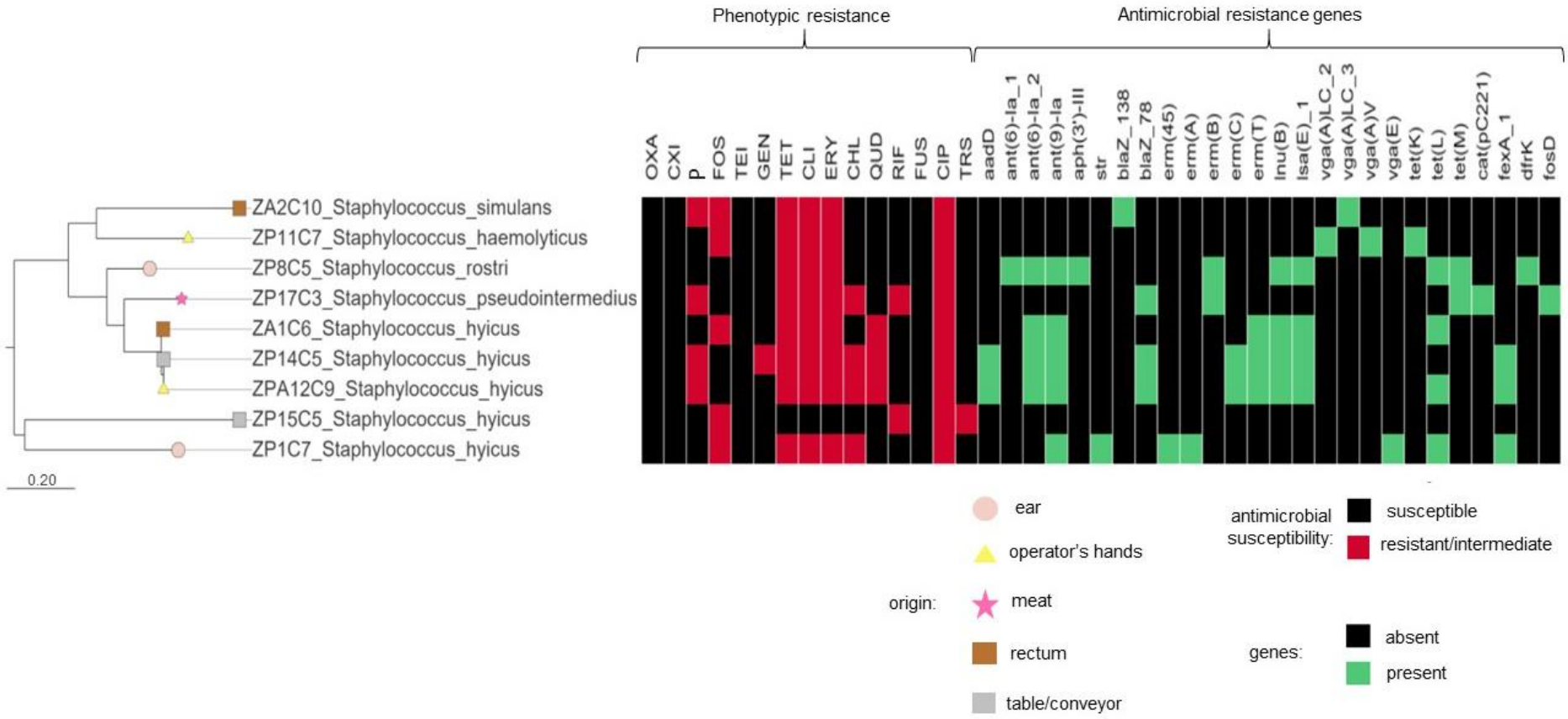


Figure 3.9. Maximum likelihood tree comparing *Staphylococcus spp*. isolates (n=9) based on SNPs identified through CSIPhylogeny v1.4 analysis. SNPs tree visualization was performed with Microreact server v. 197.0.0. Red and green boxes indicate the phenotypic resistance to antibiotic and the presence of acquired antimicrobial resistance genes, respectively. Black boxes indicate the susceptible phenotype and the absence of genetic determinants. OXA: oxacillin; CXI: cefoxitin; P: penicillin; CIP: ciprofloxacin; GEN: gentamycin; TEI: teicoplanin; CLI: clindamycin; ERY: erythromycin; QUD: quinupristin-dalfopristin; TET: tetracycline; CHL: chloramphenicol; FUS: fusidic-acid; FOS: fosfomycin; RIF: rifampicin; TRS: trimethoprim-sulfamethoxazole (EUCAST, 2020a).

3.1.1.10 Assessment of cross-transmission of *Staphylococcus* spp. bacteria in the pig production chain

To understand if staphylococci were being transmitted along the pig production chain, it was compared the whole genome of the nine staphylococcal isolates by SNP analysis, excluding recombination. This analysis identified *S. hyicus* isolates that were highly closely related, with SNP differences ranging from zero to two. It was found a strain pair from the equipment-worker (ZP14 C5/ZPA12C9) had no SNP difference. Furthermore, two pairs of isolates from pig-worker (ZA1C6/ZPA12C9) and pig-equipment (ZA1C6/ZP14C5) had two SNP differences each, suggesting the occurrence of cross-transmission of *S. hyicus* in the slaughterhouse (two events) (Figure 3.10). From the nine strains sequenced, five of them (56%, n=5/9) differed from each other in less than 104 SNPs, suggesting the same strain was circulating between the live pig, the worker and the slaughterhouse equipment.

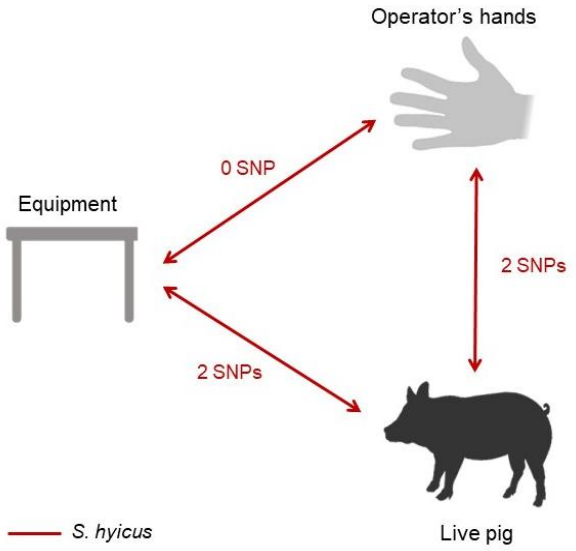


Figure 3.10. A proposed model for the dissemination of *S. hyicus* along the pig production chain. The arrows represent the transmission of *S. hyicus* isolates between live pigs, the operator's hands, and equipment.

These three pairs of isolates, besides differing in a low number of SNPs in their core genome, also appear to be similar at the level of the accessory genome. All of them carried similar antibiotic resistance genes content as defined by WGS data analysis (Figure 3.9). They also shared six different resistance determinants (*ant(6)-Ia*, *ant(9)-Ia*, *erm(T)*, *lnu(B)* and *lsa(E)*). In particular, the pair of the highly related isolates (0 SNP), from worker and equipment, were indistinguishable regarding their antibiotic resistance and they harboured nine similar genes such as *blaZ*, *aadD*, *ant(6)-Ia*, *ant(9)-Ia*, *erm(C)*, *erm(T)*, *lnu(B)*, *lsa(E)* and *fexA.*

It was observed that cross-transmission events, not only occur during the direct handling of animals, but also can persist over time since we could recover close-related *S. hyicus* isolates (0-2

SNPs) from the two different sampling periods (Annexe 1). The two pairs were recovered from pig/worker and pig/equipment, which implies that there was cross-transmission from the pig to the equipment and to the worker, during the slaughterhouse processing.

To understand if staphylococcal strains identified in the swine production chain were related to strains previously found to cause infection in humans, it was additionally determined the MLST for strains belonging to the two species for which an MLST scheme was previously developed (n=1 *S. pseudintermedius*, n=1 *S. haemolyticus*). However, the two strains belonged to new STs, suggesting that they are distantly related to any strain previously described to cause infections in humans and/or animals and for which STs were previously defined.

3.2 Evaluation of diversity in bacterial species in a single sample

To understand the bacterial population and diversity in the samples, we accessed the number of identified species in each of the studied samples (Table 3.4). In some of them, it was only identified one bacterial species. While, in others, multiple bacterial species were detected. In some of the samples collected from clean cutting tables, meat, and clean and dirty workers' hands we only found *S. hyicus* (ZP15), *S. pseudintermedius* (ZP17), *K. pneumoniae* (ZP28) and *S. haemolyticus* (ZP11) species, respectively.

It was found that live pig (ear and rectum) and some equipment samples (dirty cutting tables) were contaminated with both Gram-negative bacteria and *Staphylococcus* spp. In particular, one pig ear sample (ZP1) was simultaneously contaminated with *S. hyicus*, *S. simulans*, *K. pneumoniae* and *P. mirabilis*. The dirty cutting table sample ZP14 was contaminated with *S. hyicus*, *E. kobei* and *E. ludwigii.* Additionally, the same meat, equipment, and workers' samples were contaminated with more than one species of *Enterobacteriaceae* or *Staphylococcus* spp. For example, the meat sample ZP16 was contaminated with four different *Enterobacteriaceae* species (*E. asburiae*, *E. kobei*, *K. pneumoniae*, *E. coli*). Also, the pig ear sample ZAP1 was contaminated with *Staphylococcus* spp., *S. aureus* and *M. sciuri*. The finding of different species within the same samples suggests that different bacterial species are frequently in close contact, which can be an opportunity for genetic exchange.

Table 3.4. Diversity of bacterial species across sample types.

Sample	Sample source	Gram-negative bacterial species	Staphylococcal species
ZA2	Pig rectum	*E. coli*	*S. simulans*
ZAP1	Pig ear	-	*S. aureus, M. sciuri*
ZP1	Pig ear	*K. pneumoniae*, *P. mirabilis*	*S. hyicus*, *S. simulans*
ZP2	Pig ear	*K. pneumonia*e, *E. coli*	-
ZP13	Clean cutting tables	*E. hormaechei*, *E. coli*	-
ZP15	Clean cutting tables	-	*S. hyicus*
ZP14	Dirty cutting tables	*E. kobei*, *E. ludwigii*	*S. hyicus*
ZP16	Meat	*E. asburiae*, *E. kobei*, *K. pneumoniae*, *E. coli*	-
ZP17	Meat	-	*S. pseudintermedius*
ZP18	Meat	*E. kobei, K. pneumoniae*	-
ZP27	Dirty hands	*K. pneumoniae, E. coli, P. mirabilis*	-
ZP28	Clean hands	*K. pneumoniae*	-
ZP11	Clean hands	-	*S. haemolyticus*

4. DISCUSSION & CONCLUSIONS

4.1 Resistance to last-resort antibiotics was observed in different *Enterobacteriaceae* genera from the pig processing chain

In this study, a total of 45 Gram-negative bacteria collected from the swine processing chain and resistant to tigecycline and/or colistin were identified (Figure 3.1). These include *K. pneumoniae*, *E. coli, Enterobacter* spp., *P. mirabilis* and *A. pittii*. Colistin and tigecycline resistance in *Enterobacteriaceae* (mainly in *E. coli* and *K. pneumoniae*) and in some *Acinetobacter* spp. from pig farms, pig carcasses and pork were previously reported (Sun et al., 2020; Pungpian et al., 2021; J. Wang et al., 2020; Carvalheira et al., 2017a). However, as far as we are concerned, tigecycline-resistance *A. pittii* isolates were not previously described in the pork production chain. Even so, the presence of tigecycline-resistant determinants in *A. pittii,* such as the *tet*(X3), was already detected in hospital isolates in Columbia (He et al., 2019).

The bacterial species identified in our study as having resistance to last-resort antibiotics were all previously found to cause infections in humans. In particular, *E. coli* and *K. pneumoniae* are well-known MDR pathogenic species and have been reported to cause a wide variety of infections such as UTIs (Behzadi et al., 2010; Bischoff et al., 2018), intestinal diseases (Afset et al., 2003), pneumonia and bloodstream infections (Weiner et al., 2016). All the *Enterobacter* spp. identified in our study, namely *E. asburiae*, *E. kobei*, *E. ludwigii* and *E. hormaechei,* belonged to the *E. cloacae* complex (Hoffmann & Roggenkamp, 2003), which includes the species within the genus which are most frequently found in nosocomial infections (Hoffmann et al., 2005; Beyrouthy et al., 2018).

Regarding *Acinetobacter*, most nosocomial infections are due to *A. baumannii* (Sieniawski et al., 2013). However, there have been increasing reports of hospital-acquired infections caused by *A. pittii* (Chusri et al., 2014). Several non-susceptible colistin and tigecycline isolates were already reported to cause human infections particular in the hospital environment. A study performed in an Athens (Greece) hospital reported an increased emergence of colistin-resistant *K. pneumoniae* between 2004 and 2005, particularly from UCI, that were recovered from SSTIs, VAP and bacteriemia infections (Antoniadou et al., 2007). Another study reported the emergence of *E. hormaechei* with reduced susceptibility to tigecycline in a patient with hepatic failure in a France hospital in 2007, after treatment with tigecycline (Daurel et al., 2009). In 2017, a tigecycline-resistant *E. coli* isolate was recovered from human faeces of a patient with an intra-abdominal infection admitted to the Hangzhou hospital in China, during tigecycline treatment (Q. Wang et al., 2018). This isolate, besides being tigecycline resistant (MIC 8 mg/L), also *harboured mcr-1* gene (Q. Wang et al., 2018). Tigecycline and colistin-resistant bacteria were also reported in species from the ACB complex, mainly *A. baumannii*, from wounds, respiratory tract, blood, urine, and cerebrospinal fluid samples collected in 2003 from inpatients of a medical centre

in Israel (Navon-Venezia et al., 2007). More recently, in 2018, the first colistin and tigecycline-resistant *A. baumannii* isolate was described in Portugal in a Hospital in Lisbon from an infected wound of a patient with renal insufficiency (Caneiras et al., 2018).

The finding of colistin and tigecycline resistant *Enterobacteriaceae* and *Acinetobacter* spp. in the pig processing chain is worrisome, because if proper infection control measures are not complied these bacteria can be disseminated to humans, by direct contact of the workers with contaminated live pigs, meat, and equipment. Actually, according to our study, the main reservoirs of bacteria resistant to last-resort antibiotics were the workers' hands and live pigs, but resistant bacteria were also found in meat and equipment in lower frequencies (Figure 3.3). These results suggest that live pigs may be the origin of these bacteria that are then transmitted to workers, meat, and equipment. The colonization of workers with tigecycline and colistin-resistant bacteria can be a means of dissemination of these bacteria in the community through the transmission to households. Moreover, if colonized humans are in contact with hospitals and become immunocompromised, these bacteria can become a serious infectious agent with few treatment options.

4.2 There was a high prevalence of resistance to last-resort antibiotics among *Enterobacteriaceae* from the pig processing chain

Considering the initial number of putative Gram-negative bacteria that were isolated (n=121), overall, it was found a frequency of 34% tigecycline-resistant (n=41/121) and 13% (n=16/121) colistin-resistance isolates among our collection. Also, as much as 37% (n=45/121) of the samples contained at least one bacterial isolate resistant to either colistin or tigecycline (Figure 3.2). The species more frequently found associated with resistance to colistin and/or tigecycline were *E. coli, K. pneumoniae* and *Enterobacter* spp.

The rates of resistance to last-resort antibiotics in the swine processing chain found in this study are alarmingly high. Currently, the number of epidemiological studies focusing on tigecycline resistance in livestock are still scarce and there are insufficient estimative regarding the frequency of resistant isolates in other countries. Most of the studies were performed in China and are focused on the detection of antimicrobial-resistant determinants such as *tet(*X) and *mcr* genes and their variants. The few studies in animals include a study in companion animals wherein a tigecycline non-susceptible of 20% (n=20/86) was reported in *K. pneumoniae* and *K.quasipneumoniae* (Sato et al., 2018). This study included isolates collected from different clinical samples of both dogs and cats admitted to a veterinary clinic in Japan between 2003 and 2016 (Sato et al., 2018). Moreover, the *tet(*X4) gene, associated with tigecycline resistance, had a prevalence among pigs (faecal swabs) isolates from farms and slaughterhouses that ranged from 0% to 50% among *E. coli* isolates in different Chinese provinces collected during the year 2018 (Sun et al., 2020). The percentage of tigecycline-resistant *E. coli* isolates was also high among chicken cloacal swabs in some provinces (44% (n=30/60) in Shaanxi)) (Sun et al., 2020). Another study found that the prevalence of *tet*(X) genes in *Acinetobacter* spp. from humans, migratory birds, pigs and surrounding environmental samples in China between 2015 and 2018 was 5% (C. Chen et al., 2020). Furthermore, a 3% prevalence of *Acinetobacter* spp. non-susceptible to

tigecycline was found in a multicentre study including hospitals from thirty-two countries (located in North America, Europe, Latin America, and Asia-Pacific) in 2005 and 2009 (Mendes et al., 2010).

Most tigecycline-resistant isolates from our study carried the *oqxA and oqxB* genes, encoding for a multidrug efflux pump, that was previously associated not only with resistance to tigecycline but also to quinoxalines, quinolones, nitrofurantoin, several detergents and disinfectants (J. Li et al., 2019). All the sequenced *Enterobacteriaceae* isolates (n=14) also harboured *acrA* and *acrB* genes (Figure 3.5), and the *A. pittii* isolated carried *adeF, adeG* and *adeH.* Overexpression of both OqxAB and AcrAB efflux pumps have been previously associated with tigecycline resistance on *K. pneumoniae* clinical isolates collected from a Chinese hospital between 2009 and 2013 (Zhong et al., 2014). Thus, the obtained results suggest that OqxAB and/or the AcrAB efflux pumps might be responsible for the resistance observed in the *Enterobacteriaceae*. Other tigecycline resistance determinants, previously associated with tigecycline, were not carried by strains of this study, namely *tet*(X) genes (Sun et al., 2020; Dong et al., 2021). The AdeFGH pump was previously reported as playing a key role in MDR *A. baumannii* (Yoon et al., 2013). *A. baumannii* mutants with increased expression of this operon had high-level resistance to clindamycin and fluoroquinolones and decreased susceptibility to tigecycline (Coyne et al., 2010). Since *acrA, acrB, oqxA* and *oqxB* genes were not present in the *A. pittii* isolate, the tigecycline resistance might be related to AdeFGH overexpression.

To establish if tigecycline resistance is, in fact, associated with these efflux pumps we would have to perform assays in which the efflux pump would be inhibited and the consequent effect on antibiotic resistance would be analysed. An alternative mechanism that might be responsible for tigecycline resistance in strains from our study carrying *tet* genes, is that associated to the overexpression of *tet* genes, such as *tet*(A), (Linkevicius et al., 2016; J. Xu et al., 2021), but this was not further explored in our study.

Regarding colistin, a high prevalence of colistin-resistant *E. coli* isolates was found among several livestock animals, in particular from pigs on farms (24.1%) and slaughter (9.5%) in China between 2013 and 2014, (Huang et al., 2017). In Malaysia, 28.3% of the *K. pneumoniae* isolates collected from swine rectum, faeces and nasal and oral cavities in 2013-2015 were colistin-resistant (Mobasseri et al., 2019). Moreover, a high prevalence (22%) of colistin resistance was found in *Enterobacter* spp. (including in *E. kobei, E. ludwiigii*, among others) in a tertiary hospital in China between 2011 and 2020 (W. Liao et al., 2022). According to with BSAC surveillance programme, the prevalence of colistin resistance among bloodstream *E. cloacae* complex isolates in the UK and Ireland heath facilities increased from 5.7% and 8.1% in 2011 and 2012, respectively, to 15.9% and 13.4% in 2016 and 2017, respectively (Mushtaq et al., 2020). These frequencies were even higher than those verified for the bloodstream and lower respiratory tract infections caused by *E. coli* and *Klebsiella spp.* which were below 2% (Mushtaq et al., 2020). However, as far as we are concerned, there are no reports on the presence of colistin-resistant *Enterobacter* spp. in farm animals. Although colistin-resistant *Acinetobacter* spp. were not detected among our samples, a previous study performed in Portugal reported that 41.7% of the *Acinetobacter* spp. isolated from the raw pork steaks, beef, turkey and chicken meat were colistin-resistant

(Carvalheira et al., 2017a). In particular, 16.7% of *A. pittii*, (n=2/12) isolates collected from turkey and chicken were colistin-resistant isolates (Carvalheira et al., 2017a).

The *mcr* gene was not detected in any colistin-resistant isolates expressing phenotypic resistance in our study. Mutations in the *mgrB* gene, encoding a small transmembranar protein constitute an alternative mechanism of colistin resistance (Cannatelli et al., 2013), however, mutations in this gene were not looked for in this study. The colistin-resistant phenotype could also be due to mutations in enzymes involved in lipid A biosynthesis (P. Zhou & Zhao, 2017) resulting in lipid A inactivation or complete LPS suppression (Moffatt et al., 2010; Lin et al., 2017). This mechanism has been shown to occur in colistin-resistant derivatives of *A. baumannii* ATCC 19606 (Moffatt et al., 2010). Furthermore, the same study reported the first LPS complete spontaneous deletion in a clinical *A. baumannii* isolated from a bronchoalveolar lavage fluid of an ICU patient in an Australian hospital (Moffatt et al., 2010). LPS loss due to *lpx* genes deletions was later reported in MDR *E.coli* obtained from infections from different clinical specimens such as blood, urine, or wounds in an Iranian hospital in 2017 (Moosavian et al., 2020). Also, polymorphisms in the two-component systems proteins PmrAB and PhoPQ might be involved in colistin resistance (Adams et al., 2009; Jayol et al., 2014). In fact, modification of the lipid A portion of LPS through the addition of PEtN and l-Ara4N due to specific mutations in PhoP/PhoQ and PmrA/PmrB or environmental stimuli has been reported as the most common mechanisms of colistin resistance in *E. coli* and *Salmonella* spp. (Bergen et al., 2012; Kempf et al., 2013; Olaitan et al., 2014). So, we hypothesise that colistin resistance might be caused by mutations in *mgrB* or in the two-component systems PhoP/PhoQ and PmrA/PmrB. However, it would be necessary to screen for mutations in these specific genes, to confirm this hypothesis.

The reason for the high frequencies of colistin and tigecycline resistance observed in our study among *Enterobacteriaceae* and *Acinetobacter* spp. from the pig production chain is not obvious, because the use of these antibiotics in animals has been highly regulated in Europe. According to EMA, tigecycline is classified as “category A” (avoid) antibiotics meaning they are not authorized for veterinary use (EMA, 2019). However, they might be used in individual companion animals according to the prescribing “cascade” (EMA, 2019). The “cascade” use of drugs corresponds to the use of unauthorized medicines in veterinary or their use in non-target animals or following a different indication treatment (DGAMV & DSMDS, 2009). This norm allows the veterinarian to use unauthorized medicines in exceptional cases, such as the case in which there is no alternative treatment available or when an animal's suffering cannot be avoided any other way (DGAMV & DSMDS, 2009). In animals for food consumption, the use of category A antibiotics can only occur up to an established maximum residue limit (EMA, 2019).

On the other hand, polymyxins, including colistin and polymyxin B, are classified as “category B” antibiotics (EMA, 2019), but are considered as “Reserved group”, as well as tigecycline, by the WHO (WHO, 2021). This means that polymyxins are authorized to be used in the treatment of animal infections in Europe, although their use is restricted (EMA, 2019). It is also known that colistin was extensively used in the past for the treatment and prevention of Gram-negative infections and also as animal growth promoters propose (Kirchhelle, 2018; Schoenmakers, 2020). The use of antibiotics as

feed additives was eventually banned in European countries in 2006 (EU, 2005), but in other countries such as China, the use of colistin as a growth promoter was only banned recently, in 2016 (Walsh & Wu, 2016), after the discovery of the *mcr*-1 gene (Y.-Y. Liu et al., 2016, p. 1). Furthermore, polymyxins are still one of the most sold antibiotics for veterinary purposes in some European countries (EMA, 2021b). Polymyxins sales in Portugal represented more than 5% of the total sales in 2020 (EMA, 2021a), which is more than double of the overall polymyxins rates sales in the remaining countries (EMA, 2021b). This could be an explanation for the colistin-resistance levels verified in our study and calls for the need for an urgent reduction in the use of polymyxins in animals.

As previously mentioned, currently, these antibiotics can only be used in exceptional cases reducing the selective pressure and the possibility of resistance emergence. It is critical maintaining the effectiveness of these antibiotics because of their importance to human public health. They are considered the last resort for the treatment of life-threatening infections caused by MDR bacteria in humans (WHO, 2021). Even not being authorized for veterinary use, a high prevalence of tigecycline associated with plasmid-encoded *tet*(X) genes and their variants have been reported in *Enterobacteriaceae* (Rodríguez-Baño et al., 2018) and *Acinetobacter* spp. (J. Wang et al., 2020) from human and animal origin across China (Dong et al., 2021). It is also possible that resistance rates observed result from the lack of compliance with the rules established.

Alternative explanations include the possibility that indirect selective pressure might be inducing resistance to these antibiotics. This includes the overuse of other antibiotics and drugs, like disinfectants and metals that are transported by the same multidrug efflux pumps as tigecycline and colistin and that might be inducing mutations that lead to the overexpression of these systems. Actually, the expression of multidrug efflux pumps such as the OqxAB was previously shown to decrease the susceptibility to heavy metals and disinfectants in bacteria (J. Li et al., 2019) as well as to chloramphenicol, quinolones and fluoroquinolones and trimethoprim (J. Li et al., 2019). Another possibility is the fact that colistin and tigecycline resistant genes might be transported within the same plasmid as genes conferring resistance to other antibiotics or drugs, leading to a co-selection of resistance. For example, on IncHI2 plasmids found in *E. coli* of avian origin *oqxAB* genes co-exist with other resistance genes (e.g., *blaCTX-M, rmtB* and *aac(6')-Ib*), virulence genes and heavy metals resistance genes *pco* and *sil* operons, responsible for high $CuSO_4$ and $AgNO_3$ MICs (L. Fang et al., 2016).

Isolates resistant to colistin and tigecycline were also resistant to other antimicrobials. The most common resistances were found for ticarcillin (47%) and amoxicillin-clavulanic acid (33%) (Figure 3.4). Hight frequencies of resistance to amoxicillin-clavulanic acid and ticarcillin of 87% and 100% respectively, were previously reported in ESBL-producing *E. coli* isolated from faecal samples of pig farms in Latvia harbouring *bla*TEM (94%, n = 47), *bla*CTX-M (86%, n = 43) and *bla*SHV genes (48%, n = 24) (Gāliņa et al., 2021). According to this study, the high resistance rates to these antibiotics might be due to the overuse of these antibiotics in the veterinary setting (Gāliņa et al., 2021).

Despite the finding that *Enterobacteriaceae* from the pig production chain are resistant to last-resort antibiotics, in this study we could not find evidence for the occurrence of transmission of these antibiotic-resistant bacteria from animals, meat or surfaces to humans. However, the transmission of

bacterial species such as *Salmonella* spp. (C. N. Wilson et al., 2020; González-Santamarina et al., 2021) and *E. coli* (P. Zhang et al., 2021) has been previously detected in the food production chain. It is possible that, if the number of isolates from the same species compared by SNPs analysis had been higher, epidemiological links between the different steps of the chain would be detected. Nevertheless, some antibiotic resistance determinants (e.g., *oqxA, oqxB, acrA,acrB*) were present in different bacterial species from different sample sites. For example, the *oqxA* and *oqxB* genes were present in *K. pneumoniae* isolates from pig ears and operators' hands, in *E. kobei* from equipment and meat and in *E.asburiae* from meat (Figure 3.5). These results indicate that horizontal transfer of these antibiotic resistance genes might have occurred in the slaughterhouse environment.

4.3 No resistance to last-resort antibiotics was found among staphylococci from the pig processing chain

None of the staphylococci collected from the pig production chain analysed in this study were tigecycline or linezolid resistant. This is in line with the low frequencies of linezolid and tigecycline resistance previously reported for both *S. aureus* and CoNS of both animal and human clinical origin. According to a metanalysis performed recently, tigecycline resistance among isolates causing infections in humans (e.g. bacteraemia, skin infections, hospital-acquired pneumonia) was reported in 1.6% of CoNS and only 0.1% of the *S. aureus* (Shariati et al., 2020). However, there are relatively few studies in the veterinary sector that assess the prevalence of *Staphylococcus* spp. resistance to these antibiotics, especially to tigecycline.

The first linezolid-resistant staphylococcal isolate reported in Portugal was in 2011, an MRSA collected from severe otitis in a dog (Seixas et al., 2011). After that, another study performed in the same Lisbon slaughterhouse as that analysed in our study, reported no linezolid-resistant *S. aureus* isolates among any of the pig production chain samples (Bouchami et al., 2020). However, in a more recent study, wherein poultry samples that were collected in a Portuguese slaughterhouse in 2020, it was reported one *M. sciuri* isolate carried the *cfr* gene (1.3%) (Silva et al., 2022). In other countries, such as South Korea, an overall 2.3% rate of *S. aureus* isolates resistant to linezolid was found among pig carcasses between 2010 and 2017 (Kang et al., 2020). However, in the year 2012, a higher frequency of linezolid-resistant in pig carcasses was detected (9.8%) (Kang et al., 2020). *S. aureus* isolates resistant to these antibiotics were already reported in pigs in South Africa, reaching 10% and 50%, respectively (Sineke et al., 2021).

After twelve years of linezolid use in clinical practice (CDER, 2000), the frequency of linezolid resistance among staphylococci was reported to be also very reduced among clinical isolates obtained from patients in hospitals in several countries between 2012 and 2018 (Gu et al., 2013; Shariati et al., 2020). Overall, the frequency of linezolid-resistant *S. aureus* changed from 0.05 % in 2012 to 0.1% in 2018 and decreased from 1.4% to 0.3% in CoNS (Gu et al., 2013; Shariati et al., 2020). Some countries like Poland did not detect any linezolid resistant isolate among MRSA in several hospitals between 2015-2017 (Kot et al., 2020). Still, there were countries like Pakistan, in which high rates of *cfr*-carrying

linezolid resistant staphylococci were described, reaching 48.1% in MRSA and 29.2% in MSSA collected from wounds, ear, and skin swabs from inpatients (Azhar et al., 2017). In Portugal, there are only a few reported cases of resistance to linezolid in staphylococci from clinical samples. In 2012, five linezolid resistant *S. epidermidis* isolates were recovered from blood and catheters of human inpatients (Barros et al., 2014). However, it was only in 2019 that the first *cfr*-positive MRSA was notified in Portugal (Silva et al., 2019). In this recent study, about 10% of the MRSA (n=3/28) isolates recovered from diabetic foot ulcers were linezolid resistant and all of them carried the *cfr* gene (Silva et al., 2019).

4.4 Staphylococci from the pig processing chain were multidrug-resistant

The staphylococcal species most commonly isolated in the pig production chain was *S. hyicus* (54%) (Figure 3.7), a species previously reported to be the main etiological agent of EE in piglets (L. Schwarz et al., 2021). The remaining species were found in similar frequencies (8-15%). All six *Staphylococcus* spp. identified in this study were already associated with live pigs, raw meat or the farm environment in previous studies (Nagase et al., 2002; Riesen & Perreten, 2010; G. Y. Lee et al., 2019; L. Chen et al., 2021; L. Schwarz et al., 2021; G. Y. Lee & Yang, 2021).

Although staphylococci from the pig production chain were not resistant to last-resort antibiotics, they showed an MDR profile. The highest resistance rates were found for tetracyclines (77%), clindamycin (77%) and erythromycin (69%) (Figure 3.8). Other studies have also reported high rates of resistance to tetracycline, clindamycin and erythromycin in staphylococci from production animals including Portugal (Bouchami et al., 2020) and South Africa (Sineke et al., 2021). Both tetracyclines and macrolides were heavily used in the past as AGPs (EMA, 2010; Nordmann & Poirel, 2016). Furthermore, tetracyclines continue to be one of the most used antibiotics classes in veterinary settings (EMA, 2021b; FDA, 2021), especially in pig farming (Lekagul et al., 2019; FDA, 2021), which might have selected for resistance.

S. hyicus was the species showing resistance to the highest number of different antibiotics with resistance to up to six different antibiotics (ciprofloxacin, clindamycin, erythromycin, tetracyclines, chloramphenicol and fosfomycin) (Figure 3.9 and Annexe 3). Other studies in which *S. hyicus* have been isolated also showed that this species it is an important reservoir of antibiotic resistance genes in this setting. For example, samples collected from skin lesions of pigs with EE in Denmark between 1996 and 2001 reported the presence of several antimicrobial resistance genes including genes encoding resistance to macrolides (*erm(A), erm(B)* and *erm*(C)), penicillin (*blaZ*), streptogramin (*vat, vga, vga(B), vat(B), vat(D) and vat(E)*), and tetracycline resistance (*tet*(K), *tet*(L), *tet*(M) and *tet*(O)) (Aarestrup & Jensen, 2002). Most of these genes were also found among staphylococci isolated in this study (Figure 3.9). The MDR profile shown by *S. hyicus* isolates might represent a challenge during the treatment of infections caused by *S. hyicus* in pigs, namely in the treatment of EE, and represent an additional burden for pigs and costs for animal producers.

4.5 There was a transmission of staphylococci between the different steps of the pig processing chain

By comparing the core genome of staphylococci by SNPs analysis, we found that three *S. hyicus* isolates from different sources were highly related (between 0 and 2 SNPs), suggesting the occurrence of transmission between the pig-equipment and also between the animals and the equipment to humans (Figure 3.10). This is worrisome because these bacteria are MDR and when transmitted to humans that are immunocompromised can originate infections that are particularly difficult to treat. Although rarely, *S. hyicus* was previously found to cause infections in humans, always with the previous contact with animals. The first reported case was in 1997, in Sweden, in which a woman had a wound infection caused by *S. hyicus* after a donkey bite (Österlund & Nordlund, 1997). Later, in 2011, a bacteraemia caused by *S. hyicus* was reported in a Switzerland farmer, which has been in close contact with piglets, and was admitted to hospital with signs of sepsis and cellulitis in the foot (Casanova et al., 2011). In both cases, the patients were immunocompetent and were successfully treated (Österlund & Nordlund, 1997; Casanova et al., 2011).

4.6 Limitations of the study

The source of *Enterobacteriaceae*, *Acinetobacter* spp. and *Staphylococcus* spp. for this study were samples collected from the ear and rectum of live pigs, surfaces, meat, and hands of human slaughterhouse workers. To be able to isolate individual colonies for downstream analysis, it was necessary to dilute the initial bacterial population sampled and use chromogenic media, from which only a few colonies were selected to test for resistance to last-resort antibiotics. Also, antibiotic concentrations above the EUCAST breakpoints were used in other to ensure that the isolates were, indeed, resistant and avoid false positives. Given that the frequency of resistance to last-resort antibiotics is low, it is possible that by using this methodology some isolates resistant to last-resort antibiotics were missed.

To understand the prevalence of clinically relevant genetic determinants conferring resistance to last-resort antibiotics, like *mcr* (colistin), *cfr* (linezolid), *tetX* (tigecycline), it would be interesting to detect their presence and relative proportion in samples' total DNA extracts. Additionally, a shotgun metagenomics approach in which all the DNA present in the original samples would be analysed for species and the presence of resistant determinants could provide a more accurate prevalence of resistance to last-resort antibiotics among *Enterobacteriaceae* and *Staphylococcus*.

Another factor that might have influenced the rates of resistance to last-resort antibiotics determined is the fact that many of the bacterial isolates analysed in this study belonged to species usually found in environmental and animal samples and rarely found in clinical human samples (e.g. *S.rostri*, *S. hyicus*). This is important because the breakpoints used to classify a bacterial species as resistant or susceptible are not established and validated as for clinically relevant species and might not be the most appropriate to use with the species identified in our study.

To assess the transmission of antibiotic-resistant *Enterobacteriaceae* and *Staphylococcus* isolates in the pig production chain, WGS of a low number of isolates of each species were sequenced

and compared. For that reason, some epidemiological links might be missed between the different steps of the production chain. The comparison of a higher number of isolates of each species would circumvent this limitation. In this regard, another limitation is the fact that no cut-offs are defined for some species (e.g. *S. hyicus*) for the number of SNPs that should be considered for an isolate to belong to the same chain of transmission. Although we used the cut-off defined for *S. aureus* (Goyal et al., 2019) of 20 SNPs, this was not previously validated for *S. hyicus*. A more extensive study focused on *S. hyicus* molecular epidemiology by WGS would have to be done to define this cut-off.

4.7 Conclusions

Our study showed that there was a high frequency of resistance to tigecycline and colistin among *Enterobacteriaceae* collected from the swine production chain. Resistance to these last-resort antibiotics were probably associated with the overexpression of efflux pumps and mutations in genes involved in lipid A biosynthesis. The finding of such high rates of resistance to the last resort antibiotics among *Enterobacteriaceae* from the pig production chain is worrisome because these bacteria can potentially be transmitted to human workers and households and be a cause of future infections. Although resistance to last-resort antibiotics was not detected among staphylococci collected in the pig production chain, these bacteria were MDR and had the ability to disseminate to human workers, also constituting a potential risk for human health.

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ANNEXES

Annexe 1- Slaughterhouse samples analyzed in this study (n=24) collected in 2016 from different sampling sites.

#	Sample ID	Isolation source	Isolation year
1	ZP15	Clean cutting table (1000 cm2)	Summer 2016
2	ZP28	Clean hands operator 1	Summer 2016
3	ZP29	Clean hands operator 2	Summer 2016
4	ZP13	clean treadmill (1000 cm2)	Summer 2016
5	ZP14	dirty cutting table (1000 cm2)	Summer 2016
6	ZP27	dirty hands operator 3 (gloves)	Summer 2016
7	ZP11	operator hands (washed)	Summer 2016
8	ZP1	pig ear	Summer 2016
9	ZP2	pig ear	Summer 2016
10	ZP8	pig ear	Summer 2016
11	ZP9	pig ear	Summer 2016
12	ZP21	Pig shoulder (400cm2)	Summer 2016
13	ZP24	Pig shoulder (400cm2)	Summer 2016
14	ZP16	streaky pork (500 cm2)	Summer 2016
15	ZP17	streaky pork (500 cm2)	Summer 2016
16	ZP18	streaky pork (500 cm2)	Summer 2016
17	ZP19	streaky pork (500 cm2)	Summer 2016
18	ZT1	clean treadmill (Zone of streaky pork)	Winter 2016
19	ZT2	dirty treadmill (Zone of streaky pork)	Winter 2016
20	ZPA12	operator 1 hand dirty	Winter 2016
21	ZAP1	pig ear	Winter 2016
22	ZA1	pig rectum	Winter 2016
23	ZA2	pig rectum	Winter 2016
24	ZA3	pig rectum	Winter 2016

Annexe 2 - Phenotypic resistance profile of putative Gram-negative (n=45) bacteria isolated from the slaughterhouse

#	Isolate ID	Species identification (16S rRNA)	Origin	Isolation source	TIG	COL	AMC	PIT	TIC	TEM	CTV	CEP	IMI	MER	CIP	GEN	TRS
1	ZP29 W1	*A. pittii*	operator	clean hands operator 2	R	S	-	-	-	-	-	-	-	-	-	-	-
2	ZP29 W2	*A. pittii*	operator	clean hands operator 2	R	S	-	-	-	-	-	-	-	-	-	-	-
3	ZP29 W3 [W]	*A. pittii*	operator	clean hands operator 2	R	S	NA	NA	S	NA	NA	S	S	S	I	S	S
4	ZP16 B1.1 [W]	*E. kobei*	meat	streaky pork (500 cm2)	R	R	R	S	S	I	S	S	S	S	S	S	S
5	ZA2 P3	*E. coli*	live pig	pig rectum	R	S	-	-	-	-	-	-	-	-	-	-	-
6	ZA2 P4	*E. coli*	live pig	pig rectum	R	S	-	-	-	-	-	-	-	-	-	-	-
7	ZA2 W3 [W]	*E. coli*	live pig	pig rectum	R	S	S	S	R	I	S	S	S	S	S	S	R
8	ZP13 P1 [W]	*E. coli*	equipment	clean treadmill (1000 cm2)	R	S	S	S	S	I	S	S	S	S	S	S	S
9	ZP16 P2 [W]	*E. coli*	meat	pig shoulder (400 cm2)	R	S	S	S	I	I	S	S	S	S	S	S	S
10	ZP16 P3	*E. coli*	meat	pig shoulder (400 cm2)	R	S	-	-	-	-	-	-	-	-	-	-	-
11	ZP16 P4	*E. coli*	meat	pig shoulder (400 cm2)	R	S	-	-	-	-	-	-	-	-	-	-	-
12	ZP2 P1	*E. coli*	live pig	pig ear	R	S	-	-	-	-	-	-	-	-	-	-	-
13	ZP2 P2 [W]	*E. coli*	live pig	pig ear	R	S	R	S	S	I	S	S	S	S	S	S	S
14	ZP27 P4	*E. coli*	operator	dirty hands operator 3 (gloves)	R	R	-	-	-	-	-	-	-	-	-	-	-
15	ZP27 P5	*E. coli*	operator	dirty hands operator 3 (gloves)	R	R	-	-	-	-	-	-	-	-	-	-	-
16	ZP13 B4 [W]	*E. hormaechei*	equipment	clean treadmill (1000 cm2)	R	S	R	S	S	I	S	S	S	S	S	S	S
17	ZP29 B2 [W]	*E. hormaechei*	operator	clean hands operator 2	R	S	R	S	S	I	S	S	S	S	S	S	S
18	ZP29 B5	*E. hormaechei*	operator	clean hands operator 2	R	S	-	-	-	-	-	-	-	-	-	-	-
19	ZP29 B6	*E. hormaechei*	operator	clean hands operator 2	R	S	-	-	-	-	-	-	-	-	-	-	-
20	ZP29 B7	*E. hormaechei*	operator	clean hands operator 2	R	S	-	-	-	-	-	-	-	-	-	-	-
21	ZP13 B1	*E. hormaechei*	equipment	clean treadmill (1000 cm2)	R	S	-	-	-	-	-	-	-	-	-	-	-
22	ZP13 B2	*E. hormaechei*	equipment	clean treadmill (1000 cm2)	R	S	-	-	-	-	-	-	-	-	-	-	-
23	ZP13 P2	*E. hormaechei*	equipment	clean treadmill (1000 cm2)	R	S	-	-	-	-	-	-	-	-	-	-	-
24	ZP14 B1 [W]	*E. kobei*	equipment	dirty cutting table (1000 cm2)	S	R	R	S	S	I	S	S	S	S	S	S	S

#	Isolate ID	Species identification (16S rRNA)	Origin	Isolation source	TIG	COL	AMC	PIT	TIC	TEM	CTV	CEP	IMI	MER	CIP	GEN	TRS
25	ZP16 B2	*E. kobei*	meat	streaky pork (500 cm2)	R	R	-	-	-	-	-	-	-	-	-	-	-
26	ZP18 B3 [W]	*E. kobei*	meat	streaky pork (500 cm2)	S	R	R	S	S	I	S	S	S	S	S	S	S
27	ZP14 B2	*E. ludwigii*	equipment	dirty cutting table (1000 cm2)	S	R	-	-	-	-	-	-	-	-	-	-	-
28	ZP14 B3	*E. ludwigii*	equipment	dirty cutting table (1000 cm2)	S	R	-	-	-	-	-	-	-	-	-	-	-
29	ZP1 B1	*K. pneumoniae*	live pig	pig ear	R	S	-	-	-	-	-	-	-	-	-	-	-
30	ZP1 B2 [W]	*K. pneumoniae*	live pig	pig ear	R	S	S	S	R	I	S	S	S	S	S	S	S
31	ZP1 B3A	*K. pneumoniae*	live pig	pig ear	R	S	-	-	-	-	-	-	-	-	-	-	-
32	ZP16 B3 [W]	*K. pneumoniae*	meat	streaky pork (500 cm2)	R	S	S	S	R	I	S	S	S	S	S	S	S
33	ZP18 B5	*K. pneumoniae*	meat	streaky pork (500 cm2)	R	S	S	S	R	I	S	S	S	S	S	S	S
34	ZP2 B1	*K. pneumoniae*	live pig	pig ear	R	R	-	-	-	-	-	-	-	-	-	-	-
35	ZP2 B2	*K. pneumoniae*	live pig	pig ear	R	S	-	-	-	-	-	-	-	-	-	-	-
36	ZP2 B3 [W]	*K. pneumoniae*	live pig	pig ear	R	S	S	S	R	I	S	S	S	S	S	S	S
37	ZP2 B4	*K. pneumoniae*	live pig	pig ear	R	R	-	-	-	-	-	-	-	-	-	-	-
38	ZP27 B2 [W]	*K. pneumoniae*	operator	dirty hands operator 3 (gloves)	R	R	S	S	R	I	S	S	S	S	S	S	S
39	ZP27 B3	*K. pneumoniae*	operator	dirty hands operator 3 (gloves)	R	S	-	-	-	-	-	-	-	-	-	-	-
40	ZP27 W5	*K. pneumoniae*	operator	dirty hands operator 3 (gloves)	R	R	-	-	-	-	-	-	-	-	-	-	-
41	ZP29 B1 [W]	*K. pneumoniae*	operator	clean hands operator 2	R	S	S	S	R	I	S	S	S	S	S	S	S
42	ZP1 W1	*P. mirabilis*	live pig	pig ear	R	R	-	-	-	-	-	-	-	-	-	-	-
43	ZP1 W5	*P. mirabilis*	live pig	pig ear	R	R	-	-	-	-	-	-	-	-	-	-	-
44	ZP27 B4	*P. mirabilis*	operator	dirty hands operator 3 (gloves)	R	R	-	-	-	-	-	-	-	-	-	-	-
45	ZP27 W3	*P. mirabilis*	operator	dirty hands operator 3 (gloves)	R	R	-	-	-	-	-	-	-	-	-	-	-
46	ZP29 B3	*E. faecalis*	operator	clean hands operator 2	-	-	-	-	-	-	-	-	-	-	-	-	-
47	ZP29 B4	*E. faecalis*	operator	clean hands operator 2	-	-	-	-	-	-	-	-	-	-	-	-	-
48	ZA2 B1A	*E. casseliflavus*	live pig	pig rectum	-	-	-	-	-	-	-	-	-	-	-	-	-
49	ZA2 B1B	*E. casseliflavus*	live pig	pig rectum	-	-	-	-	-	-	-	-	-	-	-	-	-
50	ZA2 B2A	*E. casseliflavus*	live pig	pig rectum	-	-	-	-	-	-	-	-	-	-	-	-	-

#	Isolate ID	Species identification (16S rRNA)	Origin	Isolation source	TIG	COL	AMC	PIT	TIC	TEM	CTV	CEP	IMI	MER	CIP	GEN	TRS
51	ZP27 P3	*E. casseliflavus*	operator	dirty hands operator 3 (gloves)	-	-	-	-	-	-	-	-	-	-	-	-	-
52	ZP2 W4	*S. borealis*	live pig	pig ear	-	-	-	-	-	-	-	-	-	-	-	-	-
53	ZP1 B4	*S. simulans*	live pig	pig ear	-	-	-	-	-	-	-	-	-	-	-	-	-

[W] – WGS performed for this isolate; operator 1- bleeding; operators 2 & 3 – cutting meat pieces; NA- not aplicable for this species; no breakpoint avaible;

S (green colour) - susceptible; R (red colour) - resistant; I (yellow colour) - intermediate ; AMC: amoxicillin-clavulanic acid; PIT: piperacillin-tazobactam; TIC: ticarcillin; TEM: temocillin; CEP: cefepime; CTV: ceftazidime-avibactam; IMI: imipenem; MER: meropenem; CIP: ciprofloxacin; GEN: gentamicin; TRS: trimethoprim-sulfamethoxazole.

Annexe 3 - Antimicrobial susceptible testing for the putative *Staphylococcus* spp. identified by *tuf* sequencing (n=25)

#	Isolate ID	Species identification (*tuf*)	Origin	Isolation source	OXA	P	CXI	CIP	GEN	TEI	CLI	ERY	QUD	TET	TIG	CHL	LIN	FUS	FOS	RIF	TRS
1	ZAP1 C10	*S. aureus*	live pig	pig ear	R	R	R	R	R	S	R	R	I	R	S	S	S	S	S	S	S
2	ZP11 C7 [W]	*S. haemolyticus*	operator	operator hands (clean hands)	S	S	S	R	S	S	R	R	S	R	S	S	S	S	R	S	S
3	ZP11 C8	*S. haemolyticus*	operator	operator hands (clean hands)	S	S	S	R	S	S	R	S	S	R	S	S	S	S	R	S	S
4	ZA1 C6 [W]	*S. hyicus*	live pig	pig rectum	S	S	S	I	S	S	R	R	I	R	S	S	S	S	R	S	S
5	ZA1 C2	*S. hyicus*	live pig	pig rectum	S	S	S	I	S	S	S	S	S	S	S	S	S	S	R	S	S
6	ZP1 C7 [W]	*S. hyicus*	live pig	pig ear	S	S	S	R	S	S	R	R	S	R	S	R	S	S	R	S	S
7	ZP14 C5 [W]	*S. hyicus*	equipment	dirty cutting table (1000 cm2)	S	R	S	I	I	S	R	R	I	R	S	R	S	S	S	S	S
8	ZP14 Cd	*S. hyicus*	equipment	dirty cutting table (1000 cm2)	S	S	S	I	S	S	S	S	S	S	S	S	S	S	R	S	S
9	ZP15 C5 [W]	*S. hyicus*	equipment	clean cutting table (1000 cm2)	S	S	S	I	S	S	S	S	S	S	S	S	S	S	R	R	R
10	ZPA12C9 [W]	*S. hyicus*	operator	operator 1 hand dirty	S	R	S	I	S	S	R	R	I	R	S	R	S	S	S	S	S
11	ZP17 C3 [W]	*S. pseudintermedius*	meat	streaky pork (500 cm2)	S	R	S	I	S	S	R	R	S	R	S	R	S	S	S	R	S
12	ZP8 C5 [W]	*S. rostri**	live pig	pig ear	S	S	S	R	S	S	R	R	S	R	S	S	S	S	S	S	S
13	ZA2 C10 [W]	*S. simulans*	live pig	pig rectum	S	R	S	I	S	S	R	R	S	R	S	S	S	S	R	S	S
14	ZAP1 C1	*M. sciuri**	live pig	pig ear	R	S	R	I	S	S	R	S	I	R	S	R	S	R	S	S	S
15	ZP21 C4	*V. lutrae*	meat	pig shoulder (400 cm2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	ZP21 C8	*V. lutrae*	meat	pig shoulder (400 cm2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

#	Isolate ID	Species identification (*tuf*)	Origin	Isolation source	OXA	P	CXI	CIP	GEN	TEI	CLI	ERY	QUD	TET	TIG	CHL	LIN	FUS	FOS	RIF	TRS
17	ZP24 C11	*V. lutrae*	meat	pig shoulder (400 cm2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	ZP27 Ca	*V. lutrae*	operator	dirty hands operator 3 (gloves)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	ZP28 C6	*V. lutrae*	operator	clean hands operator 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	ZP8 C2	*V. lutrae*	live pig	pig ear	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	ZP16 C7	*M. caseolyticus*	meat	streaky pork (500 cm2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	ZP17 C7	*M. caseolyticus*	meat	streaky pork (500 cm2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	ZP27 Cb	*M. caseolyticus*	operator	dirty hands operator 3 (gloves)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	ZT2 C3	*M. caseolyticus*	equipment	clean treadmill (zone of streaky pork)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	ZT2 C6	*M. caseolyticus*	equipment	dirty treadmill (Zone of streaky pork)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* - considering the breakpoint interpretative criteria for CoNS of EUCAST 2020.

[W] – WGS performed for this isolate.

operator 1- bleeding; operators 2 & 3 – cutting meat pieces; S (green colour) - susceptible; R (red colour) - resistant; I (yellow colour) - intermediate ; OXA: oxacillin; CXI: cefoxitin; P: penicillin; CIP: ciprofloxacin; GEN: gentamycin; TEI: teicoplanin; CLI: clindamycin; ERY: erythromycin; QUD: quinupristin-dalfopristin; TET: tetracycline; CHL: chloramphenicol; FUS: fusidic-acid; FOS: fosfomycin; RIF: rifampicin; TRS: trimethoprim-sulfamethoxazole