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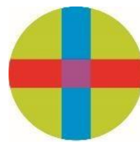

Characterization of methicillin-resistant *Staphylococcus aureus* in commercial and wild rabbits (*Oryctolagus cuniculus*) and immunological evaluation of a paternal line of commercial rabbits. Study of the pathogen-host interaction

TESIS DOCTORAL

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Fdo. Dra. Dña. Laura Selva Martínez

A mi madre

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“No hay nada más maravilloso que ser un científico, en ninguna parte preferiría estar más que en mi laboratorio, manchando mi ropa y cobrando por jugar.”

Marie Curie

SUMMARY

SUMMARY

Staphylococcus aureus is an important pathogen that is well able to develop resistance to antibiotics. One of the most remarkable clones to have spread worldwide and caused pandemic outbreaks is methicillin-resistant *Staphylococcus aureus* (MRSA), a dangerous strain that is frequently multiresistant to antibiotics. In this work, we studied the pathogen *S. aureus* by focusing on MRSA strains. We evaluated the presence of *S. aureus* on rabbit farms and in wild rabbits before carrying out an in-depth study of the isolated clones through their sequencing. Then we studied a frequent host of this pathogen, the rabbit (*Oryctolagus cuniculus*), to which this bacterium is highly adapted and is, therefore, a very useful model of infections by this pathogen. We evaluated the immune system of commercial rabbits at remarkable time points of the productive cycle and then performed experimental infection to evaluate the immune system when it faces an infectious challenge. First, an unexpected large number of MRSA strains from infrequent lesions were found on rabbit farms. This is the first time that *mec*C-MRSA strains isolated from rabbit farms are described. The MRSA strains isolated from rabbit farms showed limited genetic diversity, with ST2855 being the most prevalent clone. The majority of the tested MRSA isolates were multidrug-resistant. Second, a high percentage of *S. aureus* carriers was detected in wild rabbits and hares hunted in high-density areas in east Spain, and the ear was the main ecological niche where *S. aureus* was isolated. Another finding was the marked presence of ST1945 MRSA of the CC130 lineage containing the *mec*C gene in these animals. These strains harboured mobile genetic element SCC*mec* type XI. Therefore, farm rabbits and wild rabbits can act as a reservoir for MRSA strains being transmitted in nature. As a result of sequencing mobile genetic element SCC*mec*, we describe two new SCC*mec* cassette types: one containing the *mec*C gene and another simultaneously containing *mec*A and *mec*C genes. Two of these *mec*A-*mec*C-MRSA strains had the SCC*mec* excised from the genome. We also observed that each SCC*mec* type was associated with certain STs. The phenotypic resistance profile revealed that strains had an

antibiotic resistance profile depending on whether they had methicillin-resistance gene *mec*A or *mec*C. Nevertheless, simultaneously carrying the two genes offered no more advantages than having only the *mec*A gene in the resistance profile to the tested antibiotics. Finally, after evaluating the immune system by flow cytometry at main time points of the productive cycle of commercial rabbits the genetic selection by average daily gain did not affect rabbit females' ability to mount immune response. After evaluating lesions and immune system evolution during an experimental infection by *S. aureus* in skin, the results evidenced that this breeding programme favoured the immune system's capability to undergo an infectious challenge with *S. aureus*. To evaluate vitrification effects, a group of animals obtained from vitrified embryos was compared to another group obtained by routine artificial insemination. A long-term vitrification effect on the immune response to infection with *S. aureus* was observed insofar as the animals from the restored vitrificated population presented better performance during infection.

RESUMEN

RESUMEN

Staphylococcus aureus es un patógeno importante con gran capacidad de desarrollar resistencia a los antibióticos. Uno de los clones más notables que se ha extendido por todo el mundo y ha provocado brotes pandémicos es el *Staphylococcus aureus* resistente a la meticilina (MRSA), una cepa peligrosa que con frecuencia es multirresistente a los antibióticos. En este trabajo, estudiamos el patógeno *S. aureus* centrándonos en las cepas MRSA. Evaluamos la presencia de *S. aureus* en granjas de conejos y en conejos silvestres antes de realizar un estudio en profundidad mediante su secuenciación de los clones aislados. Luego se estudió un hospedador frecuente de este patógeno, el conejo (*Oryctolagus cuniculus*), al que esta bacteria está muy adaptada y es, por tanto, un modelo muy útil de infecciones por este patógeno. Evaluamos el sistema inmunológico de conejos comerciales en momentos notables del ciclo productivo y luego realizamos una infección experimental para evaluar el sistema inmunológico cuando se enfrenta a un desafío infeccioso. En primer lugar, se encontró una cantidad inesperada de cepas de MRSA de lesiones poco frecuentes en granjas de conejos. Además, es la primera vez que se describen cepas *mec*C-MRSA aisladas de granjas de conejos. Las cepas de MRSA aisladas de granjas de conejos mostraron una diversidad genética limitada, siendo ST2855 el clon más prevalente. La mayoría de los aislados de MRSA probados fueron multirresistentes. En segundo lugar, se detectó un alto porcentaje de portadores de *S. aureus* en conejos y liebres silvestres cazados en zonas de alta densidad del este de España, siendo la oreja el principal nicho ecológico donde se aisló *S. aureus*. Otro hallazgo fue la marcada presencia de clones ST1945 MRSA del linaje CC130 que contienen el gen *mec*C en estos animales. Estas cepas albergaron el elemento genético móvil SCC*mec* tipo XI. Por lo tanto, los conejos de granja y los conejos silvestres pueden actuar como reservorios de cepas MRSA que se transmiten en la naturaleza. Como resultado de la secuenciación del elemento genético móvil SCC*mec*, describimos dos nuevos tipos de casetes de SCC*mec*: uno que contiene el gen *mec*C y otro que contiene

simultáneamente los genes *mec*A y *mec*C. Dos de estas cepas *mec*A-*mec*C-MRSA tenían además el SCC*mec* escindido del genoma. También observamos que cada tipo de SCC*mec* estaba asociado con ciertos ST. El perfil de resistencia fenotípica reveló que las cepas tenían un perfil de resistencia a antibióticos dependiendo de si tenían el gen *mec*A o *mec*C de resistencia a la meticilina. Sin embargo, llevar simultáneamente los dos genes no evidenció más ventajas que tener solo el gen *mec*A en el perfil de resistencia a los antibióticos probados. Finalmente, tras evaluar el sistema inmunológico por citometría de flujo en los puntos principales del ciclo productivo de los conejos comerciales, la selección genética por ganancia diaria promedio no afectó la capacidad de las hembras de conejos para generar una respuesta inmune. Después de evaluar las lesiones y la evolución del sistema inmunológico durante una infección experimental por *S. aureus* en piel, los resultados evidenciaron que este programa de mejora genética favoreció la capacidad del sistema inmunológico al sufrir un desafío infeccioso con *S. aureus*. Para evaluar los efectos de la vitrificación, se comparó un grupo de animales obtenidos de embriones vitrificados con otro grupo obtenido mediante inseminación artificial rutinaria. Se observó un efecto a largo plazo de la vitrificación sobre la respuesta inmune a la infección por *S. aureus* en la medida en que los animales de la población vitrificada restaurada presentaron un mejor comportamiento durante la infección.

INDEX OF ABBREVIATIONS

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AAC	Aminoglycoside acetyltransferases
AAD	Aminoglycoside adenyltransferases
ACME	Arginine catabolic mobile element
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APH	Aminoglycoside phosphotransferases
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CAT	Chloramphenicol acetyltransferase
CC	Clonal complex
CFU	Colony forming units
CHIPS	Chemotaxis inhibitory protein of *S. aureus*
ClfA	Clumping factor A
CoPS	*Staphylococcus* coagulase positive
CoNS	*Staphylococcus* coagulase negative
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
DLV	Double locus variant
DNA	Deoxyribonucleic acid
FnbpA	Fibronectin binding protein A
HA-MRSA	Hospital-acquired methicillin-resistant *Staphylococcus aureus*
HGT	Horizontal gene transfer
IEC	Immune evasion cluster
kb	Kilobases
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
LDM	Leukocyte differentiation molecules
mAb	Monoclonal antibody
mg	Milligrams

MGE	Mobile genetic element
MHC	Major histocompatibility complex
ml	Milliliters
MLST	Multilocus sequence typing
mm	Millimeters
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NADPH	Nicotinamide adenine dinucleotide phosphate
NLR	NOD-like receptors
ºC	Degrees Celsius
PAMP	Pathogen-associated molecular pattern
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PGRP	Peptidoglycan recognition protein
PIA	Polysaccharide intercellular adhesin
PMN	Polymorphonuclear leukocyte
PSM	Phenol-soluble modulin
PRR	Pattern recognition receptor
PVL	Panton-Valentine leucocidin
RFLP	Restriction fragment length polymorphism
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SaPI	*Staphylococcus aureus* pathogenicity island
SAG	Superantigenic toxin
SAK	Staphylokinase
SCC	Staphylococcal cassette chromosome
SCIN	Staphylococcal complement inhibitor

Sdr	Serine-Aspartate Repeat Protein
SE	Staphylococcal enterotoxin
SEA	Staphylococcal enterotoxin A
SEB	Staphylococcal enterotoxin B
SEC	Staphylococcal enterotoxin C
SLV	Single locus variant
SNP	Single nucleotide polymorphism
SpA	Staphylococcal protein A
ST	Sequence type
TCR	T cell receptor
TCS	Two-component system
Th	Helper T lymphocytes
TLR	Toll-like receptor
TMP-SMX	Trimethoprim-sulfamethoxazole
TNF	Tumor necrosis factor
TSB	Tryptic soy broth
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin 1
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant *Enterococcus faecalis*
VRSA	Vancomycin-resistant *Staphylococcus aureus*

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INTRODUCTION

INTRODUCTION

In the development of diseases, the interaction established between pathogens and hosts is decisive in determining their course. In the case of *Staphylococcus aureus*, the rabbit, in addition to humans, is one of the hosts to which this bacterium is very well adapted and therefore the interaction of *S. aureus* in infections in rabbits is a very representative model of the pathogenesis of this bacterium. Within this bacterium-host binomial, we are going to start talking about the pathogen, to continue with the interaction between both and finally describe the host that has been used as a model in this study.

1. The pathogen: *Staphylococcus aureus*

The *S. aureus* bacteria was discovered in 1880 by the doctor Alexander Ogston when he observed spherical bacterial forms in clustered clusters, although Pasteur and Koch had previously observed it. Since then, it has been considered one of the most important pathogens due to its potential to produce multiple infections in humans and animals. *S. aureus* is a member of the Staphylococcaceae family (***Table 1***), it is considered the type species within its group and the most virulent due to the diversity and severity of diseases it produces and its geographical distribution is worldwide.

Rank	Specific name
Domain	Bacteria
Kingdom	Eubacteria
Phylum	Firmicutes
Class	Bacilli
Order	Baciallales
Family	Staphylococcaceae
Genus	Staphylococcus
Species	*Staphylococcus aureus*

Table 1. Taxonomy of *Staphylococcus aureus*

1.1. The Staphylococcus genus

Staphylococci are gram-positive bacteria usually living as commensals on the skin of mammals and birds. Forty-one species and several subspecies have been catalogued into the *Staphylococcus* genus. *S. aureus* and *S. epidermidis* are the most important from a human and animal health perspective.

The first division that is made between the species that make up this genus is based on the presence of the enzyme coagulase that is responsible for the passage of fibrinogen to fibrin, resulting in blood clotting (Devriese et al., 2005). As a result of this classification, we have the *Staphylococcus* coagulase positive (CoPS) and the coagulase negative (CoNS). The CoNS group is broader and more heterogeneous, however in the case of CoPS only seven species have been identified: *Staphylococcus aureus, S. intermedius, S. schleiferi subsp. coagulans, S. hyicus, S. lutrae, S. delphini*, and *S. pseudintermedius*.

1.2. General characteristics of *Staphylococcus aureus*

Staphylococcus aureus is gram-possitive cocci, which can appear in pairs, in chains or in clusters. Clustering of cocci is promoted by growth on solid medium; on occasion, the clusters may be asymmetrical. *S. aureus* is non motile, do not form spores or resting states, its size ranges from 0.5 to 1.5 microns of diameter, and some strains produce a mucoid outer capsule that increases its ability to cause infection, however, most strains produce only a microcapsule and the colonies appear non-mucoid (O'Riordan and Lee, 2004). This organism is distinguished from other staphylococcal species because of the gold pigmentation of colonies composed of carotenoids. *S. aureus* is capable of growing in a wide range of pH and temperatures and in saline solutions with a proportion up to 15% sodium chloride. *S. aureus* is part of the bacterial microbiota existing in the skin and mucous membranes of man and animals (primates, ungulates, carnivores, rodents, lagomorphs, marsupials and birds) (Williams, 1963; Kloos, 1980). Besides, it survives for weeks in carcasses, in the tissues and organs of animals

(meat) and, for days, on the skin, in the ground and on the surface of metal and glass objects. Colonies of *S. aureus* are β-hemolytic due to the production of several hemolysins: α-toxin, β-toxin, γ-toxin, and δ-toxin (Tegmark et al., 1998) (***Figure 1***).

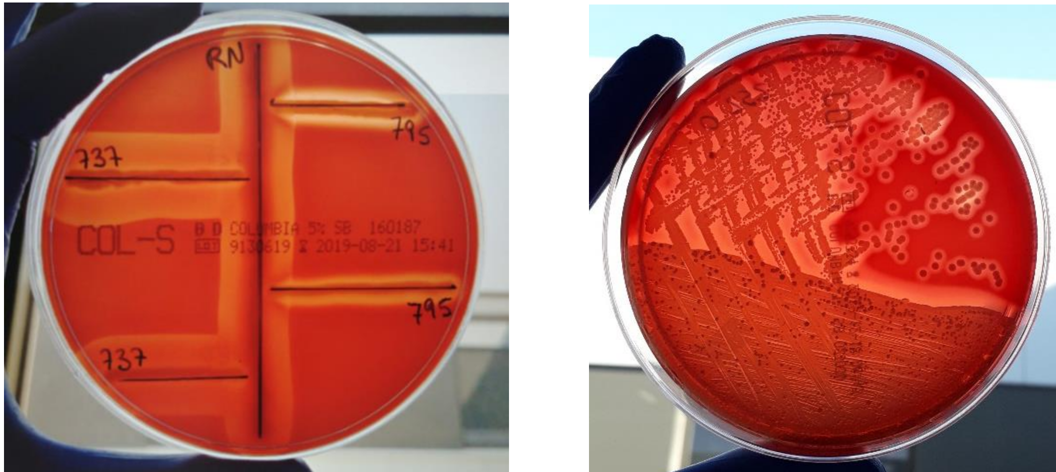

Figure 1*.* Hemolysis produced on blood agar by different strains of *S. aureus.*

In relation to its metabolism *S. aureus* is facultative anaerobic, coagulase positive (it produces a positive coagulase reaction in rabbit plasma), catalase positive (a feature that distinguishes them from the streptococci and enterococci, although occasional reports of catalase-negative strains have been described (Millar et al., 1986; Del'Alamo et al., 2007; Grüner et al., 2007), oxidase negative and it uses carbohydrates for their metabolism both by oxidation and by fermentation (Branson, 1968; Lowy, 1998). When grown on mannitol salt agar (media containing a pH-based indicator), the fermentation of mannitol that *S. aureus* produces generate a yellow zone around the colony in the red agar. In addition to mannitol, *S. aureus* can metabolize glucose, xylose, lactose, sucrose, maltose, and glycerol (Somerville and Proctor, 2009).

1.3. *Staphylococcus aureus* identification techniques

Due to the pathogenic potential of *S. aureus*, the comprehension of the population structure and evolutionary dynamics of the pathogenic strains and the identification of particularly successful clonal lineages it is a very important tool for the control and prevention of infections produced by this

pathogen. The first step is to discriminate the *S. aureus* isolates from other species of the genus; for this, various phenotypic and molecular techniques have been developed over the years to differentiate the species *S. aureus*, and within it the different bacterial strains and their phylogenetic relationships.

The previously mentioned mannitol salt agar does not discriminate all species of the genus *Staphylococcus*, but a negative reaction allows us to rule out species such as *S. epidermidis*, which produces white colonies on this agar with no color change. Recently, various types of commercial media have been described, some of which can also differentiate methicillin-resistant *S. aureus* (MRSA) from methicillin-sensitive *S. aureus* (MSSA) (Perry et al., 2004) (***Figure 2***); they are more expensive but highly specific for presumptive identification.

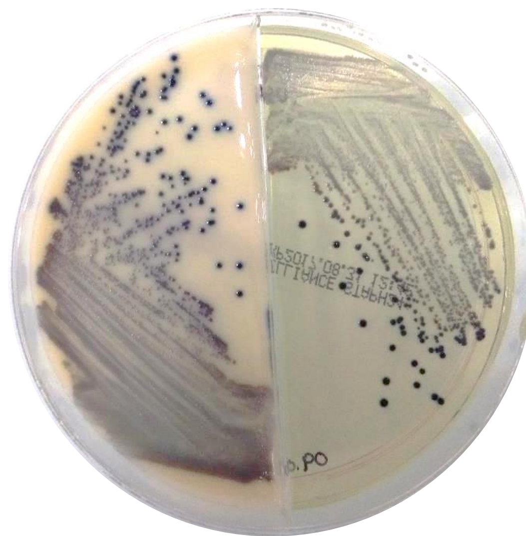

Figure 2*.* Medium Thermo Scientific™ Brilliance™ MRSA 2/Brilliance™ Staph 24 that discriminates methicillin resistant *S. aureus* (MRSA) and coagulase positive staphylococci.

Since the development of molecular diagnosis, there are several genotypic identification methods, such as those based on 16s rRNA sequencing and real-time polymerase chain reaction (RT-PCR)-based diagnostic platforms.

One of the most outstanding discoveries was the development of Pulse Field Gel Electrophoresis (PFGE) method during the mid to late 1980s. This method employs restriction enzymes that recognize an informative number of sites that are used to generate large fragments that are resolved by electrophoresis (Schwartz and Cantor, 1984; Goering, 1993), thereby generating a strain-specific restriction fragment length polymorphism (RFLP) banding pattern. This method can detect variation between closely related strains due to point mutations, insertions, and deletions resulting in loss or gain of individual restriction sites and alteration of fragment sizes. Therefore is still the method of choice for comparing closely related strains in which is highly discriminative. The enzyme of choice in the case of *S. aureus* is *Sma*I because it recognizes GC-rich sequences, and staphylococcal species have low GC content (Poddar and McClelland, 1991; Linhardt et al., 1992; Pantůček et al., 1996). Despite its usefulness the difficulties associated with interlaboratory comparisons, the difficulty to perform PFGE on large number of samples, have led to search for alternative methods for large-scale genotyping.

Since 1998, the multilocus sequence typing (MLST) has become the gold standard for describing the population structure of prokaryotes (Maiden et al., 1998; Enright et al., 2000). It is a DNA sequence-based method that employ seven conserved housekeeping genes that in the case of *S. aureus* are *arc* (carbamase kinase), *aro*E (shikimate 5-dehydrogenase), *glp*F (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triose phosphate isomerase) and *yqi*L (acetyl-Coa acetyltransferase). Each unique sequence recovered for a particular gene is denoted as a specific allele and the concatenation of a given allelic profile is assigned a particular sequence type (designed ST followed by a number). The variation in the sequence types is a result of point mutations within these genes (Feil et al., 2003) and allows the determination of macro or long-term genetic variation and evolution. This method allows to assess the highly clonal population structure of *S. aureus* (Robinson and Enright, 2004). The *S. aureus* MLST database is one of the largest of bacteria

database. The single nucleotide polymorphisms (SNPs) are described as single-locus variants (SLVs) or double-locus variants (DLVs). The sequence type with the greatest number of SLVs is assumed to represent the 'founder' of a given cluster, with large clusters termed 'clonal complexes' (designated CC followed by a number).

Taken together, PFGE and MLST represent give information about different aspects of the genotypic diversity: MLST can capture the large-scale population structure of an entire species but is unable to discriminate between closely related isolates, whereas PFGE is the gold standard for indexing microvariation yet is unable to assess the overall population structure. To find a middle point between the two methods the *spa*-typing emerged (Shopsin et al., 1999; Harmsen et al., 2003).

The *spa*-typing is a single-locus sequence typing method based on sequencing of a hypervariable repeat region within the coding sequence of protein A. This technique is useful for indexing microvariation and macrovariation of the strains and is being used for the analysis of the global epidemiology of *S. aureus* as well as for strain comparison in infection control and localized geographic studies.

Other method consisting of double-locus sequence typing includes two families of surface proteins in the variable genome of *S. aureus*, which are *spa* (Koreen et al., 2004), as in the *spa*-typing, and *clf*B (clumping factor B) (Koreen et al., 2005). This method combines sequencing of repeats in both genes based on sequence variation of variable repeat regions extending from the cell surface.

1.4. Cell wall, cell membrane and surface proteins

The cell wall is made up of 50 percent peptidoglycan, approximately 30 percent theicoic acid and polysaccharides, and contains the high osmotic pressure of the cytoplasm of staphylococci. The peptidoglycan may have endotoxin-like activity, stimulating the release of cytokines by macrophages, activation of complement, and aggregation of platelets. Differences in the

peptidoglycan structure of staphylococcal strains may contribute to variations in their capacity to cause disseminated intravascular coagulation (Kessler et al., 1991). Theicoic acids are approximately 30% of the dry cell wall weight. They take part in cell division, autolytic activity, and biofilm formation (Jorasch et al., 2000).The polysaccharide intercellular adhesin (PIA) is required for the maturation of biofilms (Heilmann et al., 1996) and is important for evasion of the host innate immune system (Vuong et al., 2004a and 2004b).

Penicillin-binding proteins (PBPs) are involved in the biosynthesis of cell wall peptidoglycan; they catalyze the last step of peptidoglycan biosynthesis (Frère et al., 1992). PBPs are of particular importance because MRSA strains have acquired resistance to all β-lactam antibiotics due to a modified PBP2 called PBP2a, which is capable of producing cell wall biosynthesis in the presence of high concentrations of antibiotics due to its low affinity for β-lactam antibiotics (Hartman and Tomasz, 1984; Reynolds and Brown, 1985).

Most staphylococci produce microcapsules made of polysaccharide that increases its adherence capacity, prevents it from being recognized, as well as reinforces the anti-phagocytic effect (Nilsson et al., 1997).

S. aureus has a large array of surface proteins and carbohydrates that enable binding to a broad range of host tissues, including platelets, epithelial cells, endothelial cells and intercellular matrix proteins (Harraghy et al., 2006). Some of the most important *S. aureus* surface proteins are protein A, coagulase (a prothrombin activator that converts fibrinogen to fibrin), and the clumping factor. The synthesis of many of the surface proteins is dependent on the growth phase and is controlled by regulatory genes such as *agr*. These proteins are usually synthesized during the exponential-growth phase. However, many of the secreted proteins are synthesized in the stationary phase, such as Enterotoxin B, TSST-1 (toxic shock syndrome toxin 1) and α-Toxin. The *agr* gene is a global virulence regulator that induces the expression of exoprotein (extracellular protein) while

suppressing the expression of surface protein. This sequential expression of genes may have clinical importance. In the early stages of infection, activation of surface proteins allows host tissues to be successfully colonized while exoprotein synthesis allows the spread to adjacent tissues. This hypothesis is supported by studies in animals showing that the inactivation of regulatory genes reduces bacterial virulence (Cheung et al., 1994). Nearly all strains secrete a group of enzymes and cytotoxins that have the main function of converting local host tissues into nutrients required for bacterial growth, destroy tissue and they have very diverse mechanisms of action. Some of these enzymes and cytotoxins are four hemolisins (alpha, beta, gamma and delta), nucleases, lipases, proteases, hyaluronidase and collagenase. Some strains produce other additional exoproteins, some of which include toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH, and SEI), and leucocidin. Their primary function in vivo is to inhibit host immune responses to *S. aureus*, thus these toxins have potent effects on cells of the immune system, but many of them have other biological effects as well.

1.5. Genomes

Genome sequencing has enabled investigators to explore questions of virulence, resistance, physiology, and host interactions; and has provided insights into their success as significant bacterial pathogens. The first *S. aureus* genome sequences were published in 2001 comparing the genomes of two methicillin-resistant strains, N315 and Mu50 (Kuroda et al., 2001). Subsequently many other genomes of *S. aureus* were published as MW2 (Baba et al., 2002), MRSA252 and MSSA476 (Holden et al., 2004), COL (Gill et al., 2005), USA300 (Diep et al., 2006), ET3-1 (Herron-Olson et al., 2007), JH1 and JH9 (Mwangi et al., 2007), and Newman (Baba et al., 2008).

The *S. aureus* genome consists of a circular haploid chromosome of approximately 2.8 to 2.9 Mb encoding 2563-2721 proteins (Kuroda et al., 2001; Baba et al., 2002; Diep et al., 2006; Herron-Olson et al., 2007) which

contains two distinct regions. The first region called core genome, represent approximately 80% of the bacterial genome and contains conserved genes that encode essential metabolic and regulatory functions, surface proteins with roles in tissue adhesion and surface architecture as well as virulence-associated factors that are expressed by nearly all strains, such as protein A (*spa*), clumping factor (*clfAB*), coagulase (*coa*), fibrinogen-binding protein A (*fnbA*), α-toxin (*hla*), lipase (*lip*), and superoxide dismutase (*sodM*). A region called "*ori*C environ" situated downstream from the origin of replication includes important *S. aureus* genes such as *spa* (encoding protein A) and *coa* (encoding coagulase) and is thought to be related with the evolution and differentiation of the staphylococcal species. Phylogenetic classification indicated that over 50% of predicted proteins encoded by the *S. aureus* genome are most similar to those in *Bacilllus subtilis* and *Bacillus halodurans* (Kuroda et al., 2001). Blast homology search for the most closely related orthologues indicates that the genetic background of *S. aureus* has been vertically transmitted from a common ancestor that subsequently diverged to *Bacillus* and *Staphylococcus* species (Ito et al., 2003). The other region called accessory genome (dispensable genetic material), performs about 20% of the genome and is composed of other variable genes that often show variation associated with lineage. These variable regions may be more prone to diversifying events such as single nucleotide polymorphisms (SNPs), partial or complete gene deletions likely by recombination events, and repeat variation. This is the region with the greatest interest for the study and is mainly composed of MGEs that are integrated throughout the genome and carry about 50% of *S. aureus* virulence factors.

The MGEs of *S. aureus* are pathogenicity islands, bacteriophages, chromosomal cassettes, genomic islands, plasmids and transposons. These MGEs play significant roles in the evolution of *S. aureus* virulence and emergence of new strains with clinical implications because they are regions of the genome that undergo continuous mutations (Lowy, 1998; Baba et al., 2008; Chen et al., 2018). The MGEs can transfer horizontally

between strains differentially, from high frequency to low frequency, while some do not transfer at all. In general, there are three mechanisms of lateral gene transfer in bacteria: conjugation, transformation, and transduction (Thomas and Nielsen, 2005). The horizontal gene transfer (HGT) is of considerable importance in medicine because is the major route by which bacteria acquire virulence factors and antibiotic resistance. MGE-mediated movement of toxin genes is associated with particular lineages that may dominate in carriage and disease (Moore and Lindsay, 2001; Peacock et al., 2002). Comparative analysis of the S. *aureus* genome sequences has given evidence of substantial homologous recombination within MGEs, such that each MGE is composed of multiple short mosaic fragments that are randomly spread through other MGEs of the same type (phage or SaPI). MGEs strongly associated with a particular lineage are presumable stable and transmitted by vertical transmission to next generation bacterial cells. In contrast, MGEs that are randomly distributed among multiple lineages are likely to be transferred horizontally. Combinations of virulence factor carried by the mobile genetic elements (MGEs) and their exchange among strains results in evolution of clones able to infect selected hosts and cause specific diseases (Herron-Olson et al., 2007).

The former virulence and resistance genes tend to be found in *S. aureus* pathogenicity islands (SaPI) whereas the latter rely on SCC, plasmids and transposons for transfer.

1.5.1 Bacteriophages

Staphylococcus aureus harbor temperate and lytic bacteriophages (Kwan et al., 2005) that play key roles in mobilization of virulence factors between strains and genome evolution of virulent isolates. Bacteriophages are the most abundant gene-transfer particles, and phage transduction is generally regarded as the most important mechanism of horizontal gene transfer between bacterial cells. A recent study reports that *S. aureus* temperate bacteriophages engage in a form of transduction different from HGT termed

lateral transduction (Chen et al., 2018) and it can be a universal mechanism of gene transfer present in other bacterial species.

There are two groups of *S. aureus* bacteriophages that are likely responsible for the widespread horizontal gene transfer between strains; they are the generalized transducing phage (Iandolo et al., 2002) and the temperate phage associated with SaPI (Novick and Subedi, 2007; Tallent et al., 2007). The SaPI element encodes proteins that remodel the phage capsid to accommodate the smaller SaPI genome (Tormo et al., 2008).

A subgroup of temperate *S. aureus* bacteriophages produces the inactivation of virulence genes by inserting into these genes, this phenomenon is called lysogenic conversion. An example is the serogroup F bacteriophages which insert into and inactivate the β-hemolisyn (*hlb*) gene. The Hlb-converting phages are most frequently associated with clinical isolates of *S. aureus*. Phages encoding immune evasion molecules (SCIN, CHIP) which integrate specifically into the *hlb* gene in *S.aureus*, are widely distributed. The dynamics of these phages during host infection lead to the splitting of phage population into two populations exhibiting different virulence potentials: phages that produce Hlb and phages that produce immune evasion molecules (SCIN and CHIP) (Goerke et al., 2006).

Another mechanism that leads to the emergence of virulent *S. aureus* clinical isolates is the mobilization of bacteriophages encoding specific virulence factors. For example, Panton-Valentine leucocidin (PVL) is encoded by bacteriophages from Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates associated with soft tissue infections and necrotizing pneumonia in children (Gillet et al., 2002).

1.5.2. Pathogenicity islands

Pathogenicity islands (SaPIs) have a general genomic organization extremely conserved and like that of temperate phages. They also depend on staphylococcal phages to carry out excision, replication, packaging into infectious small-headed phage-like particles, and finally mobilization into recipient strains (Tormo et al., 2008) resulting in extremely high transfer

frequencies. SaPIs are highly common in *S. aureus* and are composed from 15 to 17 kb. At least 16 SaPIs have been found on the *S. aureus* chromosome. They are named according to the loci of the chromosome to which the islands are integrated; SaPI1 is considered as the prototype (Novick, 2003; Subedi, 2007). Core genes include two open reading frames encoding transcriptional regulatory proteins and a region encoding integrase, Rep protein, and terminase. Some SaPIs confer pathogenicity while others confer antibiotic resistance. The two SaPI families, SaPI2 and SaPI3, encode virulence genes such as leucocidine (lukDE) and staphylococcal superantigens (enterotoxins and exotoxins), and all SaPIs encode enterotoxins or toxic shock syndrome toxin (TSST) (Thomas et al., 2007; Yarwood et al., 2002). Antibiotic treatment is thought to induce the mobilization of SaPIs (Úbeda et al., 2005; Maiques et al., 2006).

1.5.3. Genomic islands

In contrast to the SaPIs, the genomic islands are stably integrated in the same position in all *S. aureus* genomes and do not appear to be mobile. All sequences *S. aureus* genomes contain sets of three genomic islands that are variable between strains. Gene diversification in the island occurs as a result of gene loss and recombination (Fitzgerald et al., 2003). Given the composition of genomic islands, it is suspected that genomic islands were once mobile elements acquired by HGT (Dobrindt et al., 2004). Both flanking DNA segments contribute to the stability of genomic islands within the *S. aureus* chromosome.

1.5.4. Staphylococcal cassette chromosome

The staphylococcal cassette chromosome (SCC) is an important MGE because it contains multiple virulence and antimicrobial resistance genes. In contrast to other MGE, cassette chromosome *mec* (SCCmec; *mec* standing for methicillin resistance) preferentially carries antibiotic resistance genes rather than pathogenicity factors. SCC is characterized by a set of site-specific recombinase genes (*ccr*A and *ccr*B). The SCC*mec* element is the most common in *S. aureus*, which contains the *mec*A gene and the

recently discovered *mec*C gene (Paterson et al., 2014a) encoding methicillin resistance. The SCC*mec* elements are classified in different groups based on whether they are found in Hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA) or CA-MRSA isolates: types I-III are HA-MRSA and types IV-VII are CA-MRSA. Evolutionary studies suggest that SCC*mec* likely originated in coagulase-negative staphylococci (Yuki Katayama et al., 2001). This MGE, in contrast to other MGEs, have variable contents of insertion elements and plasmids suggesting that recombination events are modifying the element (Robinson and Enright, 2003) to convert it into a smaller element capable of being mobilized by transduction at a higher frequency.

1.5.5. ACME element

The arginine catabolic mobile element (ACME) is a genomic island in Staphylococci that contain the six-gene arc cluster that plays several roles in virulence and survival in an infection. Some authors have suggested that ACME is a new member of the SCC family. The ACME element is integrated downstream of the staphylococcal chromosomal cassette (SCC) harboring the methicillin resistance gene *mec*A (SCC*mec*) and use the same attachment site as SCC*mec* for integration within *orf*X. Similar to SCC*mec* elements, ACME is flanked by repeat sequences, and SCC*mec*-encoded cassette chromosome recombinase (*ccr*) genes catalyze integration and excision of ACME from the staphylococcal chromosome (Diep et al., 2006). ACME exists as a composite island with SCC*mec* IVa (ACME/SCC*mec*-CI) in ST8-MRSA-Iva (also known as USA300) and this strain is the predominant CA-MRSA strain in the United States. The extensive spread and success of USA300 has been partially attributed to the presence of the ACME element, which is thought to play an important role in its growth and survival (Shore et al., 2011b).

1.5.6. Plasmids

Plasmids are auto-replicating DNA molecules. *S. aureus* possess relatively few plasmids. Most of the sequenced *S. aureus* genomes contain one or

more extrachromosomal plasmids that use to contain genes encoding resistance to antibiotics and heavy metals or antiseptics and virulence factors including enterotoxins and exfoliative toxins. *S. aureus*, unlike other pathogens, utilizes transduction for horizontal transfer of plasmids between isolates. In some cases, extrachromosomal replicating plasmids have integrated into de *S. aureus* genome.

2. Interaction between the pathogen and the host: Pathogenesis of *Staphylococcus aureus*

Staphylococcus aureus is a versatile and dangerous pathogen in humans that has a wide host range, diverse cellular and environmental lifestyles, and the ability to highly develop drug-resistant forms. It is the species of the genus *Staphylococcus* that most frequently causes clinical processes in the hosts it colonizes (Witte et al., 2014). In fact, has long been recognized as a major human pathogen and remains a frequent cause of morbidity and mortality. Several studies show that *S. aureus* is one of the most common cause of nosocomial infections (Fridkin et al., 2002). It can be transmitted by direct contact or through secretions or fomites. Despite of the high pathogenicity of *S. aureus*, it is an opportunistic pathogen. This means that it is a commensal microorganism of the skin and mucous membranes that awaits favorable conditions, like wounds, immunosuppression or other concomitant diseases, to multiply and to produce an infection (Kobayashi et al., 2015).

2.1. *Staphylococcus aureus* colonization and infection

S. aureus infections in humans differ in community-acquired and hospital-acquired staphylococcal infections. Besides, *S. aureus* has two distinct lifestyles: a commensal asymptomatic state, and acute state where it invades specific tissues and pathogenicity ensures (Lowy, 1998). Humans are a natural reservoir of *S. aureus*; in healthy individuals, it can colonize the skin, nails and nostrils without causing any type of lesion. These individuals are known as carriers, which can be persistent or intermittent.

Colonization with *S. aureus* does not have a protective effect; on the contrary, it increases the risk of infection by this pathogen (Wenzel and Perl, 1995). Nevertheless, one study shows that after nosocomial infection, colonized individuals have less severe *S. aureus* disease compared with noncolonized individuals (Wertheim et al., 2004).

When immunosuppression occurs (due to stress, other diseases, etc.) or wounds on the skin and mucosa, the bacteria can multiply and invade the tissues, usually producing suppurative lesions. *S. aureus* causes a broad range of infections in humans that are varied in their severity and tissue tropism ranging from relatively mild conditions including folliculitis, superficial and deep skin abscesses, skin and soft tissue infections, to more severe disease such as wound infections, impetigo, osteomyelitis, pneumonia, suppurative arthritis, pleural emphysema, meningitis, which can be complicated by septicemia and endocarditis, toxic shock (Lowy, 1998). It is obvious that there is host susceptibility and host-pathogen dynamics at play to determine if colonization or infection occurs, although these phenomena are poorly understood.

Nasal carriage is the most frequent form of colonization of *S. aureus*, a high percentage of the population is carrier without showing symptoms. However, carriers have been found in the throat and on the skin, with negative culture in the nose (von Eiff et al., 2001; Kluytmans and Wertheim, 2005). Therefore, anterior nares cultures will detect most, but not all, people who are colonized with *S. aureus* (Shurland et all., 2009). Host immune deterrents for bacterial nasal colonization include antimicrobial peptides, lysozyme, lactoferrin, and IgA (Wertheim et al., 2005). However, little is known of the critical host defenses against *S. aureus* colonization.

A higher incidence rate of nasal carriage of community-acquired *S. aureus* (CA-MRSA) has also been associated with individuals having frequent contact with animals, suggesting that they can be vectors in the spread of CA-MRSA (Hanselman et al., 2006; Weese et al., 2006; Weese and Lefebvre, 2007).

Infection is often preceded by colonization or direct or indirect contact with the pathogen and occurs when *S. aureus* enters tissues and establish an invasive infection on breaches in the skin or mucous membranes (Lowy, 1998). *S. aureus* has a versatile tissue tropism attributed to its remarkable set of virulence factors involved in pathogenesis.

2.2. *Staphylococcus aureus* immune evasion: Virulence factors

This organism produces more than 30 virulence factors that provide the ability to colonize the host, adhere to surfaces as biofilm, invade or evade the immune system, develop resistance to multiple antibiotics and cause toxicity to the host that lead pathogenic bacteria adapt to survival in their host. One of the mechanisms of *S. aureus* to cause disease is evasion of innate host defense, which includes resistance to antimicrobial peptides (AMPs) and killing by phagocytic leukocytes. Two of the *S. aureus* two-component systems (TCSs) GraSR/VraFG, play an essential role in antimicrobial peptide resistance (Li et al., 2007; Meehl et al., 2007). Initial exposure of *S. aureus* to host tissues beyond the mucosal surface or skin is thought to trigger up-regulation of virulence genes (Novick, 2003). *S. aureus* has an astounding repertoire of immune evasion factors that try to subvert host defense mechanisms. Although the pathogen produces a proinflammatory response, it generates several molecules that block chemotaxis of polymorphonuclear leukocytes (PMNs).

Phagocyte function may be subverted at many different stages. *S aureus* may hide from recognition by producing protective coats, such as capsular polysaccharide or biofilm. Further, they produce or secrete specific molecules to block phagocyte receptor function. After ingestion, the bacteria use mechanisms to decrease the efficiency of antimicrobial mechanisms, which likely account for noted post phagocytosis survival (Voyich et al., 2005). Finally, they often produce toxins that lyse phagocytes, thus using the same kind of weapon that neutrophils use to kill bacteria.

There are numerous virulence factors and mechanisms of *S. aureus* to evade the host innate immune response that determines the success or

failure of the pathogen in an infection. The most important virulence factors in *S. aureus* are listed below.

2.2.1. The accessory gene regulator *agr*

Among the genes that control *S. aureus* colonization and virulence, the best global regulator is the accessory gene regulator *agr*. Although it is not directly a virulence factor, this gene is a quorum sensing locus which directly controls expression of a high number of virulence and colonizing factors such the coagulases, protein A and haemolysins (Wolz et al., 1996). Down-regulation of *agr* is associated with colonization and activation of *agr* with host invasion (Traber et al., 2016).

2.2.2. Polysaccharide intercellular adhesion

Biofilm formation is the main pathogenetic mechanism leading to the chronicity and irreducibility of infections. The extracellular polymeric substances of staphylococcal biofilms are the polysaccharide intercellular adhesin (PIA), extracellular-DNA, proteins, and amyloid fibrils (Arciola et al., 2015). Polysaccharide intercellular adhesion (PIA) is a biofilm-related extracellular matrix substance with a positive net charge whose synthesis is mediated produced by the *ica*ADBC locus, and its main function is protecting from neutrophil phagocytosis and AMPs (Vuong et al., 2004b). The *ica* locus was found present in biofilm producing *S. aureus* strains responsible for catheter and implant infections (Arciola et al., 2001).

2.2.3. The staphylococcal immune evasion cluster

The immune evasion cluster (IEC) located on β-haemolysin-converting bacteriophages (βC-Φs), harbor *scn* and a different combination of *sak*, *chp* and *sea* (or *sep*) genes that encode the human-specific immune modulators including staphylococcal complement inhibitor (SCIN), staphylococcal enterotoxin A (SEA), staphylokinase (SAK) and chemotaxis inhibitory protein of *S. aureus* (CHIPS). The SCIN secreted *S. aureus* molecules block the complement system, thereby reducing phagocytosis by PMN after

opsonization and neutrophil chemotaxis (Wamel et al., 2006). SEA is involved in the down-regulation of chemokine receptors of monocytes (Balaban and Rasooly, 2000). SAK achieves its function primarily by forming a plasminogen activating complex together with plasmin itself, which prevents biofilm formation (Kwiecinski et al., 2016), and also initiates the fibrinolytic cascade to help the invading bacterium move deeper into tissues (Bergmann and Hammerschmidt, 2007). SAK is also able to neutralize host antimicrobial peptides (AMPs) (Rooijakkers et al., 2005). Finally, CHIPS is a molecule that blocks receptor-mediated recognition of formylated peptides, which are PAMPs secreted by bacteria and central for phagocyte detection of bacterial invaders (De Haas et al., 2004).

2.2.4. The microbial surface component recognizing adhesive matrix molecules (MSCRAMM)

The ability to adhere effectively to multiple host tissues is a critical factor for the pathogenic success of *S.aureus*. The microbial surface component recognizing adhesive matrix molecules (MSCRAMM) are adhesin proteins that mediate the initial attachment of bacteria to host tissue, providing a critical step to establish infection. Nevertheless, MSCRAMM proteins provide an excellent target for immunological attack by antibodies because the highly specific MSCRAMM antibodies prevent microbial adherence (McDevitt et al., 1995; Rennermalm et al., 2001), as well as recolonization of host tissues or biomaterials; and because the increased level of MSCRAMM protein antibodies bound to the bacterial cell wall facilitates rapid clearance of the organism through opsonophagocytosis (Rozalska and Wadstrom, 1993; Nilsson et al., 1998). Some of these proteins include protein A, clumping factor A (ClfA), fibronectin binding protein A (FnbpA) and SdrG (Foster et al., 2014). All these MSCRAMMs bind to fibrinogen except protein A, that binds IgG.

Clumping factor A (ClfA) is an MSCRAMM protein expressed by *S. aureus* that promotes binding of fibrinogen and fibrin to the bacterial cell surface (McDevitt et al., 1994). This protein mediate direct binding to human

platelets (Siboo et al., 2001) and protects bacteria from neutrophil phagocytosis through the recruitment of fibrinogen to the bacterial cell surface (Higgins et al., 2006).

The staphylococcal protein A (SpA), encoded by the *spa* gene, has several functions. The interaction of protein A with the Fc part of IgG molecules enables *S. aureus* to sequester nonspecific antibodies on its surface, which protects efficiently from attacks by the innate and acquired immune systems (Forsgren and Nordström, 1962). Protein A also has a more specific role in the pathogenesis of airway infections by interacting with the tumor necrosis factor (TNF) a receptor on airway epithelia which leads to the activation of intracellular signaling, the expression of cytokines and the recruitment of neutrophils (Gómez et al., 2004).

Staphylococcus aureus has been shown to adhere to and invade human endothelial cells, potentially the first step in the pathogenesis of disseminated infection. *S. aureus* fibronectin-binding protein (FnBPA) is central to the invasion of endothelium, fibronectin forming a bridge between bacterial fibronectin-binding proteins and host cell receptors. FnBPA is capable of conferring both adherence to fibronectin and endothelial cells, and endothelial cell invasion (Massey et al., 2001).

The Serine-Aspartate Repeat Proteins (Sdr) are encoded by the *sdr*C, *sdr*D, and *sdr*E genes located in the sdr locus. At least two *sdr* genes are present in all tested *S. aureus* strains (Josefsson et al., 1998) and always include *sdr*C (Peacock et al., 2002). The Sdr proteins are characterized by the presence of an R region containing various numbers of the Ser-Asp dipeptides and have a similar structural organization. A signal peptide is followed by an A region which is similar in size among the different members of the Sdr family. The function of Sdr proteins in *S. aureus* remains unknown; however, there have been a few studies which reported a strong correlation between *sdr* genes and certain human diseases (Peacock et al., 2002; Trad et al., 2004; Sabat et al., 2006). The presence of insertion mutation and deletion mutation in the *sdr* genes suggests that they are

variable (Xue et al., 2011). These findings lead to better understanding the emergence of traits such as increased virulence or antibiotic resistance.

2.2.5. Toxic shock syndrome toxin (TSST)

Potentiation or overstimulation of the immune response is a very effective way of interfering with the human immune system. *S. aureus* produces many superantigenic toxins (SAGs), a class of secreted toxins that activate T-cells without the need for the presence of an antigen on an antigen-presenting cell (McCormick et al., 2001). These toxins include the toxic shock syndrome toxin (TSST), a low-molecular-weight single peptide toxin secreted by some strains of *S. aureus* that is highly associated with menstrually related toxic shock syndrome (TSS) and is considered a likely causative toxin. These superantigens bypass the normal pathway for activation of T cells resulting in over-activation of cytokines and inflammatory cells what produces symptoms like fever, rash formation, and hypotension that can lead to multiple organ failure and lethal shock, as well as desquamation in patients that recover (Wiesenthal et al., 1985). Nonmenstrual-associated staphylococcal TSS is normally associated with TSST-1, staphylococcal enterotoxin (SE) serotype B (SEB), or SEC (Bohach et al., 1990).

2.2.6. Phenol-soluble modulins and α-toxin

S. aureus has a great number of immune evasion passive mechanisms to hide from recognition or blocking receptors or effectors involved in the elimination of the bacteria. But it also produces toxins that directly attack white and red blood cells. These toxins include the large family of leukocidins and α-toxin (also known as α-hemolysin), phenol-soluble modulins (PSMs), and other hemolysins.

The PSMs (PSMα1, PSMα2, PSMα3, PSMα4) are secreted peptides that interpose in the acute response of the host against *S. aureus*, diminishing the response of neutrophils. PSMs contribute significantly to increase virulence of CA-MRSA relative to HA-MRSA (Wang et al., 2007).

S. aureus α-toxin (toxin A, hemolysin A (Hla)) is a major virulence-associated factor contributing significantly to the pathogenicity of the respective *S. aureus* strains that plays an essential role in pneumonia. Hla activates defensive mechanisms of the epithelium (Räth et al., 2013) and affects activation or inhibition of different intracellular signaling pathways that triggers necrotic or apoptotic cell death (Below et al., 2021).

2.2.7. Panton-Valentine leukocidin

Panton-Valentine leukocidin (PVL) has an epidemiologic correlation with community-associated MRSA because some CA-MRSA harbor a phage harboring PVL genes, and they are thought to be responsible at least in part for the increased virulence of CA-MRSA. This is because *pvl* has been associated with epidemic CA-MRSA strains causing skin and soft tissue infections, necrotizing pneumonia and lethal necrotizing fasciitis (Mongkolrattanothai et al., 2003; Miller et al., 2005). Some studies show that there is a high association between MRSA USA300 nasal/axillary carriage and presence of PVL in persons with abscesses (Immergluck et al., 2017). The subunits of the PVL are encoded by the genes *luk*S-PV and *luk*F-PV (*pvl*). PVL is a bicomponent, pore-forming leukotoxin with ability to lyse leukocytes or cause apoptosis (depending on the concentration). But unlike other *S. aureus* pore-forming leukocidins, PVL is not hemolytic (Boyle-Vavra and Daum, 2007). Regardless, the precise role of PVL in pathogenesis remains to be elucidated.

2.2.8. Coagulases

Coagulation is an ancient innate defence mechanism against microbial pathogens that traps and immobilizes invading bacteria in a clot (Loof et al., 2011), however coagulation is also the target of bacterial immune evasive strategies. To date, two coagulases have been described in *S. aureus*: coagulase (Coa) and von Willebrand Factor Binding Protein (Vwbp); both promote similar modifications of the coagulation cascade during host infection. The coagulase protein is encoded by the *coa* gene and is a

polypeptide that bind to and activate prothrombin, thereby converting fibrinogen to fibrin and promoting the clotting of plasma or blood; this leads to the formation of abscesses and bacterial persistence in host tissues and also enables the pathogen to cause lethal sepsis (McAdow et al., 2012). The Vwbp, that was discovered at the beginning of this century (Bjerketorp et al., 2002), binds to the von Willebrand factor and also to the prothrombin, triggering the coagulation as well. The Vwbp protein is encoded by the *vwb* gene. When both *coa* and *vwb* genes are deleted, the bacterium is unable to coagulate the plasma and, therefore, the pseudocapsule of the abscess is not formed and the lesion is less severe than those produced by the wild type. But the effect of *coa* is greater than *vwb*, as deletion of the *coa* gene makes the bacteria take longer to coagulate the plasma than deleting only the *vwb* (Cheng et al., 2010).

2.2.9. Haemolysins

Haemolysins also play an important role in the development of the infection, as they cause the lysis of blood cells. *S. aureus* can produce four different haemolysins (alpha, beta, delta, and gamma).

A high percentage of strains produce alpha-hemolysin, encoded by the *hla* gene, that is the most characterized virulence factor of *S. aureus* This haemolysin is toxic to a wide range of mammalian cells, and it is also dermonecrotic and neurotoxic. The defining characteristic of alpha-toxin is its ability to lyse erythrocytes. In particular, rabbit erythrocytes are extraordinarily susceptible to hemolysis by alpha-toxin, at least 100 times more so than other mammals and 1,000 times more than human erythrocytes (Bhakdi et al., 1984). Alpha-hemolysin produces pores on the cell surface leading to necrotic death of the target cell (Vandenesch et al., 2012).

S. aureus β-hemolysin is selectively cytotoxic to monocytes and is inactive against lymphocytes, granulocytes, and fibroblasts (Walev et al., 1996). It is highly hemolytic for sheep but not rabbit erythrocytes and it is not dermonecrotic in guinea pigs, and it is not lethal in mice. The gene that

encodes alpha-hemolysin is *hlb* gene. Beta-hemolysin is produced in large quantity by a high number of *S. aureus* strains, particularly animal isolates. The role of β-hemolysin in disease is not clearly understood, but this high level of expression in animal strains indicates that β-hemolysin producers have some selective advantage from toxin secretion (Dinges et al., 2000).

Delta-hemolysin, encoded by the *hld* gene, causes membrane damage in a variety of mammalian cells. Delta-hemolysin is capable of lysing erythrocytes and other mammalian cells, as well as subcellular structures such as membrane-bound organelles, spheroplasts, and protoplasts (Freer and Birkbeck, 1982). However, its importance in disease etiology remains unclear.

Two types of bicomponent toxins are synthetized by *S. aureus*, gamma-hemolysin and PVL. Gamma-hemolysin is produced by almost every strain of *S. aureus*, while only 2 to 3% of strains secrete PVL. These toxins affect neutrophils and macrophages, and gamma-hemolysin is additionally able to lyse many varieties of mammalian erythrocytes (Dinges et al., 2000). Gamma-hemolysin is not identifiable on blood agar plates, due to the inhibitory effect of agar on toxin activity (Prevost et al., 1995).

2.3. Host defense against *Staphylococcus aureus*

The crucial role of innate host defense is eliminating invading *S. aureus*. In humans, the primary cellular defense against *S. aureus* infections are the polymorphonuclear leukocytes (PMNs), also called neutrophils or granulocytes, which constitute the greatest number of leukocytes in people (DeLeo et al., 2009). A first step in the eradication of invading microorganisms is active recruitment of PMNs to the site of infection by chemotaxis. The host- and pathogen-derived chemotactic factors make neutrophils being mobilized from peripheral blood or bone marrow. It has been demonstrated that stimulation of CD4+ T-cells by *S. aureus* capsular polysaccharide leads to production of chemokines that recruit neutrophils to the site of infection (Tzianabos et al., 2001; McLoughlin et al., 2006) .

Many chemoattractants are priming agents (rather than activating agents) for neutrophils. Neutrophils can be primed for enhanced adhesion, phagocytosis, production of reactive oxygen species (ROS), cytokine secretion, leukotriene synthesis, degranulation, and bactericidal activity. Many neutrophil priming agents are host-derived molecules such as cytokines, chemokines, and growth factors (Kobayashi et al., 2005). Primed neutrophils produce mobilization of secretory vesicles (and thus up-regulation of specific cell surface receptors, eg, CD11b/CD18) and secretion of cytokines, but fails to trigger release of azurophilic granules or elicit production of superoxide that is produced by fully activated neutrophils (DeLeo et al., 1998). In summary, chemotactic/priming agents ultimately promote efficient clearance of invading microorganisms.

One of the most important processes made by neutrophils is phagocytosis. It consists of binding and ingesting invading microorganisms. It is a critical step in the removal of bacteria during infection. PMNs recognize many surface-bound or freely secreted molecules produced by bacteria known as pathogen-associated molecular patterns (PAMPs). They interact with pattern recognition receptors expressed on the neutrophil cell surface, including TLRs. Neutrophil TLRs activate signal transduction pathways that contribute to microbicidal activity (Hayashi et al., 2003). Peptidoglycan recognition protein (PGRP) is a secreted host protein that contributes to intracellular killing of gram-positive bacteria by neutrophils. In contrast to TLRs, which promote recognition of bacteria, PGRP-S contributes directly to bactericidal activity (Liu et al., 2000). The concerted action of pattern recognition receptors/molecules and antibody and complement receptors promotes efficient phagocytosis of microbes (***Figure 3***).

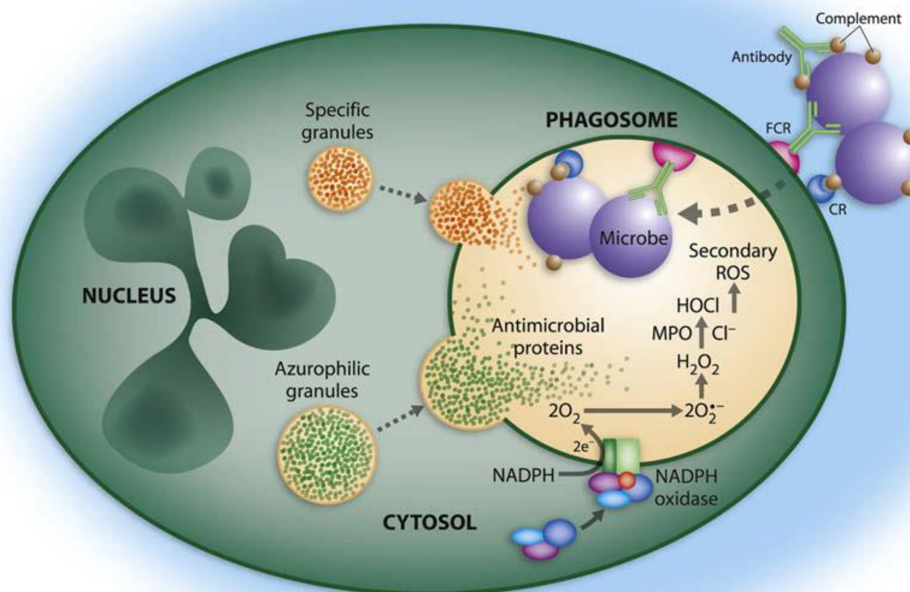

Figure 3. Polymorphonuclear leukocytes phagocytosis and microbicidal activity. Abbreviations: CR, complement receptor; FCR, Fc receptor; MPO, myeloperoxidase. (Adapted from Quinn et al., 2006).

Once *S. aureus* is phagocytosed, the phagosome is formed within the PMNs. Then, a membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase is activated, this process is traditionally called respiratory burst, and generates high levels of superoxide (Quinn et al., 2006). Next, degranulation occurs when neutrophil cytoplasmic granules fuse with bacteria-containing phagosomes. Fusion of azurophilic granules with phagosomes enriches the vacuole lumen with numerous antimicrobial peptides (AMPs) and antimicrobial proteins (**Figure 3**). The PMNs are able to produce structures called neutrophil extracellular traps (NETs), composed of chromatin, histones, and azurophilic granule proteins, which have the capacity to kill bacteria, including *S. aureus* (Amulic et al., 2012).

Finally, neutrophil turnover must be regulated highly during infection because host tissues can be damaged by the inadvertent release of

cytotoxic components. Normal turnover of aging neutrophils occurs by spontaneous apoptosis and in the absence of an activating agent, but neutrophil apoptosis is accelerated significantly following phagocytosis and this phenomenon appears critical to the resolution of the inflammatory response (Kobayashi et al., 2002). Phagocytosis and killing of bacteria culminate with induction of neutrophil apoptosis (also called phagocytosis-induced cell death) and subsequent removal by macrophages, ultimately resulting in the resolution of infection.

It is therefore evident that the phagocytosis produced by PMNs is very important and decisive in the resolution of the infection.

2.4. Resistances to antibiotics in *Staphylococcus aureus*

Widespread antimicrobial resistance among *S. aureus* isolates is cause for considerable concern and has resulted in substantial increases in the cost of treatment associated with *S. aureus* infection. It is well known that the organism acquires resistance soon after the introduction of new antibiotics (Lyon and Skurray, 1987). Penicillin-resistant *S. aureus* was reported within 4 years of the introduction of penicillin G into clinical use in 1941. Then, other antibiotics such as erythromycin, tetracycline, and aminoglycosides were used for the treatment of patients infected by penicillinase-producing *S. aureus* only to result in the appearance of multi-drug resistant *S. aureus* by the 1950s.

Antibiotic resistance per se does not contribute to virulence it is simply a form of adaptation to the environment that the bacteria develop. The adaptive capacity of *S. aureus* is very important for the development of antibiotic resistance. The mechanisms of acquisition of resistance in *S. aureus* are classified into two main categories: mutation of a bacterial gene on the chromosome and acquisition of a resistance gene from other organisms by some form of genetic exchange (conjugation, transduction, or transformation). In the case of resistance acquisition, exogenous antibiotic resistance genes are found on some mobile genetic elements (plasmids, insertion sequences, transposons, or genomic islands) of resistant bacteria.

Since the appearance of bacteria resistant to antibiotics various mobile genetic elements carrying antibiotic resistance genes in staphylococci have been investigated (Projan, 2000). In *S. aureus* there are numerous genes that confer resistance to different antibiotics: penicillins (*bla*Z), aminoglycosides (*aac*A-D), tetracyclines (*tet*K and *tet*M), macrolides (*erm*A, *erm*B, *msr*A, *msr*B and *mef*A), fluoroquinolones (*gyr*A and *grl*A), lincosamides (*lin*A), folate inhibitors (*dfr*A1), phenicols (*cfr*), and ansamycins (*rpo*B) (Rahi et al., 2020).

Some of the mechanisms of resistance to antibiotics in *S. aureus* are explained below.

2.4.1. Resistance to macrolides

Emergence of resistance to macrolides occurred in staphylococci shortly after the therapeutic use of erythromycin (Chabbert, 1956). The mechanisms responsible for resistance to erythromycin in staphylococci are target site modification (Leclercq and Courvalin, 1991; Weisblum, 1995) and active drug efflux and enzymatic inactivation of the drugs. Resistance to macrolide, lincosamide and streptogramin B (MLS_B) antibiotics is mediated by the presence of *erm* genes (*erm(*A), *erm(*B) and *erm(*C)) that are responsible to the target site modification mechanism. Macrolide efflux is produced by membrane proteins encoded by the *msr(*A)/*msr(*B) genes and is specific for macrolides and streptogramin B (MS phenotype) (Lina et al., 1999; Roberts et al., 1999). The *mph(*C) gene, coding for macrolide phosphotransferase C (Mph(C)), has recently been detected in low-level resistant staphylococcal isolates of bovine (Lüthje and Schwarz, 2006) and equine (Schnellmann et al., 2006) origin.

2.4.2. Resistance to tetracyclines

Tetracycline resistance is widespread in *S. aureus* clinical strains. There are four tetracycline-resistance determinants, *tet*K, *tet*L, *tet*M and *tet*O. Most of the resistant strains carry either *tet*K or *tet*M gene and rarely possess *tet*L gene (Martin et al., 1986; Schmitz et al., 2001). The transposon Tn5801 carries *tet*M gene and is responsible of its dissemination. Two mechanisms

of tetracycline resistance have been identified in *Staphylococcus* species. The first mechanism involves the genes *tet*M or *tet*O which encode a protein that confers resistance to tetracycline as well as minocycline by interacting with the ribosome and promoting the release of bound tetracycline (Burdett, 1991, 1996). These genes are located in a transposon or in the chromosome. Some studies suggest that all *tet*M-positive isolates by polymerase chain reaction (PCR) are resistant to all tetracyclines including minocycline (Trzcinski et al., 2000). The second mechanism involves the *tet*K gene which encodes a protein that enhances the efflux of tetracycline; it is usually carried by small-sized plasmids, and one of them, pT181, is found inserted in type-III SCC*mec*. *S. aureus* strains carrying *tet*K only have been described as tetracycline resistant, but minocycline susceptible.

The tetM and tetK genes are the most prevalent single tetracycline resistance determinants in MRSA and MSSA. Furthermore, isolates harboring both the *tet*K and *tet*M genes display higher MIC values than the isolates containing just one of the genes (Schmitz et al., 2001).

2.4.3. Resistance to aminoglycosides

Aminoglycosides are rarely used as monotherapy against *S. aureus* due to the risk of development of resistance and toxic effects. Despite this, *S. aureus* has developed mechanisms of resistance to aminoglycoside antibiotics such as gentamicin, streptomocin and neomycin. The most widespread mechanism of resistance to aminoglycosides is the modification of the antibiotics by cellular enzymes, such as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyltransferases (AAD) and aminoglycoside phosphotransferases (APH) (Shaw et al., 1993). Further, in *S. aureus*, chromosomal mutation affecting ribosomes may confer a high level of resistance to the streptidine-containing aminoglycoside streptomycin (Lacey and Chopra, 1972), and mutations which affect the cellular permeability may provide low-level cross resistance to most aminoglycosides (Shannon and Phillips, 1982). As a treatment, it has been shown that some substance capable of carrying out transposon inactivation

of genes (i.e. *atp*A, *atp*B, *atp*G or *atp*H) encoding the subunits of the ATP synthase increase the efficacy of gentamicin against *S. aureus* (Vestergaard et al., 2016)

2.4.4. Resistance to vancomycin

The first study of *S. aureus* with reduced susceptibility to vancomycin (MIC of 8 lg/mL) was reported in Japan (Hiramatsu et al., 1997). Subsequently, eight isolates of vancomycin-intermediate *S. aureus* (VISA) were documented in the United States (Centers for Disease Control and Prevention, 1997). VISA strains have abnormal, thickened cells walls in the presence of vancomycin. Two mechanisms of resistance have been described in the strains: affinity trapping of vancomycin molecules by cell wall monomers and clogging of the outer layers of peptidoglycan by bound vancomycin molecules, and change in the structure or metabolism of teichoic acids. (Appelbaum and Bozdogan, 2004).

In 2002 occurred the emergence of the first vancomycin-resistant *S. aureus* (VRSA) in Michigan as a result of interspecies conjugative transfer of the transposon Tn1546 carrying *van*A gene between vancomycin-resistant *Enterococcus faecalis* (VRE) and *S. aureus* co-isolates in a polymicrobial infection (Weigel et al., 2003) under such selective pressure. In *S. aureus* the Tn1546 (*van*A) element is integrated into a multirresistant conjugative plasmid. The transfer of Tn1546 (*van*A) is an uncommon event with very few isolates identified, most of them from Michigan. All the donor VRE isolates analyzed from Michigan contained an Inc18- like *van*A conjugative plasmid that is the likely factor in the geographically restricted emergence of VRSA (Zhu et al., 2008). These developments are of great concern, and every effort should be made to prevent further development and spread of vancomycin resistance in staphylococci.

2.4.5. Resistance to bacitracin

Bacitracin is a polypeptide antibiotic produced by *Bacillus subtilis* and *Bacillus licheniformis* (Johnson et al., 1945; Azevedo et al., 1993), is active against gram-positive bacteria and used to prevent and treat skin and

ophthalmic infections. It binds to undecaprenyl pyrophosphate (UPP), resulting in inhibition of cell wall biosynthesis (Stone and Strominger, 1971). Some two-component systems (TCSs) have been demonstrated to affect the susceptibility to antibacterial agents. Gram-positive bacteria have developed several mechanisms of bacitracin resistance but the BceSR two-component system (TCS)/BceAB ABC transporter is the most efficient and well-studied. Several studies show that the TCS *Bce*RS is associated with an increase susceptibility to bacitracin, implying that this TCS may be involved in bacitracin sensing and also resistance to bacitracin. *Bce*RS senses bacitracin and also positively regulates the expression of two ABC transporters that function in bacitracin efflux (Yoshida et al., 2011). On the other hand, it has been hypothesized that *bac*A encodes undecaprenol kinase, and that when this enzyme is overproduced it is able to produce sufficient supplies of UP from undecaprenol to overcome the effects of UPP sequestration, thereby causing bacitracin resistance. The gene is not essential for in vitro growth, and seem to be implicated in virulence during an infection (Chalker et al., 2000).

2.4.6. Resistance to trimethoprim-sulfamethoxazole

Bacterial resistance to trimethoprim-sulfamethoxazole (TMP-SMX) has not been fully elucidated but it may develop independently of TMP and SMX. While SMX is a sulfonamide, TMP is a diamino pyridine and both belong to the peptide family like vancomycin and bacitracin. Sulfonamide resistance among *S. aureus* may be attributed to two mechanisms: Most resistance is likely due to chromosomally mediated overproduction of para-aminobenzoic acid (Grim et al., 2005). Although a sulfonamide-resistant plasmid has also been described in *S. aureus*, the mechanism of resistance is unclear (Lyon and Skurray, 1987). Resistance to TMP also may be chromosomally or plasmid mediated (Zinner and Mayer, 2000).

2.4.7. Resistance to chloramphenicol

Chloramphenicol shows activity against many gram-positive and gram-negative bacteria and is licensed exclusively for use in veterinary medicine.

Bacterial resistance to chloramphenicol and thiamphenicol is most commonly mediated by mono- and diacetylation via chloramphenicol acetyltransferase (CAT) enzymes. Acceptor site for acetyl groups is structurally altered in florfenicol. The resistance genes in staphylococci that confers resistance to chloramphenicol and florfenicol are *cfr* and *fex*A genes (Schwarz et al., 2004). These resistance genes are located on mobile genetic elements which may carry additional resistance genes that might facilitate their dissemination. One of the difficulties in knowing the true incidence of strains resistant to chloramphenicol is that it is not approved for the control of staphylococcal infections in animals and hence staphylococcal isolates are not routinely tested for their susceptibility to chloramphenicol. It is because of that, an unknown number of staphylococcal isolates with elevated MICs for florfenicol might remain undetected.

2.4.8. Resistance to fluoroquinolones

The increased use of fluoroquinolones has led to increasing resistance to these antimicrobials. Resistance to fluoroquinolones typically arises as a result of alterations in the target enzymes (DNA gyrase and topoisomerase IV) and of changes in drug entry and efflux. Three mechanisms have been described to justify resistance to quinolones: mutations in the genes encoding the DNA gyrase and topoisomerase IV A subunits (*gyr*A and *grl*A, respectively) (Sreedharan et al., 1990; Goswitz et al., 1992); mutations in *gyr*B, the DNA gyrase B-subunit gene; and overexpression of *nor*A, the gene encoding the NorA protein that is a membrane-based multidrug efflux protein capable of transporting fluoroquinolones as well as several other structurally unrelated compounds from the cell (Yoshida et al., 1990; Kaatz and Seo, 1995). More than one resistance mechanism may be present in a single strain. Resistance to quinolones can also be mediated by plasmids that produce the Qnr protein; although Qnr by itself produces only low-level resistance, its presence facilitates the selection of higher-level resistance mutations, thus contributing to the alarming increase in resistance to quinolones.

2.4.9. Resistance to penicillin

Penicillin was the first antibiotic mass produced for use in humans. It was initially highly effective for treatment of *S. aureus* infections, but today over 90% of human *S. aureus* strains are resistant to this antibiotic (Olsen et al., 2006). β-lactamase is an enzyme that inactivates penicillin and confers penicillin resistance by hydrolyzing the b-lactam ring of penicillin thereby inactivating the antibiotic, and/or production of a low-affinity penicillin-binding protein (PBP2a) encoded by the *mec*A gene (Chambers and DeLeo, 2009). In *S. aureus*, β-lactamase is encoded by the *bla*Z gene and the closely linked regulatory genes, blaI and blaR (Hackbarth and Chambers, 1993). This β-lactamase may be located on a plasmid, transposons or within chromosomal DNA (Malachowa and DeLeo, 2010).

2.4.10. Methicillin-resistant *Staphylococcus aureus*

Antibiotic therapy is a selective force for bacteria so that only cells that bear advantageous properties can persist, replicate and disseminate, subsequently predominating in the population. *S. aureus* is a pathogen capable of developing highly drug-resistant forms that can cause serious diseases with epidemic potential. The control and prevention of *S. aureus* infections have been complicated in by the general spread of multidrug-resistant *S. aureus* strains known as methicillin-resistant *S. aureus* (MRSA). The introduction of methicillin into clinical use in 1961 for the treatment of multi-drug resistant *S. aureus* resulted in the increase of the occurrence of MRSA in healthcare institutions worldwide (Lowy, 1998). In the same year, Jevons discovered methicillin-resistant *S. aureus* (MRSA), which by 1970s became spread all over the world (Jevons, 1961).

MRSA are increasingly resistant to all available classes of antibiotics and that make treatment of infections caused by these strains limited to vancomycin, linezolid, and daptomycin. However, resistance to these three drugs has been reported (Tsiodras et al., 2001; CDC, 2002; Mangili et al., 2005), making MRSA a tremendous public health challenge. Both methicillin-sensitive and methicillin-resistant isolates are persistent

colonizers (Casewell and Hill, 1986; Sanford et al., 1994) which makes the fight against them more difficult.

At first, MRSA infections was restricted to nosocomial environments and individuals associated to healthcare institutions, but below this ecological niche has expanded giving place to the Community-acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) (Saïd-Salim et al., 2003). Since 1990s, there has been an increase in infections caused by *S. aureus*, mostly caused by MRSA, in community settings among healthy individuals (Naimi et al., 2003; Fridkin et al., 2005; Zetola et al., 2005) and some of them causing serious medical complications with poor clinical outcomes (Miller et al., 2005). The number of CA-MRSA infections has risen and, in some places, has become epidemic. CA-MRSA isolates are typically less resistant to other non-β-lactams than hospital-acquired MRSA (HA-MRSA) but this is changing; some epidemic clones of CA-MRSA are increasingly being isolated from healthcare settings reducing the differences between community- and hospital-acquired infections, thereby complicating the epidemiology and control of this pathogen (Saïd-Salim et al., 2003; Chambers, 2005; Klevens et al., 2006; Moran et al., 2006; Klevens et al., 2007).

As said before, although the appearance of MRSA occurred in hospital, CA-MRSA has become a great concern around the world. Most of CA-MRSA strains exhibit typical heterogeneous type methicillin resistance, and some of the strains appear highly virulent and produce Panton–Valentine leukocidins (Gillet et al., 2002). The genetic backgrounds of HA-MRSA are rather limited (Enright et al., 2002); conversely, the diversity of CA-MRSA genotypes is comparable to the diversity of *S. aureus* in the community. All this indicate that CA-MRSA strains are not descendants of extant health-care-associated MRSA strains and have different origin of derivation (Okuma et al., 2002).

β-lactam antibiotics (eg. methicillin) are a broad group of molecules that are naturally produced by different organisms (molds belonging to *Penicillium*

spp. and *Cephalosporium spp.* for penicillins and cephalosporins, respectively, and bacteria belonging to different species for monobactams and carbapenems). The antibiotics of the β-lactam family are enzyme inhibitors, and their mechanism of action involves inhibition of bacterial transpeptidases (also called penicillin binding proteins (PBP)). This proteins are enzymes located in the cytoplasmic membrane catalyzing cell-wall assembly (De Rosa et al., 2021). PBPs are involved in the final steps of peptidoglycan biosynthesis, and they are the targets of the β-lactam antibiotics. One of the main factors of methicillin resistance in *S. aureus* is the penicillin-binding protein PBP2a encoded by the *mec* genes located on the chromosome of MRSA. It has been suggested that PBP2a takes over the biosynthesis of peptidoglycan in the presence of lethal doses of β-lactam antibiotics (Pinho et al., 2001). Because β-lactams remain the primary therapeutics for treating bacterial infections, understanding the molecular mechanism of resistance to these antibiotics is essential.

2.4.10.1. SCC*mec* cassette

The emergence of methicillin-resistant staphylococcal lineages (MRSA) is due to the acquisition and insertion of the SCC*mec* element into the chromosome of susceptible strains (MSSA). In MRSA, the acquisition of the mobile genetic element SCC*mec* leads to the acquisition of resistance to β-lactam antibiotics because this element carries the *mec* gene. This MGE also carries other genes for resistance to antibiotics and heavy metals depending on the type of SCC*mec* cassette. SCC*mec* may have been acquired by *S. aureus* from *S. sciuri* (Wu et al., 1996; Severin et al., 2005).

SCC is known to be a heterogeneous mobile genetic element that specifically integrates at the *orf*X site with unknown function located near the origin of replication. This may provide advantage to the instant utilization of imported antibiotic resistance genes. Its specific insertion or excision is mediated by the recombinase chromosome cassette complex (*ccr*) and by the presence of direct and inverted repeats at the ends of the SCC*mec* (Wang and Archer, 2010). *Ccr* recombinases are encoded by three

phylogenetically distinct *ccr* genes, *ccr*A, *ccr*B, and *ccr*C, with nucleotide similarities among them below 50%. In general, *ccr* genes with more than 85% of nucleotide identities are assigned to a same allotype. Thanks to DNA sequencing, new allotypes of the three types of recombinases are continually being discovered that give rise to new types of SCC*mec* cassette (Wu et al., 2015).

The combination of classes of *mec* gene complexes and *ccr* gene complexes forms the basis of their classification (Ito et al., 2004; IWG-SCC, 2009), and are essential elements for the functionality of the SCC*mec*. The SCC*mec* element contains the *mec* gene (*mec*A, *mec*B and *mec*C) along with the genes that control its expression, *mec*R1 (which encodes the signal that transduces the MecR1 protein) and *mec*I (encoding the repressor protein MecI), and acts as a carrier to exchange genetic information between *Staphylococcus* strains.

In addition, in this mobile genetic element the so-called non-essential, very heterogeneous J-regions (J1-3) are located, varying in length and composition and separated by the *mec* gene complex and the *ccr* complex. These regions contain various genes or pseudo genes whose presence does not appear essential or useful for the bacterial cell; the notable exceptions are resistance genes for non-β-lactam antibiotics or heavy metals, some of which are derived from plasmids or transposons.

Since the initial description of SCC*mec* in 1999 (Katayama et al., 2000), up to 14 different SCC*mec* have been described in MRSA (labeled I-XIV) and subtypes have been described, but the extreme plasticity of the SCC element results in the continual permutation and identification of new “types” (Hanssen and Ericson Sollid, 2005; Deurenberg et al., 2007; Lakhundi and Zhang, 2018; Urushibara et al., 2020). Numerous multiplex PCR schemes have been proposed for rapid typing (Oliveira and De Lencastre, 2002; Kondo et al., 2007; Milheiriço et al., 2007a; Stephens et al., 2007). Subsequently an International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWC-SCC) has been

assembled, and Oliveira et al. (Oliveira et al., 2006) have created a web-based resource for SCC*mec* typing by comparative sequencing (http://www.ccrtyping.net).

While health-care-associated MRSA strains carry either type-I, -II or -III SCC*mec*, CA-MRSA strains typically carry SCCmecIV, V, or VII elements. Type-IV SCC*mec* (defined by carriage of class B *mec* gene complex and type-2 *ccr* gene complex) rarely seen in health-care-associated MRSA strains, is the dominant SCC*mec* in CA-MRSA. No resistance genes against non-β-lactam antibiotics are found in types-IVa and -IVb SCC*mec*. Several strains isolated from outpatients' clinic in 1981 in Japan possessed type-IV SCCmec, but it was distinct from the above subtypes; this new subtype having greater size than subtypes IVa and IVb, was designated IVc. So far, no CA-MRSA strains have been associated with this subtype of type-IV SCC*mec* (Ito et al., 2003). ***Table 2*** shows the general characteristics of differentiation between MRSA isolates.

HA-MRSA	Frequently associated genetic lines: ST5, ST22, ST36, ST45, ST239, ST250. History of hospitalization or recent surgical intervention. SCC*mec* type I, II, III. Not related to an increased frequency of PVL.
CA-MRSA	Frequently associated genetic lines: ST1, ST8, ST59, ST80, ST93. No history of hospitalization but close contact between individuals. Related to a higher frequency of PVL.
LA-MRSA	Frequently associated genetic lines: ST5, ST9, ST97, ST130, ST398. Carriers, use of antimicrobials. Not related to an increased frequency of PVL.

Table 2. General characteristics of differentiation between MRSA isolates. (Adapted from: Porrero, 2014a).

2.4.10.2. The recently discovered *mec*C gene

The *mec*C gene represents a fourth of the hitherto known phylogenetic branches of the *mec* genes. In 2011, the novel SCC*mec* element type XI in

S. aureus was discovered carrying the *mec*C gene (García-Álvarez et al., 2011; Shore et al., 2011a). SCC*mec* XI has structural similarities to other known SCC*mec* elements and is integrated within the same chromosomal locus, *orf*X site. The *mec*C gene only shares 70% identity with *mec*A gene on the nucleotide level and the proteins encoded by these genes PBR2a and PBP2c show 63% identity; despite this both can mediate beta-lactam resistance in *S. aureus*. Besides, *mec*C mediates oxacillin and cefoxitin resistance, like *mec*A, irrespective of the genetic strain background. Transcription of *mec*C has been observed to be oxacillin inducible, indicating the presence of a functional beta-lactam-dependent regulatory system in *S. aureus* strains harboring the SCC*mec* XI element (Ballhausen et al., 2014).

The majority of known *mec*C-MRSA belongs to the ST-CC130/spa-CC 1535 clonal lineage with t843 as the most prominent spa type (Becker et al., 2014). The occurrence of *mec*C as part of the SCC*mec* XI element in different not closely related CCs indicates that SCC*mec* XI might have been independently acquired several times. It has been observed that *mec*C-MRSA strains exhibit the usual, strain-dependent pathogenic potential as exhibited by other *S. aureus* lineages. Human *mec*C-MRSA strains belonging to MLST CC130, CC1934 and CC599 carried typical *S. aureus* virulence markers including genes encoding hemolysins (*hla*, *hlb* and *hld*), immune evasion factors (*aur*, *spl*A, *spl*B and *spl*E) and biofilm factors (*ica* operon).

2.5. *Staphylococcus aureus* and MRSA in veterinary medicine

S. aureus is not solely a human bacterial pathogen but colonizes and causes infections in multiple hosts including dairy cows, sheep, poultry and rabbits (Fitzgerald, 2012). Even though *S. aureus* has been found to be rather host specific (Herron-Olson et al., 2007), it has been reported that a single mutation, which occurred naturally, was sufficient to allow a radical change in bacterial host tropism in *S. aureus* (Viana et al., 2015a). Therefore, the capacity for pathogens to switch host species leading to

epidemic spread in new host populations is a major veterinary and public health concern.

In veterinary medicine, *S. aureus* stands out for its involvement in infections of the mammary gland of lactating females that results in significant economic loss to the dairy industries. In sheep and goats, it is responsible for acute gangrenous mastitis, as well as skin disorders such as dermatitis and folliculitis. In cattle, breast infection tends to progress to chronic or subclinical forms. *S. aureus* also causes serious economic losses in rabbit breeding, since it affects all production levels, mainly causing mastitis and pododermatitis in breeding females and pyoderma in rabbits, although otitis and rhinitis are also common (Corpa et al., 2009). It is transmitted between animals by direct contact and through secretions or utensils. The high prevalence of carriers could increase the incidence of staphylococcus on farms (Selva et al., 2015) since these animals do not have lesions and are not usually eliminated.

There are numerous strains of *S. aureus*, which can be characterized by different techniques. Traditionally, it has been differentiated between high and low virulence strains, the most frequent clones in rabbit breeding being ST121 and ST96 respectively (Vancraeynest et al., 2006; Viana et al., 2011). However, in recent years, both veterinarians and farmers have expressed concern about the appearance of particularly virulent staphylococcal outbreaks and the difficulty of treating these outbreaks, probably due to the great adaptability of *S. aureus*, leading to the appearance of new clones. This seems evidence that the classification is more complex than described.

Population genetic studies from multiple hosts and geographic locales reveal a clonal population structure, with strong evidence of host-specialization among clones (Reinoso et al., 2004). Clones with distinctive genetic backgrounds are responsible for most infections within a host type. The molecular basis of host-specialization in *S. aureus* remains unknown despite its clinical importance from a human and animal health perspective.

2.5.1. *Staphylococcus aureus* in rabbit production

In the last 30–40 years, rabbit farming intensification has coincided with a highly virulent epidemic clone of *S. aureus* emerging, that it has already mentioned, known as ST121, which is associated with the most chronic staphylococcal rabbit infections, mainly skin abscesses, pododermatitis and mastitis on commercial rabbitries. This strain does not affect only rabbits, Clonal Complex 121 strains are also associated with distinct clinical entities in humans (Kurt et al., 2013), and are a particular common cause of human skin and soft tissue infections (McCaig et al., 2006). However, other less common lineages can be involved in staphylococcal rabbit infections, such as ST96, which is the second commonest lineage defined by multi-locus sequence typing (MLST) involved in rabbit staphylococcal infections. In experimental infections of the dermis in rabbits, *S. aureus* ST96 strains are not capable of producing abscesses, however, these strains are isolated from lesions in farms. In experimental mammary gland infections in lactating females, inoculation of ST96 clones did cause infection but this infection generated different immune response than the one produced by ST121 strains and the injuries were less severe too (Penadés et al., 2020). This suggests that certain conditions exist in rabbit farms that make it easier for *S. aureus* to cause infection in animals. These conditions can be both animal conditions (immunosuppression due to disease or production stress, treatments with antibiotics or other substances…), and environmental conditions (temperature changes, cleaning and disinfection of farms...).

Farmers and veterinarians have also warned about an increase in the frequency and virulence of staphylococcal infections in rabbit farms in the last years. This increase in virulence may be due to the appearance of new clones of *S. aureus* with virulence factors that favor its adaptation to the host and the appearance of antibiotic resistant strains that may be selected when treating animals with antibiotics. Epizootic rabbit enteropathy (ERE) is a fatal condition to infected animals and threaten the rabbit production industry (Puon Peláez et al., 2018). This disease makes antibiotic treatment necessary in the fattening phase of rabbits even when there is a trend in the

reduction in the use of antibiotics in animal production, and therefore this generalized use of antibiotics favors the appearance of resistant strains of *S. aureus*, which is a public and animal health problem.

2.5.2. MRSA in livestock and wild animals

As explained above, one of the most important resistances, especially in this bacterium, is resistance to methicillin. The clonal complex of *S. aureus* resistant to methicillin (MRSA), widely related to livestock (LA-MRSA), is CC398. This clonal complex was first described in 2005, it usually colonizes the pig (Armand-Lefevre et al., 2005; Conceicão et al., 2017), but it has also been isolated in staphylococcal infections in horses, cows and chickens (Graveland et al., 2011). Before this, finding MRSA infections have been considered sporadic infections produced by isolates of human origin (Vanderhaeghen et al., 2010; Fitzgerald, 2012; Chatterjee and Otto, 2013), and due to persistent contact between individuals (Weese, 2010; Fitzgerald, 2012; McCarthy et al., 2012). The importance of the finding of this clone MRSA CC398 related to the swine sector is that it was the first description of a MRSA reservoir in slaughter animals (Fluit, 2012; Petinaki and Spiliopoulou, 2012) thus this clone plays an important role as a reservoir of transmission to humans (Frana et al., 2013). It belongs to a differenced genetic line previously no detected and it represents the ability of MRSA clones to adapt to other hosts. Currently it is mainly associated with occupational exposure but can be highly pathogenic. In addition, its presence in foods of animal origin has been investigated, and although it has been detected, contact transmission is considered to be the most frequent mechanism and therefore constitutes an occupational risk (Verkade and Kluytmans, 2014). In industrial rabbit farming, only one case of MRSA has been described, in 2014 in a farm in Italy, produced by the clonal complex associated with CC398 livestock (Agnoletti et al., 2014).

The great dissemination of LA-MRSA ST398 raised the need to know its origin. The first data pointed to a porcine origin since ST398 MSSA was detected in pigs (Armand-Lefevre et al., 2005; Guardabassi et al., 2007).

However, subsequent massive sequencing studies suggest that the ancestral origin of CC398 is human, having subsequently adapted to pigs (Price et al., 2012). Other clones of MRSA for which inter-species jumps have been observed and that define slaughter animals as a reservoir of MRSA have been CC5 (in poultry), CC97 and CC130 (both in cattle).

In Europe, a broad animal host-spectrum of MRSA comprising companion and wild-living animals as well as livestock have been discovered posing the question of the impact of those strains as source for zoonotic infections. In the case of cattle, CC97 is a genotype whose frequency of isolation is increasing in humans and its origin is bovine (Spoor et al., 2013). Besides CC130 is one of the genetic lines in which the *mec*C gene has been most frequently detected in both humans and animals, having recently analyzed the potential transmission between small ruminants and people (Harrison et al., 2013). In the last years, this *mec*C gene type has been found so widespread among livestock, companion, and wildlife animals. Following the first description of *mec*C-MRSA in specimens of bovine sources (dairy cattle, beef cattle and milk), it was found also in isolates recovered from specimens of other livestock animals, such as sheep and commercial rabbits. The most widely distributed clonal complex in *mec*C-MRSA is CC130; it has been found in several animal species including those with economic impact such as cattle and other commercial animals. Also, other CCs that have been found in *mec*C-positive isolates, such as CC49 and CC425, have been associated with infections and colonization in animals (García-Álvarez et al., 2011; Meemken et al., 2013; Simpson et al., 2013; Vandendriessche et al., 2013).

Staphylococci harboring *mec*C, seem to be widely distributed among wildlife animals too. Those isolates were recovered from animals colonizing quite different habitats and belonging to different classes like birds and mammals. This reinforces the hypothesis that wild rodents, insectivores and small carnivores may serve as reservoir for MRSA and that cassettes of the SCC family may be introduced into livestock or companion animals e.g. via rodent-colonizing bacterial species (Becker et al., 2014). Moreover, it has

been demonstrated by analysis of the soil resistome that bacteria that express resistance to antibiotics are widespread.

The ability of MRSA to adapt to more than one host, including the human species, together with a high prevalence of MRSA in certain animal species, suppose a risk to the population as slaughter animals act as a reservoir of MRSA (32). Therefore, studies of the dissemination of virulence factors and resistance to antibiotics are necessary to explain the appearance of new *S. aureus* clones and the success in their dissemination.

3. The host: Characteristics of the rabbit as a production animal

Rabbit meat production worldwide is not very important, assuming less than 0.5% of world production. However, according to FAO sources, Spain is among the 4 largest producers of rabbit meat behind China, Italy and France. Rabbit meat production is located mainly in the Mediterranean, where there is a greater demand for rabbit meat: Italy, Spain and France are the main producers and consumers. In Spain and Portugal, this production has become more concentrated and intensified in recent years, it has evolved from more or less traditional production systems to other more intensive ones due to relevant advances in genetic selection, reproductive management and feeding systems (Pascual, 2010). Farms now house large numbers of animals that are exposed to sanitary problems.

Due to their growth capacity, reproductive characteristics, and ease of handling on farms, medium-sized breeds of rabbit are the most widely used in the production of rabbit meat. The California and White New Zealand breeds are the most used. Rabbit breeding is done primarily by selecting lines for desired characters. The different rabbit lines arise from small populations in which the founder animals are animals with very outstanding values for the desired characters, coming from several populations with a good productive level or a large commercial population. These lines are generally subjected to a selection program with clear selection objectives and effective selection methods. These are lines in which there is more uniformity than in a breed, the results are more predictable, are productively

specialized and are usually owned by companies or institutions. Genetic selection for reproduction has worked, with programs resulting in an effective increase of between 0.05 and 0.13 live-born kits per generation of selection (Gómez et al., 1996; García and Baselga, 2002a, 2002b).

The Spanish rabbit production is evolving to farms of more than two hundred does and it is compulsory to increase productivity, because production costs and sale prices are very close. Nowadays, the use of more productive animals is increasing (Rosell, 1996); the demand of crossbred does and the use of males from specialized paternal lines is becoming higher and higher. Two public institutions work since 1976 in the development of new lines of rabbits under the scope of their use in a three-way scheme of crossbreeding; they are the Unit of Rabbit Science (Barcelona, I.R.T.A.) and the Department of Animal Science (Valencia, U.P.V.). Consequently, the efforts have been focused on the foundation and selection of maternal and paternal lines. Specialized maternal lines are devoted to produce crossbred does by crossing two of them, while paternal lines supply the sires to be mated to the crossbred does (Baselga et al., 1999). The rabbit lines selected in Spain currently are: Line A (UPV), Line C (IRTA), Line P (IRTA), Line R (UPV), Line V (UPV), Line HH (UPV) and line LP (UPV).

Litter size and food conversion rate are still the most important traits in rabbit meat production. Litter size is difficult to improve by selection and intensive research is being carried out to increase it. Food conversion rate is expensive to measure, but further research is needed about implementing records of this trait in selection programmes. The R line (Pink), is a paternal line selected for 25 generations for its average daily gain of the kits between the 4th and 9th weeks of life that is related to food conversion and easier to measure. However the selection by certain parameters has sometimes been accompanied by undesired side effects (Rauw et al., 1998) such as higher disease incidence (Dourmad et al., 1994) or reproductive problems. In a study in which reproductive parameters were evaluated in R line, it was observed that the rabbits in production showed serious reproductive problems that were attributed to the effect of selection by GMD (Vicente et

al., 2012). In addition to the productive objectives, health may be considered one of the main concerns of current rabbit production under commercial conditions, with high replacement rates and the frequent appearance of digestive disorders (Rosell and de la Fuente, 2009). In fact, one of the main objectives in animal production is the maintenance of a balance between productivity and animal health. Therefore, studies are needed in production animals to evaluate whether selection for productive parameters affects their health.

3.1. Cryopreservation in genetic selection

Embryo cryopreservation is a mechanism that consists of freezing embryos at low temperatures to keep them viable in the long term and it is used to carry out the selection of production animals among other reasons because it allows to conserve unique genetic material which might otherwise be lost. In addition to this, cryopreservation makes it possible to evaluate the genetic improvement of the selected traits and of other traits that may be indirectly altering. This is because thanks to cryopreservation, animals of any generation can be obtained at any given time.

However, the cryopreservation procedure may induce differences in environmental effects that in fact could cause changes in embryo, fetuses, and postnatal development (Armbrust and Eisen, 1994). The effect of the genotype on the efficiency of cryobanked embryos in the rat, mouse, and rabbit has been observed (Rall et al., 2000; Lavara et al., 2011). As an example, cryopreserved embryos from paternal rabbit lines (selected for growth characteristics) showed reduced survival rate compared with cryopreserved embryos from maternal lines (selected for reproductive characteristics) (Vicente et al., 2003). Even though, little is known about the possible effect of embryo cryopreservation and transfer on postnatal growth development in mammals and these effects may affect the results when cryopreservation is used to evaluate some parameter.

3.2. The flow cytometry tool as a way to assess the immune response

Fluorescence-activated flow cytometry is one of the most powerful technologies that is routinely used in immunology. It enables identification, characterization, and isolation of defined leukocyte subsets. This technology has also revealed that there are hundreds of phenotypically distinct cell types in the peripheral blood (De Rosa and Roederer, 2001). The ability to discriminate between these cell types is crucial to our understanding of cellular immunity and disease pathogenesis.

Two physical parameters (forward and side scatter) and two fluorescence parameters are sufficient for simple discrimination between peripheral blood cells (Lanier et al., 1983). Forward- and side-scatter characteristics can be used to distinguish monocytes and granulocytes (high side scatter and high forward scatter) from platelets and erythrocytes (low side scatter and low forward scatter). Lymphocytes and circulating dendritic cells show intermediate forward and side scatter and can be further segregated by lineage (that is, B cells, T cells or natural killer (NK) cells) using one or more fluorescence parameters. Within T-cell populations, various markers have been proposed to distinguish effector- and memory-cell populations. These markers are often paired (Davis et al., 1995; Perfetto et al., 2004), (e.g. CD14 with CD45 expressed by granulocytes). Panels of well characterized monoclonal antibodies (mAb) provide a powerful tool to analyse leukocyte populations.

The rabbit is an example of a species where there is a critical need for flow cytometry mAb reagents (NCBI Rabbit Genome Resources, USA). To date, however, only a few mAbs have been developed to meet this need and this makes necessary to look for antibodies from other species to complete the panel of mAbs. The probability of finding a mAb that recognizes an epitope conserved on orthologous leukocyte differentiation molecules (LDM) is greater between closely related species than between distantly related species (Brodersen et al., 1998) and although they are few, some mAbs recognize cell receptors from another species and allow LDM studies to be

carried out in a broader range of species (Davis and Hamilton, 2008). The use of flow cytometry for the evaluation of the immune system of rabbits is much less widespread than in other species due to this limitation in the existing monoclonal antibodies (mAbs) for this species, but more and more studies show its usefulness for the use of the rabbit as a research animal (Davis and Hamilton, 2008) for the study of diseases that affect rabbits (Guerrero et al., 2011) and for the comparison of different lines of selected rabbits based on different productive parameters (Ferrian et al., 2013).

3.3. Immune system and its relation to the success of pathogen infections

The immune system must ensure effective defence against pathological microbes and toxic or allergenic proteins and avoid inflammatory responses that produce excessive damage of self-tissues or that might eliminate beneficial, commensal microbes (Josefowicz et al., 2012). During the early stages of infection, the effective destruction of pathogens depends on the adequate coordination of a complex series of cellular and molecular processes in the host, given the logarithmic growth of microorganisms during these first steps of infection (Rigby and DeLeo, 2012). The immune system uses a complex array of protective mechanisms to control and usually eliminate these organisms and toxins.

The immune response includes the innate and adaptive systems, and although the innate mechanisms were considered part of a nonspecific inflammatory response (including neutrophil and macrophage phagocytosis and complement activation) it has considerable specificity. This is because it is directly directed towards molecular components present on the surface of microorganisms known as pathogen associated molecular patterns (PAMPs) recognized through interaction with the pattern recognition receptors (PRRs). Effective sensing of PAMPs rapidly induces host immune responses via the activation of complex signalling pathways that culminate in the induction of inflammatory responses mediated by various cytokines

and chemokines, which subsequently facilitate the eradication of the pathogen (Takeuchi and Akira, 2010).

The innate immune system also mounts an effective defence against infectious agents through the initiation of adaptive immunity, which is long-lasting and has immunological memory. Adaptive immunity is mediated via the generation of pathogen (antigen)-specific B and T lymphocytes through a process of gene rearrangement of germ-line gene elements to form intact T cell receptor (TCR) and immunoglobulin (B cell antigen receptor; Ig) genes (Chaplin, 2010).

In *S. aureus* infections, the innate response is the most important in determining the evolution of an infection since adaptive immunity alone is not sufficient for optimal defense against *S. aureus* infections (Chan et al., 2017).

The innate and adaptive immune systems usually act together, with the innate response representing the first line of host defense, and with the adaptive response becoming prominent after several days, as antigen-specific T and B cells have undergone clonal expansion. Synergy between both is essential for an intact, fully effective immune response even when they immune responses are fundamentally different in their mechanisms of action (Chaplin, 2010).

3.3.1. Polymorphonuclear neutrophils and its role in the innate immune response

The innate immune system includes different cell types, such as blood monocytes and tissue macrophages, polymorphonuclear cells (neutrophils, eosinophils, and basophils), dendritic cells, and NK cells, and although these are all considered the host's first line of defense, neutrophils play the central role of bacteria-host interaction (Kin and Sanders, 2006).

Polymorphonuclear neutrophils (PMN) belong to the innate immune system and constitute the main defense against invading bacteria and fungi. Neutrophils are short-lived granulocytes derived from pluripotent

hematopoietic stem cells in the bone marrow (Weissman et al., 2001) and they are classically underappreciated professional phagocytes. The killing mechanisms of PMN are: phagocytosis, degranulation and NETs (Kolaczkowska and Kubes, 2013).

During the PMN recruitment, some circulating neutrophils roll along the walls of postcapillary venules, by interacting with endothelial cells, surveying connective tissue, mucosal membranes, skeletal muscle, and lymphatic organs for signs of tissue damage, inflammation, or invading microorganisms (Alexander and Klaus, 2012). This PMN searches for the presence of host- and/or pathogen-derived chemotactic signals or chemoattractants that are produced by a variety of host cells in response to damage or the presence of invading pathogens. These signals direct neutrophil chemotactic movement out of intravascular circulation to sites of damage or infection within tissues, resulting in a rapid influx and accumulation of PMN (Rigby and DeLeo, 2012).

Once at the site of infection, the PMN bind and ingest invading microorganisms by a process known as phagocytosis, a critical first step in removal of bacteria during infection. Neutrophils recognize PAMPs that are recognized directly by PRRs expressed on the surface of the neutrophil. Engagement of such receptors activates signal transduction pathways that prolong cell survival, facilitate adhesion and phagocytosis, induce release of cytokines and chemokines, elicit degranulation, and promote reactive oxygen species (ROS) production and release, ultimately contributing to microbicidal activity (Kobayashi et al., 2005).

The process of neutrophil phagocytosis triggers synthesis of a number of immunomodulatory factors (Kobayashi et al., 2003) that modulates subsequent neutrophil responses, and coordinates early responses of other cells types such as monocytes, macrophages, dendritic cells, and lymphocytes, thereby providing an important link between innate and acquired immune responses. Additionally, phagocytosis has been shown to accelerate programmed cell death (apoptosis) of neutrophils (Kobayashi et

al., 2003) a phenomenon that critical to the resolution of the inflammatory response (Kennedy and DeLeo, 2009).

Moreover, phagocytosis is accompanied by the generation of microbicidal ROS (oxygen-dependent) and fusion of cytoplasmic granules with microbe-containing phagosomes (degranulation). Degranulation enriches the phagosome lumen with antimicrobial peptides and proteases (oxygen-independent process), which in combination with ROS create an environment non-conducive to survival of the ingested microbe(s) (Rigby and DeLeo, 2012).

Lastly, PMNs can also release NETs (extracellular neutrophil traps), which are composed of DNA, histones, antimicrobial peptides, and proteases that envelop bacteria for destruction, minimizing tissue damage (Brinkmann et al., 2004; Fuchs et al., 2007).

3.3.2. Monocytes and macrophages

Macrophages are long-lived cells with potent endocytic, phagocytic, and secretory functions, able to modulate their properties upon contact with different cell types as well as extracellular matrix. In response to inflammation and infection, bone marrow-derived blood monocytes flock to tissues attracted by chemotactic signals, and differentiate into macrophages and dendritic cells (DCs) in situ (Gordon et al., 2014). Growth factors such as M-CSF, GM-CSF and cytokines are responsible of local proliferation and self-renewal of macrophages (Sieweke and Allen, 2013).

The effects of macrophages on the local tissues include trophic as well as cytocidal interactions with neighbouring cells, the remodelling of matrix, as well as phagocytic clearance of dying cells and other homeostatic and defense functions. Together with perivascular macrophages, tissue macrophages regulate the recruitment of PMNs and monocytes to the site of infection and participate in the elimination of dead cells. They also produce ROS and RNS, antimicrobial peptides, and chelating proteins that

deprive bacteria of essential nutrients for their metabolism (Brandt et al., 2018).

3.3.3. T and B lymphocytes and the adaptative immune response

T and B lymphocytes play a crucial role in the cellular and humoral immunity, respectively and T lymphocytes influence the immune response of B lymphocytes (Xing et al., 2017). B lymphocytes are involved in humoral immunity by secreting antibodies (Parra et al., 2016) and can differentiate into plasma cells and memory B lymphocytes cell types. Activated B lymphocytes can behave as antibody-producing cells when binding to an antigen, but they can also participate as antigen presenting cells (APCs). In addition, they also intervene in immune defense through the secretion of cytokines (Drouet-Viard and Fortun-Lamothe, 2002; Hua and Hou, 2013).

T lymphocytes are involved in cellular immune response and are subdivided into helper T lymphocytes (Th) and cytotoxic T lymphocytes (CTLs), defined by the expression of CD4 and CD8 glycoprotein, respectively (Xing et al., 2018). CD25 is strongly expressed on CD4-positive regulatory T cells and is not detected on resting CD8-positive cells. However, all activated T cells express the CD25 protein. Helper T lymphocytes are activated by recognizing antigenic peptides bound to major histocompatibility complex (MHC) class II molecules, and exhibit predominantly helper functions. Furthermore, Th cells play a central role in defense against a wide variety of pathogenic microorganisms, helping B cells to produce antibodies and regulating CTL and macrophages (Cosmi et al., 2014). On the contrary, cytotoxic T lymphocytes are restricted to recognition by MHC class I molecules, and perform cytotoxic functions (Xiong and Bosselut, 2012); some of the most important functions are avoiding autoimmune reactions and stopping the effector response against exogenous antigens, when the response itself becomes dangerous for the host (Cosmi et al., 2014).

In summary, when an infection occurs there are many factors that determine its success or failure. The two central elements are the pathogen and the host, but many more intrinsic and external factors intervene. That is why if we study the pathogen or the host alone, we would lack a large amount of information derived from the interaction between the two. In fact, the evolution of organisms individually would not make sense if the environment in which they are found is not taken into account. This evolution also occurs in parallel and simultaneously, when an organism develops a virulence factor, its host develops over time other mechanisms to defend itself. Therefore, a continuous fed-back occurs between the pathogen and the host and that is why more studies that associate both elements are necessary.

OBJECTIVES

OBJECTIVES

The pathogen *S. aureus* is most important in human and veterinary medicine, and specifically in rabbit farming. This situation is further aggravated by the emerging increase in antibiotic-multiresistant bacteria. One of the most important antibiotic-multiresistant bacteria is the MRSA strain that appeared first in hospitals, and later in livestock animals, wild animals and the environment. In rabbits this dangerous clone has not been described in detail, and only one MRSA isolate has been reported on a farm in Italy (Agnoletti et al., 2014). On rabbit farms, other strains like ST121 clones are more prevalent (Viana et al., 2011), but the widespread use of antibiotics and the spread of new mutants of these clones (Pérez-Fuentes, 2019) may change this situation. Moreover, *S. aureus* (and even some MRSA) have been described in wild animals (Loncaric et al., 2013). Given the high percentage of rabbit *S. aureus* carriers on farms (Selva et al., 2015) and the problems that bacteria cause, it would also be interesting to know what happens in wild rabbits because they could play an important epidemiological role as reservoirs for *S. aureus*.

Finally, not only bacteria, but also the host and its immune response, play a key role in infections developing. In fact it has been described how different genetic lines of rabbits show differences in their immune system, and even distinct behaviour, when facing differing immunological challenges (Guerrero et al., 2011; Ferrian et al., 2012). Therefore, the study of the immune system and its response when facing experimental *S. aureus* infection would provide us with valuable information to generate more resistant rabbit genetic lines for staphylococcal infections.

In order to face this serious problem, four works were we carried out in this thesis whose objectives are detailed below:

1. Characterisation of livestock-associated methicillin-resistant *Staphylococcus aureus* isolates obtained from commercial rabbitries.

S. aureus is a widely distributed bacterium that is extremely capable of adapting. The amount and variability of virulence factors make it an able bacterium to cause many serious diseases. Its great resistance in the environment and the presence of carriers make *S. aureus* a bacterium that affects public and animal health. As described above, in recent years there has been increasing concern about the spread of virulent and resistant *S. aureus* strains, such as LA-MRSA, among farm animals. Apart from losses in animal production, this concern is due to the fact that they represent a reservoir of transmission to man. The appearance of these virulent and resistant strains in recent years coincides with the growing concern about staphylococcus due to the appearance of outbreaks on rabbit farms that are more frequent, virulent and difficult to eliminate. For all these reasons, the aims of this study were to:

- evaluate the presence of MRSA in a *S. aureus* collection obtained from staphylococcal lesions on different Spanish and Portuguese rabbit farms;

- compare the isolated strains using MLST, SCC*mec* and *agr* typing;

- determine the antibiotic resistance profile and virulence factors.

2. Characterisation of Methicillin-resistant *Staphylococcus aureus* in wild lagomorphs located in high density areas

However, commercial rabbits are not the only places affected by *S. aureus* as *mec*C-MRSA strains have also been isolated in wild animals. Wild rabbits could act as a reservoir for possible transmissions to wild fauna, production rabbits and as a reservoir of transmission to hunters. The role of *S. aureus* as a possible pathogen or coloniser in wild populations of lagomorphs has not yet been studied in a mass systematic manner. In high density areas, the interaction between rabbits and other animals is more marked and,

therefore, the probability of *S. aureus* transmission increases. For these reasons, the following objectives were set in this study to:

- know the role of wild rabbits and hares as a reservoir of *S. aureus* in wild lagomorph high density areas;

- characterise the *S. aureus* strains isolated from wild rabbits and hares to compare them to those obtained previously on commercial farms;

- study the prevalence of MRSA strains in wild populations.

3. Phenotypic and genotypic study of the SCC*mec* cassette element in MRSA and MSSA strains isolated from rabbits

The emerging increase in antibiotic-resistant bacteria in recent years has raised global concern as clones of bacteria resistant to all antibiotics have already appeared among farm animals given their widespread use. This very much complicates their treatment and creates dangerous clones that could act as a reservoir of human transmission. *S. aureus* is a dangerous bacterium because of its excellent capacity to develop resistance to antibiotics. In this species, one of the clones that caused most concern was MRSA strains, not only because they present resistance to methicillin, but also because these clones are usually multiresistant. Given this increasing importance of antibiotic resistances, especially methicillin resistance, and its relation with mobile genetic elements, the objectives of this work were:

- the genotypic study of mobile genetic element SCC*mec* in a selection of *S. aureus* strains isolated from rabbits;

- the phenotypical study of the antibiotic resistances presented by strains MSSA and MRSA.

4. Effect of selection by growth rate and vitrification of embryos on the rabbit (*Oryctolagus cuniculus*) immune system and its response after a *Staphylococcus aureus* experimental infection

On rabbit farms, it is very important to maintain a balance between productivity and animal health to obtain good results. Animals' susceptibility to suffer from a disease depends on many factors, among which the most important are the productive state and genetics (selection to which animals are subjected). This selection for specific characters sometimes causes other characteristics to be indirectly altered, such as health. Therefore, it is necessary to control other relevant aspects when selecting animals. One of the mechanisms that controls the effects of selection on selected characters and other characters is embryo cryopreservation, which allows animals from previous generations to be obtained. However, this technique has been found to affect the animals obtained by it and, therefore, its effects must also be monitored. With the R line, a paternal line of rabbits selected by daily weight gain, increased frequency of diseases has been observed compared to other maternal lines. Therefore, the following objectives were set in this work to:

- evaluate the effect of selection by growth rate on the immune system at two levels (maintaining immune competence under conventional conditions and mounting immune response to an immunological challenge with *S. aureus*);

- evaluate the effect of embryo vitrification when mounting the immune response when facing an immunological challenge with *S. aureus.*

RESEARCH WORKS

RESEARCH WORKS

1. Characterisation of livestock-associated methicillin-resistant *Staphylococcus aureus* isolates obtained from commercial rabbitries.

The increase in antibiotic-resistant bacteria results in a generalised growing concern about using antibiotics, especially with livestock animals. *S. aureus* is a bacterium that is very well able to adapt to any environment, and one of its characteristics is its excellent capacity to develop mechanisms of resistance to antibiotics. Strains that are resistant to almost all families of antibiotics have been isolated, which poses a major public health problem because of the difficulty of establishing treatment against an infection caused by a multidrug-resistant strain. In addition, *S. aureus* has many virulence factors that determine its success in infections.

One of the strains that has caused the most problems worldwide is MRSA. These bacteria pose a very high risk because apart from them resisting methicillin, they also carry virulence genes and resistance genes to other families of antibiotics, which makes them multiresistant and very virulent. The recent dissemination of MRSA clones in slaughtered animals and a first report describing the presence of MRSA clone ST398 on a rabbit farm and its workers, led us to suspect that the dissemination of MRSA clones among commercial rabbits could be wider than initially expected. For this reason, together with the growing concern of veterinarians and rabbit farmers who observe increased frequency and virulence of *S. aureus* infections and an increasing difficulty in treating them due to increased resistance, we proposed carrying out a phenotypic study of resistance to different families of antibiotics together with a genotypic study of the presence of the methicillin-resistance gene, which determines that strains are MRSA and other genes coding for important virulence factors in *S. aureus*.

Work 1: Characterization of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Isolates Obtained From Commercial Rabbitries Located in the Iberian Peninsula

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1.1. Summary

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) have been a growing problem in human medicine since the 1960s, and more recently in veterinary medicine with the appearance of livestock-associated MRSA (LA-MRSA). Nevertheless, information about the presence of MRSA in rabbits is quite scarce since only one LA-MRSA identification has been previously reported. The present study aimed to determine genotypic characterization by verifying the presence of resistance determinants, virulence, and toxin genes of different *S. aureus* strains that cause lesions in rabbits, and their phenotypic traits based on the antimicrobial susceptibility profile. The analysis of 240 *S. aureus* isolates obtained from different lesion types collected from 89 Spanish and Portuguese rabbit commercial farms in the last 4 years (2014–2017) was performed. The methicillin-resistant gene *mec*A was found in 11.25% of the studied isolates (27 of 240) from 19 farms (13 Spanish and 6 Portuguese). Staphylococcal cassette chromosome *mec* (SCC*mec*) typing predominantly revealed type III (n = 15). Additionally, three MRSA isolates carrying the *mec*C gen were detected in samples from three different farms (two Spanish and one Portuguese). None of the 30 MRSA isolates was PVL-positive or *tst*-positive. After the multilocus sequence typing (MLST) procedure, 16 belonged to ST2855, 6 to ST146, 6 to ST398, and 2 ST4774. No ST121

isolate was *mec*-positive. ST398 and ST4774 isolates lacked the immuneevasion-cluster (IEC) genes. ST2855 strains were associated with the presence only of the *sak* gene, and ST146 isolates were ascribed to IEC type E. Therefore, this is the first description of LA-MRSA from rabbits belonging to ST2855. Interestingly, one ST2855 and two ST4774 isolates were *mec*C-positive, which could act as a *mec*C-MRSA reservoir. More studies are needed to further characterize these isolates and their relationship with humans and other animal species.

Keywords: *Staphylococcus aureus*, MRSA, LA-MRSA, rabbit, lesions

1.2. Introduction

Staphylococcus aureus multi-resistant to antibiotics is a leading cause of bacterial infections in hospitals and communities. Specifically, methicillin-resistant *S. aureus* (MRSA) has spread worldwide in the second half of the 20th century and is now considered endemic in healthcare facilities in all industrialized countries (Kobayashi et al., 2015). In 2005 livestock-associated MRSA (LA-MRSA) emerged in pigs (Armand-Lefevre et al., 2005), where it plays an important role a reservoir of infection to humans (Frana et al., 2013). It was later reported in different farm animals, including horses, cattle or poultry worldwide (Graveland et al., 2011; Aires-de-Sousa, 2017). *Staphylococcus aureus* frequently infects commercial rabbits (Corpa et al., 2009), but there is only one reported case in which LA-MRSA was identified in this animal species, and was also isolated from farmers and their relatives (Agnoletti et al., 2014).

The results of population genetics studies have shown that most *S. aureus* strains are host-specific, which indicates low frequency of cross-species transmission (Fitzgerald, 2012). However, more recent studies that employed the multilocus sequence typing (MLST) have identified several sequence types (ST) that are associated with multiple host species. This finding implies either zoonotic transmission or a recent common ancestor (Spoor et al., 2013).

Several *S. aureus* MLST lineages have been associated with animals, including clonal complexes (CC): CC1 (livestock), CC5 (avian), CC130 (multi-host), CC133 (ruminants), CC151 (ruminants), CC398 (livestock), and CC425 (ruminants and wild mammals) (Harrison et al., 2017). *S. aureus* CC121 has a multihost tropism (Viana et al., 2015a), including humans, where it is considered a globally disseminated hypervirulent clone, although 90% of ST121 strains are methicillin-sensitive (Rao et al., 2015). In commercial rabbits, ST121 is by far the most frequently isolated clone, with prevalences close to, and even higher than, 90% (Viana et al., 2011; Guerrero et al., 2015). This causes important economic loss associated with several lesions, such as mastitis, multisystemic abscessation and pododermatitis (Corpa et al., 2009). However, no information in rabbits about the susceptibility of ST121 strains to methicillin is available.

Due to the increasing presence of LA-MRSA strains in different animal species and the scarcity of available information on commercial rabbits, the aims of this study were to: (1) evaluate the presence of MRSA in a *S. aureus* collection obtained from staphylococcal lesions on different Spanish and Portuguese rabbit farms; (2) compare the isolated strains using MLST, SCC*mec*, and *agr* typing; (3) determine antibiotic resistance profile and virulence factors.

1.3. Materials and methods

1.3.1. Isolation and characterization of *Staphylococcus aureus* isolates

Two hundred and forty *S. aureus* isolates from rabbit clinical samples obtained on 89 rabbitries located in Spain (n = 82) and Portugal (n = 7), were studied in our laboratories in the last 4 years (2014–2017). These selected samples came from rabbits with different lesion types, including mastitis (n = 86), subcutaneous abscesses (n = 33), pododermatitis (n = 31), dermatitis (n = 21), otitis (n = 13), metritis (n = 12), conjunctivitis (n = 11), pneumonia (n = 9), rhinitis (n = 3), hepatitis (n = 3), peritonitis (n = 2), pericarditis (n = 1), and osteomyelitis (n = 1). Fourteen *S. aureus* isolates from nasal carriers were also analyzed.

Samples were inoculated on blood-agar (BioMérieux, Marcy l'Etoile, France) and incubated aerobically at 37ºC for 24–48 h. *S. aureus* strains were identified on the basis of morphological growth characteristics and hemolytic properties (Devriese et al., 1996). To perform PCR, genomic DNA was extracted from each isolate with a Genelute Bacterial Genomic DNA kit (Sigma), according to the manufacturer's protocol, except for bacterial cells, which were lysed by lysostaphin (12.5 mg/ml, Sigma) at 37ºC for 1 h before DNA purification. Isolates were genotyped by MLST (Enright et al., 2000).

All the strains were checked for the presence of the *mec*A/*mec*C genes by PCR, as previously described (Geha et al., 1994; Khairalla et al., 2017). Furthermore, SCC *mec* cassette element classification was carried out for all the *mec*A-positive isolates, as described elsewhere (Zhang et al., 2005; Milheiriço et al., 2007b).

1.3.2. Detection *chp, sak, sea, sep, scn, tst,* and PVL-encoding genes and *agr* typing

The MRSA isolates were subjected to a PCR assay to detect the *luk*F/S-PV genes that encode the PVL toxin and the *tst* gene that encodes the TSST-1 toxin and *agr* typing, as previously described (Viana et al., 2015b). These strains were also checked for the presence of the immune-evasion cluster (IEC) genes (*sea*, *sep*, *sak*, *chp*, and *scn*) by PCR, as formerly described (van Wamel et al., 2006).

1.3.3. Antibiotic susceptibility testing

The antibiotic susceptibility of the MRSA isolates was determined by the disk diffusion method on Mueller-Hinton agar (MHA, CONDA, Spain), according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI). The disk diffusion assay was done with 14 antibiotics: bacitracin (10 U), enrofloxacin (5 µg) (OXOID), streptomycin (10 µg), spiramycin (100 µg), sulfadiazine (25 µg), chloramphenicol (30 µg) (BD), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), neomycin (30 µg), penicillin (10 U), tetracycline (30 µg), cefoxitin (30 µg), and

trimethoprim/sulfamethoxazole (1.25 µg/23.75 µg, respectively) (BIO-RAD). Minimum inhibitory concentrations (MIC) for cefoxitin and vancomycin was determined by using MIC Test Strip (Liofilchem) on inoculated Mueller Hinton agar plates and the results were interpreted according EUCAST breakpoints. *S. aureus* strain ATCC 25923 and *Enterococcus faecalis* strain ATCC 29212 were used as controls in the susceptibility test.

1.3.4. Statistical analysis

Categorical data were compared using Fisher exact test. All reported *p*-values are two-tailed and analyses were performed using GraphPad software.[1] Variables with $p < 0.01$ were considered to be statistically significant.

[1]https://www.graphpad.com/quickcalcs/catMenu/

1.4. Results

1.4.1. Identification of MRSA and lesions

Of all the analyzed *S. aureus* isolates, 12.5% (30/240) were identified as methicillin-resistant. Of the 30 MRSA isolates, *mec*A was detected in 27 isolates. In 3 isolates *mec*C were amplified. The 30 isolates were cefoxitin-resistant, with inhibition zone diameters<22 mm (range 0–20 mm) using disk diffusion method and MIC values >4 mg/ml (range 8–256 mg/ml).

When considering lesion type, MRSA was most frequently present in hepatitis samples (100%, 3/3), followed by metritis (58.3%, 7/12), pneumonia (44.4%, 4/9), rhinitis (33.3%, 1/3), otitis (23.1%, 3/13), conjunctivitis (18.2%, 2/11), mastitis (9.3%, 8/86), dermatitis (4.8%, 1/21), and pododermatitis (3.2%, 1/31). Hepatitis and metritis were strongly associated with MRSA ($p < 0.0001$) (see ***Table 3***). When only the commonest lesions (mastitis, abscesses, and pododermatitis) were taken into account, MRSA was identified in 6% (9/150) of the cases. None of the 14 isolates from nasal carriers were MRSA.

Lesion	No. isolates	MRSA	MSSA	*p*-value
Mastitis	86	8	78	0.3127
Abscess	33	–	33	0.0192
Pododermatitis	31	1	30	0.1422
Dermatitis	21	1	20	0.4867
Otitis	13	3	10	0.2120
Metritis	12	7	5	0.0001
Conjunctivitis	11	2	9	0.6328
Pneumonia	9	4	5	0.0162
Rhinitis	3	1	2	0.3313
Hepatitis	3	3	0	0.0018
Peritonitis	2	–	2	1
Pericarditis	1	–	1	1
Osteomyelitis	1	–	1	1
Nasal carrier	14	–	14	0.2270
Total	240	30	210	

Table 3. Number of MRSA and MSSA isolates identified from different lesions.

1.4.2. Characterization of *Staphylococcus aureus* isolates

The MLST typing analysis revealed 13 different STs. The MLST type, including the largest number of isolates, was ST121 (n = 73). This was immediately followed by ST3764 (n = 57), ST96 (n = 36), ST2855 (n = 32), and ST1 (n = 13). Other less frequent STs were ST398 (n = 11), ST146 (n = 6), ST5 (n = 4), ST3761 (n = 3), ST4774 (n = 2), a novel *tpi* single locus variant of ST130, ST4473 (n = 1), a novel *arc* single locus variant of ST4470, ST407 (n = 1), and ST3759 (n = 1).

The 30 MRSA isolates from 22 farms (15 Spanish and 7 Portuguese) belonged to ST2855 (53.3%, 16/30), ST146 (20%, 6/30), ST398 (20%, 6/30), and ST4774 (6.6%, 2/30). For SCC*mec* typing, the multiplex PCR assay identified 15 of the 27 *mec*A MRSA isolates with SCC*mec* type III, 6 with SCC*mec* type IV, and 6 with SCC*mec* type V. The 3 *mec*C MRSA isolates belonged to a ST4774 and one ST2855.

All the MRSA strains belonging to the same lineage displayed an identical accessory gene regulator (*agr*) type and SCC*mec* type: ST2855 harbored *agr*III and SCC*mec*-type III; ST146 displayed *agr*II and SCC*mec*-type IV;

ST398 contained *agr*I and SCC*mec*-type V; ST4774 harbored *agr*III (***Table 4***). Methicillin susceptible *S. aureus* (MSSA) strains with *agr* type I belonged to lineage ST398 and ST407; ST5 and ST3759 harbored *agr*II; ST1, ST96, ST2855, and ST4473 displayed *agr*III; ST121, ST3764, and ST3761 contained *agr*IV.

1.4.3. Antibiotic resistance profile

The full resistance rates among the 30 MRSA isolates tested in the present study were as follows: 100% (n = 30) for cefoxitin and penicillin, 83.3% (n = 25) for tetracycline, 76.6% (n = 23) for enrofloxacin, 60% (n = 18) for erythromycin, 56.6% (n = 17) for streptomycin and spiramycin, 23.3% (n = 7) for neomycin, 13.3% (n = 4) for doxycycline and gentamicin and 6.6% (n = 2) for chloramphenicol. Conversely, all the tested isolates were susceptible to sulfonamides (sulfadiazine and trimethoprim/sulfamethoxazole) and bacitracin.

The majority of the tested MRSA isolates (n = 29, 96.6%) were multidrug-resistant (resistant to three antimicrobial classes or more). A comparison of occurrence of antimicrobial resistance among the investigated MRSA isolates in relation to different STs is presented in ***Table 4***. The ST2855 strains showed resistance to a larger number of antibiotic groups than other MRSA strains; specifically to tetracycline, macrolides (erythromycin and spiramycin) and enrofloxacin. The highest percentage of tetracyclines (100%) resistance was recorded among the ST398 strains. ST146 isolates showed resistance to a smaller number of antibiotics, of which neomycin (100%), enrofloxacin (83.3%), and tetracycline (83.3%) stood out. The two ST4774 isolates showed resistance to enrofloxacin, macrolides, and tetracycline (100%) and in one case also to streptomycin. For the remaining antimicrobial groups, no differences in resistance were observed among the STs. MIC Test Strip for cefoxitin detect a strain ST2855 with high level resistance (MIC, 256 mg/ml). None of the MRSA isolates showed resistance to vancomycin, with MICs ranged from 0.5 to 2 mg/ml.

Isolate ID	Lesion	Genotipic characteristics				MIC (µg/ml)		Phenotypic resistance profile*
		ST (MLST)	SCC*mec* type	*agr* types	IEC types	FOX	VAN	
Sp-794	Otitis	146	IV	II	E	32	0.75	ENO-N-TET
Sp-795	Otitis	146	IV	II	E	24	1.5	ENO-N-TET
Sp-986	Dermatitis	146	IV	II	E	48	1.5	N-TET
Sp-992	Otitis	146	IV	II	E	48	1.5	ENO-S-N-TET
Sp-1004	Pneumonia	146	IV	II	E	48	1.5	ENO-N-TET
Sp-1005	Pneumonia	146	IV	II	E	48	2	ENO-S-N
Sp-1006	Conjunctivitis	398	V	I	–	16	0.75	CN-TET
Sp-1007	Rhinitis	398	V	I	–	12	1	ENO-E-SPC-TET
Sp-1008	Mastitis	398	V	I	–	16	0.75	DO-ENO-E-SPC-TET
Sp-1018	Mastitis	398	V	I	–	12	0.75	ENO-E-SPC-CN-TET
Sp-1019	Conjunctivitis	398	V	I	–	12	0.75	ENO-E-SPC-CN-TET
Sp-1032	Mastitis	398	V	I	–	12	0.75	ENO-S-TET
P-988	Metritis	2855	III	III	–	16	1.5	S-TET
P-989	Mastitis	2855	III	III	–	32	1.5	ENO-E-SPC-S
Sp-990	Pneumonia	2855	III	III	–	32	1.5	ENO-E-SPC-S-TET
P-991	Metritis	2855	III	III	–	32	1.5	ENO-E-SPC-S-TET
Sp-993	Mastitis	2855	III	III	–	16	1.5	S
P-994	Pododermatitis	2855	III	III	–	32	1	C-ENO-E-SPC-TET
P-995	Hepatitis	2855	III	III	–	48	1.5	ENO-S-N
P-996	Mastitis	2855	III	III	–	32	2	ENO-E-SPC-S-TET
P-997	Mastitis	2855	III	III	–	48	2	DO-ENO-E-SPC-S-TET
P-998	Metritis	2855	III	III	–	32	1	DO-E-SPC-TET
Sp-1000	Metritis	2855	III	III	–	48	2	ENO-E-SPC-S-TET
Sp-1001	Hepatitis	2855	III	III	–	256	1.5	ENO-E-S-TET
Sp-1002	Metritis	2855	III	III	–	32	1.5	ENO-E-SPC-S-TET
P-1003	Hepatitis	2855	III	III	–	64	2	DO-E-SPC-S-TET
P-1009	Pneumonia	2855	III	III	–	48	2	CN-TET
P-985	Metritis	2855	*mec*C	III	–	12	1.5	C-ENO-E-SPC-S
Sp-987	Metritis	4774	*mec*C	III	–	8	1	ENO-E-SPC-S-TET
Sp-999	Mastitis	4774	*mec*C	III	–	12	1.5	ENO-E-SPC-TET

Table 4. Relationship among the type of lesions and genotypic (MLST, SCC*mec*, *agr* and IEC types) and phenotypic (antibiotic profile) characteristics in the MRSA isolates. * *All MRSA strains were resistant to cefoxitin and penicillin and susceptible sulfonamides (sulfadiazine and trimethoprim/sulfamethoxazole) and bacitracin. C, chloramphenicol; CN, gentamicin; DO, doxycycline; E, erythromycin; ENO, enrofloxacin; FOX, cefoxitin; MIC, Minimum inhibitory concentrations; N, neomycin; P, Portugal; S, streptomycin; Sp, Spain; SPC, spiramycin; TET, tetracycline; VAN, vancomycin.*

1.4.4. Detection of IEC cluster (*scn*, *chp*, *sak*, and *sea* or *sep*), *tst*, and PVL genes among MRSA isolates

The PCR detection of the IEC genes, *tst* and PVL-encoding genes was carried out for the MRSA isolates. None of the MRSA isolates were positive for *sea* or *sep*, *chp*, *tst*, and PVL genes. The *sea*/*sep* gene along with the *sak* (73.3%), *chp* (0%), and *scn* (20%) genes, modulators of different parts of the innate immune system, forming an immune evasion cluster (IEC) (van Wamel et al., 2006). Depending on the presence or absence of these genes and their different combinations, *S. aureus* isolates were classified into 7 different IEC types according to patters previously described. The *scn* gene is mandatory for the consideration of the IEC types (Benito et al., 2016). All ST146 isolates contained IEC type E (comprised of *sak* and *scn* genes). However, isolates belonging to ST2855 were associated with the presence only of the *sak* gene, while the strains belonging to ST398 and ST4774 did not contain IEC genes.

1.5. Discussion

In the present study, 240 *S. aureus* isolates obtained from rabbits suffering different lesions, located on 89 farms of Spain and Portugal, were analyzed between 2014 and 2017. Isolates were obtained mainly from mastitis (n = 86), abscesses (n = 33) and pododermatitis (n = 31), which were the most frequently observed lesions associated with *S. aureus* infections in commercial rabbits (Segura et al., 2007; Viana et al., 2007).

Screening for methicillin-resistant isolates allowed the identification of 30 strains as MRSA. The lesion types from which they were isolated differed compared with those upper previously indicated as more frequent (mastitis, abscesses, and pododermatitis). MRSA was detected in 100% (3 out of 3) of hepatitis cases, metritis (58.3%, 7/12), pneumonia (44.4%, 4/9), rhinitis (33.3%, 1/3), otitis (23.1%, 3/13), conjunctivitis (18.2%, 2/11), mastitis (9.3%, 8/86), dermatitis (4.8%, 1/21), and pododermatitis (3.2%, 1/31). Therefore, while the percentage of the most frequent lesions (mastitis,

abscesses, and pododermatitis) caused by *S. aureus* was 62.5% of all lesions (150/240), MRSA was involved only in 30% (9/30) of these same lesions. They were not isolated from abscesses and there was only one pododermatitis case. Hepatitis and metritis were found to be strongly associated with MRSA (hepatitis $p < 0.0018$; metritis $p < 0.0001$). This unusual lesion pattern occasioned with MRSA strains versus habitual *S. aureus* infections could indicate a different pathogenesis of MRSA infections in rabbits.

In order to understand the pathogenesis of staphylococcal infections, the correct identification of the involved strain is vital. Thus the development of high discriminatory typing techniques, such as MLST, is very important (Enright et al., 2000). In the present study, the most frequently detected MLST type was ST121 (73/240). It has been reported that the majority of chronic staphylococcal infections in rabbits are caused by high virulence strains that belong mainly to the ST121 lineage (Vancraeynest et al., 2006; Viana et al., 2011; Guerrero et al., 2015;). The ST121 lineage was also the predominant one in this study, followed immediately by ST3764 (57/240). This last lineage, together with strains ST3761, a minority in our study (n = 3), belong to clonal complex CC121. *S. aureus* CC121 has a multi-host tropism, which is a common cause of human skin and soft-tissue infections (Viana et al., 2015a). In humans, the ST121 lineage is also considered a hypervirulent clone, although approximately 90% of the ST121 strains were methicillin-sensitive (Rao et al., 2015). The present study agrees with these results since all the ST121 strains from rabbit isolates were methicillin-sensitive.

A further ten different ST types among the tested isolates were identified: ST96 (n = 36), ST2855 (n = 32), ST1 (n = 13), ST398 (n = 11), ST146 (n = 6), ST5 (n = 4), ST4774 (n = 2), ST4473 (n = 1), ST407 (n = 1), and ST3759 (n = 1). However, MRSA isolates showed limited genetic diversity (ST2855, ST146, ST398, and ST4774), being the ST2855 the most predominant clone (53.3%; 16/30). It is noteworthy that 50% (16/32) of strains ST2855

were methicillin-sensitive. The only isolate described to date to belong to this lineage is an MSSA isolate of mastitis, described in a rabbit from Italy in 2012 (Agnoletti et al., 2014).[2] All the ST146 strains isolated herein were methicillin-resistant. No data exists in the literature about *S. aureus* isolates from rabbits that belong to this lineage. ST146 belongs to clonal complex CC5. This clonal complex has been previously found in *S. aureus* isolates from rabbit carcasses, but none were methicillin-resistant (Merz et al., 2016). *S. aureus* associated with CC5 is commonly detected in humans or animal hosts, including poultry (Krupa et al., 2014). On the other hand, six of the 11 ST398 isolates were MRSA. The only LA-MRSA case to have occurred in rabbits for meat production belongs to this lineage (Agnoletti et al., 2014). Finally, the 3 *mec*C MRSA isolates belonged to ST4774, a novel *tpi* single locus variant of ST130, and one ST2855. Isolates reported to date and carrying *mec*C belonged mainly to lineages common in cattle, namely CC130, CC1943, and CC425, suggesting a zoonotic reservoir (Aires-de-Sousa, 2017). Besides cattle, *mec*C has also been found among other farm animals such isolates ST130 in sheep (Ariza-Miguel et al., 2014; Giacinti et al., 2017) and an isolate ST425 that caused a highly virulent infection in a rabbit (Paterson et al., 2012).

Other typing techniques used to characterize MRSA strains include the identification of the *agr* and staphylococcal cassette chromosome *mec* (SCC*mec*). The *mec*A-positive MRSA strains that belong to the same lineage displayed identical *agr* and *mec* types: ST2855 harbored *agr*III and *mec* type III; ST146 displayed *agr*II and *mec* type IV and ST398 contained *agr*I and *mec* type V. The *mec*C-positive isolates harbored *agr*III. The *agr* locus plays a critical role in MRSA pathogenesis and has been assumed to play a key role in human staphylococcal infections (El-baz et al., 2017). Other lineages with *agr*III include strains type ST96 or ST1. In rabbits, differences have been detected in the virulence between strains ST121 and ST96 (Guerrero et al., 2015; Viana et al., 2015b), which could be related with differences with this regulator type. Regarding the SCC*mec* type, different combinations of genes in several SCC*mec* types also leads to the

strains displaying distinct antibiotic susceptibilities. Although the majority of the tested MRSA isolates were multidrug-resistant, the ST2855 strains showed resistance to a larger number of antibiotic groups than other strains, as well as higher frequency of resistance to macrolides. This multiresistance could justify its more extended presence compared to other MRSA strains. Types II and III SCC*mec* segments have been reported to be longer and to possess multidrug resistances to the strains that carry these elements. These types have frequently been demonstrated in HA-MRSA strains (Taherirad et al., 2016). Similarly to other studies, the ST398 strains identified in this study belonged to V SCC*mec* type (Witte et al., 2007; Bardiau et al., 2013). The highest percentage of tetracyclines (100%) resistance were recorded among the ST398 strains. Resistance to tetracycline is also associated with LA ST398 (Harrison et al., 2017), and acquisition of *tet*K as part of SCC*mec* type Vc by *tet*M-positive LA ST398 has been demonstrated (Larsen et al., 2016). On the other hand, ST146 isolates showed resistance to fewer antibiotics, of which neomycin (100%), enrofloxacin (83.3%) and tetracycline (83.3%) stand out. In Spain, ST146 MRSA has been detected in nasal carriage in Primary Healthcare Center patients. This isolate was typed as ST146-CC5 SCC*mec* IVc, and it presented a multiresistance phenotype, similarly to our ST146 rabbit strains. Strains with similar characteristics are considered hospital-acquired HA-MRSA (Lozano et al., 2014). Finally, *mec*C is part of a novel SCC*mec* assigned type XI (García-Álvarez et al., 2011). Aires-de-Sousa (2017) describes that isolates of this new genetic element are not resistant to antibiotics other than beta-lactams, in contrast to our results where *mec*C strains also showed resistance to enrofloxacin, tetracycline and macrolides.

Finally, all the MRSA strains described in this study were negative for the genes that code toxins PVL and TSST-1. A comparative analysis of the accessory genomes of the ST121 strains showed that the majority of human strains contained mobile genetic elements, which encode the potent toxins involved in human disease pathogenesis, such as PVL and TSST-1. According to our study, no rabbit strains carried *luk*S/F-PV or *tst* genes,

which indicates that they are dispensable for the *S. aureus* infection of rabbits (Viana et al., 2015b). On the other hand, the presence of the immune-evasion-cluster (IEC) genes was determined for MRSA isolates to determine whether they may have human or animal origin. The presence of IEC type E in all ST146 isolates points to the possible human origin of this clone. However, ST398 and ST4774 (a single locus variant of ST130) isolates belonging to LA-lineages since were IEC negative. There are numerous reports of the presence of LA-MRSA in livestock caused by CC398 and several MRSA lineages as CC130 (Agnoletti et al., 2014; Paterson et al., 2014a; Aires-de-Sousa, 2017; Harrison et al., 2017). However, isolates belonging to ST2855 were associated with the presence only of the *sak* gene. Stegger et al., (2013) described the presence of IEC in a porcine *S. aureus* CC398 isolate within the livestock clade, which supports that reacquisition of IEC enables LA-MRSA CC398 to spread in human populations. More recently Kraushaar et al., (2017) demonstrated that lysogenic conversion of LA-CC398 strains by virulence-associated phages may occur and that new pathotypes may emerge by this mechanism.

Vancomycin is an important antibiotic to treat MRSA isolates, so the emergence of vancomycin-resistant *S. aureus* (VRSA) strains poses a serious global threat to public health. Two mechanisms, including cell wall changes and acquired van genes were involved in vancomycin resistance. None of the MRSA isolates showed resistance to vancomycin.

A cefoxitin disk diffusion test is used to predict the presence of *mec*A in *S. aureus* (Cauwelier et al., 2004; Swenson et al., 2005). All MRSA isolates were cefoxitin resistant, with inhibition zone diameters <22 mm using disk diffusion method. Therefore, this method is a good test for routine detection of all classes of MRSA. On the other hand, isolates harboring *mec*C typically yield negative results for conventional *mec*A PCR, which can translate in detection errors in a subset of MRSA isolates (Paterson et al., 2012).

Therefore, detecting *mec*A/*mec*C genes proves to be more reliable test for detecting methicillin resistance among staphylococci.

Methicillin-resistant *S. aureus* prevalence varies according to sampling type and the studied animal species. The European Food Safety Authority (European Food Safety Authority (EFSA), 2009) has reported a 26.9% MRSA prevalence, which was detected in the dust of pigs' production holdings of the European Union. Recent research into small ruminants has indicated an overall estimated prevalence of 0.70% (2/286) in milk samples in herds (Giacinti et al., 2017). In cattle a 4.4% prevalence has been reported in isolates collected from bovine mastitis cases (Bardiau et al., 2013). In the only MRSA case published in rabbits, only in 1 of 40 (2.5%) farms with clinical staphylococcosis has detected MRSA in skin samples (Agnoletti et al., 2014). Although the present study was not designed according to an epidemiological point of view, an unexpectedly large number of positive samples (12.5%; 30 of 240 isolates) from 22 different farms (22 of 89 farms) in Spain (n = 15) and Portugal (n = 7) was detected. Therefore, it can be stated that the spread of MRSA strains on Spanish and Portuguese rabbitries in the last 4 years is worrisome.

[2]https://pubmlst.org/saureus/

1.6. Conclusion

An unexpected large number of MRSA strains obtained from numerous rabbit farms, isolated from infrequent lesions in *S. aureus* infections, is herein reported. A new lineage of MRSA, ST2855 associated with livestock, has not yet been described. This is the first description of LA-MRSA in rabbits belonging to ST2855 and the first report of *mec*C MRSA in rabbit samples from Iberian Peninsula. More studies are needed to further characterize these isolates and their relationship with humans and other species.

Ethics statement: No approval from the Animal Welfare Ethics Committee was required, since all isolates were analyzed as part of microbiological diagnostics in accordance with the Spanish law RD53/2013.

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1. Characterisation of methicillin-resistant *Staphylococcus aureus* in wild lagomorphs located in high density areas

The results obtained in Work 1, where we observed an unexpected percentage of MRSA strains isolated from rabbits, confirm the hypothesis that these strains are more widespread among rabbits than previously thought. These results evidence that we are facing a problem that affects animals and humans due to the reservoir that animals can represent for them. One of the important points to help to better understand the problem involves knowing if these strains that infect farm rabbits are also present in the environment and in other free-living animals. For this reason, wild rabbits were thought to act as a reservoir for the transmission of *S. aureus* strains.

In recent years, the presence of MRSA strains has been observed not only in production animals, in which the pathogens are subjected to antibiotic pressure, but also in both wild animals and the environment (rhizomes, wastewater, etc.). Wild rabbits come into contact with hunters and their animals, and with irrigation waters of crops, and also with the other animals they live among, such as birds and rodents, which could be vectors that transmit these strains to farms. In the Valencian Community (east Spain), there are areas where the population density of wild rabbits is very high, which facilitates the transmission of pathogens. As this can be a reservoir for farm animals' contagion, (e.g. rabbits), and as information on the presence of MRSA strains in wild animals is lacking, we proposed carrying out Work 2. The objective of this second study was to evaluate the presence of MRSA and MSSA strains in wild rabbits to learn more about the extent of the problem of the high prevalence of MRSA strains, multiresistant strains and strains carrying dangerous virulence factors.

Work 2: Marked Presence of Methicillin-Resistant *Staphylococcus aureus* in Wild Lagomorphs in Valencia, Spain

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2.1. Summary

The appearance of methicillin-resistant strains of *Staphylococcus aureus* (MRSA) in several animal species (including rabbits) has set off alarms for their capacity to act as reservoirs for this bacterium. This is especially important in wild animals given its epidemiological implications. The objectives of this study were to identify and characterize *S. aureus*, specifically MRSA, strains in wild lagomorph high-density areas. Ten hares and 353 wild rabbits from 14 towns with a high rabbit density in the Valencian region (eastern Spanish coast) were sampled. Swabs from the nasal cavity, ears, perineum and lesions (when present) were taken for microbiological studies. The detection of different genes and antibiotic susceptibility studies were also carried out. Of all the animals, 41.3% were positive for *S. aureus*, of which 63.3% were MRSA. Ears were the anatomical location with more *S. aureus* and MRSA strains. The more frequently identified MLST type was ST1945 (97.1%, 136/140). The *mec*A gene was found only in one sample. The rest (n = 139) carried the *mec*C gene and were included in CC130, except one. Penicillin resistance was detected in 28 *mec*-negative isolates and, in one case, bacitracin

resistance. *mec*A isolate presented resistance to enrofloxacin and tetracycline, and 10 *mec*C isolates also showed bacitracin resistance. No MRSA isolate was positive for genes *chp*, *sea*, *tst* and PVL. Two ST1945 isolates contained IEC type E (comprising genes *scn* and *sak*). One *mec*A-isolate was positive for *bla*Z. Of the 28 MSSA strains showing resistance to penicillin, 22 carried the *bla*Z gene. These surprising results highlight the marked presence of MRSA strains in wild rabbits in high-density areas.

Keywords: methicillin-resistant *Staphylococcus aureus*; MRSA; *mec*C gene; wild rabbits; high-density areas

2.2. Introduction

Staphylococcus aureus is a widely distributed bacterium in nature and is often considered a frequent host of skin (Kloos, 1980) and mucous, mainly in the nasal cavity (Wertheim et al., 2005). Studies have shown that about 20% of humans are persistent nasal carriers of *S. aureus*, and around 30% are intermittent carriers (Wertheim et al., 2005). The percentage of nasal carriers reported in different animal species varies: 7.9% in horses (Burton et al., 2008), 29% in ewes (Vautor et al., 2005) and 32.1–53% in rabbits (Hermans et al., 2000; Vautor et al., 2005). Asymptomatic nasal carriers play a key role in the epidemiology and control of staphylococcal diseases, as the nasal cavity allows bacteria to persist over time and to multiply, which constitutes a source of infection (Wertheim et al., 2005). In human medicine, the main problem caused by *S. aureus* occurs in hospitals, with the most important cause of not only nosocomial infections (Jones, 2003), but also community-acquired infection (Francois et al., 2005). *S. aureus* is also a major pathogen in veterinary medicine that affects various animal species. In commercial rabbits, it has been signaled as one of the main causes of culling on farms (Segura et al., 2007; Rosell and de la Fuente, 2009). In these cases, staphylococcal infections in rabbitries are caused by the international dissemination of the ST121 lineage of *S. aureus*, and other less frequent lineages like ST96 (Vancraeynest et al., 2006; Viana et al., 2011).

The increase in bacterial resistance to antibiotics in recent years has become a serious health problem. *S. aureus* is perfectly capable of acquiring multiple resistance mechanisms to several antimicrobial agents (Pantosti et al., 2007; Foster, 2017), which limits their therapeutic effectiveness. One of the most important ones for its clinical repercussions is resistance to methicillin. Methicillin-resistant *Staphylococcus aureus* (MRSA) evolved from Methicillin-susceptible *Staphylococcus aureus* (MSSA) by acquiring SSC*mec* elements containing a *mec* gene (*mec*A, *mec*C), which encodes a protein with a low affinity for β-lactam antibiotics (Cuny et al., 2015).

In animals, livestock-associated MRSA (LA-MRSA) emerged in pigs in 2005 (Armand-Lefevre et al., 2005) and was later described in other animal species. The most widely related clonal complex (CC) to LA-MRSA is CC398. This CC was also isolated from farmers who had been in close contact with infected animals (Graveland et al., 2011), which indicates that this clone plays an important role as a reservoir of transmission to humans (Frana et al., 2013). Therefore, special attention has been paid to the colonization of animals with *S. aureus* because they may potentially act as a reservoir to humans (Cuny et al., 2015; Aires-de-Sousa, 2017; Angen et al., 2017) .

Rabbits are among the animal species in which LA-MRSA CC398 strains have been isolated. The first case in rabbits was reported on a commercial farm in Italy, which also involved farm workers and their families (Agnoletti et al., 2014). Recently, LA-MRSA strains have been described to belong to ST2855 (CC96), ST146 (CC5), ST398 (CC398) and ST4774 (CC130) from several rabbitries of the Iberian Peninsula (Moreno-Grúa et al., 2018). These CC have been related to illness in humans (Monecke et al., 2007; Lowder et al., 2009; Pantosti, 2012; Frana et al., 2013; Mat Azis et al., 2017) .

MRSA strains have been isolated in human hospitals and on animal farms where antibiotics are regularly used. These antibiotic-resistant organisms

can spread to communities and the environment (Smith, 2015). Therefore, free-living animals might be colonized or infected by human and livestock sources, and can be associated with contaminated environments, even though they do not directly come into contact with antimicrobial drugs (Dolejska et al., 2007).

In recent studies, a high *S. aureus* carriage rate has been detected in wild animals, including European wild rabbits. The most relevant finding was that all the isolates were MRSA (Ruiz-Ripa et al., 2019a), which suggests a wildlife MRSA reservoir. Additionally MRSA, belonging to CC130, has been detected in diseased European brown hares (*Lepus europaeus*) (Loncaric et al., 2013; Monecke et al., 2016).

The wild rabbit (*Oryctolagus cuniculus*) is an extremely abundant endemic species of the Iberian Peninsula in some areas, where conservation of endangered predators is essential, but where major crop damage is a problem (DOGV, 2018). Rabbits and wild hares are also subject to hunting and are subsequently consumed domestically, often with no adequate veterinary control or sanitary management.

The role of *S. aureus* as a possible pathogen or colonizer in wild populations of lagomorphs has not yet been studied in a massive and systematic manner. In high-density areas, the interaction between rabbits and other animals is higher and, therefore, the probability of *S. aureus* transmission will also be higher. For these reasons, this study sets out the following objectives: (1) to know the role of wild rabbits and hares as a reservoir of *S. aureus* in wild lagomorph high-density areas; (2) to characterize the *S. aureus* strains isolated from wild rabbits and hares to compare them with those obtained previously on commercial farms; (3) to study the prevalence of MRSA strains in wild populations.

2.3. Materials and methods

2.3.1. Sampling, isolation and characterization of *Staphylococcus aureus* isolates

2.3.1.1. Animals and geographical locations

Ten hares (*Lepus granatensis*) and 353 wild rabbits (*Oryctolagus cuniculus*) from one game range or more located in 14 high-density towns were sampled to check the presence of *S. aureus*. The 10 hares of the study were included because they were hunted together with rabbits and it was considered interesting to compare the findings observed in another lagomorph from the same geographical locations. Animals were donated by hunters for this study once they were dead. The study was carried out in the Valencian Region, formed by the provinces of Valencia, Castellon and Alicante (each, in turn, formed by several districts) in eastern Spain (***Figure 4***) in 2019.

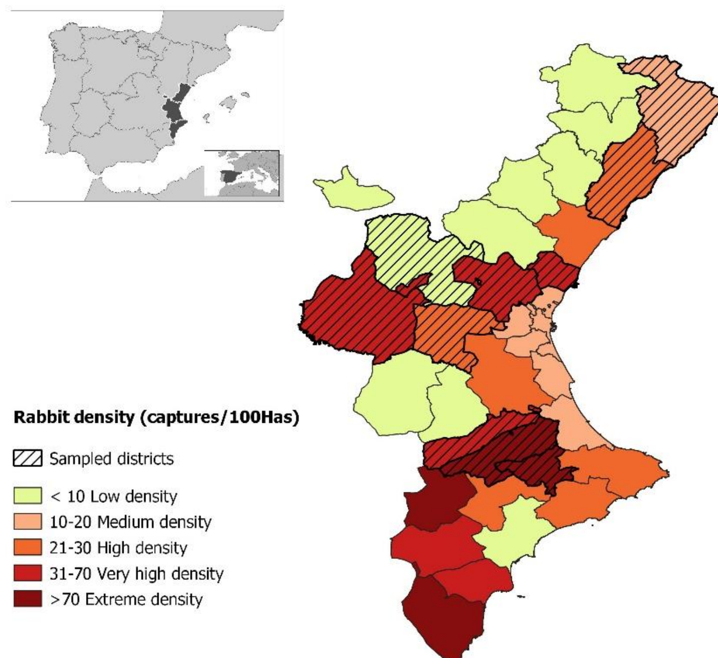

Figure 4. Districts of the Valencian Region (El Baix Maestrat (1) La Plana Alta (2), Camp de Morvedre (3), El Camp de Túria (4), Serranos (5), La Plana Utiel-

Requena (6), Hoya de Buñol (7), La Costera (8), La Vall d'Albaida (9), El Comtat (10)), colored according to rabbit density (Adapted from Moreno-Grua et al., 2020).

The lagomorph high-density towns were defined by the Valencian Regional Government (Order of June 11, 2009 and updated by Resolution of November 20, 2018 (DOGV, 2018)). The Valencian Regional Government, in collaboration with professional agricultural organizations, conducts surveys to identify those areas in different regions where the inordinate proliferation of rabbit populations associated with crop damage has been observed, and where hunting is allowed all year long.

The regional Valencian districts were classified as: extreme density (>70 rabbits/100 Has/year), very high density (31–70 rabbits/100 Has/year), high density (21–30 rabbits/100 Has/year), medium density (10–20 rabbits/ 100 Has/year) and low density (<10 rabbits/100 Has/year). This classification was based on the last 5-year (2012–2017) declared annual captures (rabbits per 100 hectares) published by the Fish and Game Service of the Valencian Regional Government (Torres et al., 2019) (***Figure 4***).

Samples from the towns of Pedralba and Vinarós were included in this study, which belong to low and medium-density districts, respectively, and are located on the borders of very high- or high-density districts, respectively, that also host high densities due to the proximity to these districts.

In order to calculate the necessary representative amount of rabbits to be sampled, the average district hunting bag of the last 5 years (2012–2017) was taken as the population data. The premise of an unknown prevalence in the *S. aureus* carrier condition was included in the analysis (WinEpi 2.0, Zaragoza, Spain). Such a restrictive criterion meant that the initially established number was not reached in several districts, namely El Baix Maestrat, El Camp de Túria, La Plana Utiel-Requena, La Costera and La Vall d'Albaida, as they lacked catches during the study period.

2.3.1.2. Microbiological studies

The studied animals were referred to the Universidad CEU Cardenal Herrera (Alfara del Patriarca, Valencia, Spain), where necropsy and sampling were carried out. Swabs from the nasal cavity, ears, perineum and lesions (whenever present) were taken.

Samples were inoculated on blood-agar (Becton–Dickinson, Sparks, MD, USA) and incubated aerobically at 37ºC for 24–48 h. *S. aureus* strains were identified on the basis of morphological growth characteristics and hemolytic properties (Devriese et al., 1996). When contamination appeared with other bacteria, Mannitol Salt Agar (Becton–Dickinson, Sparks, MD, USA) was used to obtain isolated *S. aureus* colonies. Only the plaques with 10 or more *S. aureus* CFU were considered positive. Those colonies compatible with *S. aureus* were grown in TSB (Tryptic Soy Broth) at 37ºC for 12 h with shaking. To perform PCRs, genomic DNA was extracted from each isolate by a Genelute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol, except for bacterial cells, which were lysed by lysostaphin (12.5 µg/mL, Sigma-Aldrich) at 37ºC for 1 h before DNA purification. Isolates were genotyped based on the analysis of the polymorphic regions of genes *coa* and *spa* as previously described (Viana et al., 2007). Multilocus-sequence typing (MLST) was performed in the selected isolates (Enright et al., 2000). According to sequence type (ST), isolates were ascribed to the different clonal complexes (CC). CC were assumed for some isolates according to their specific genotype (Moreno-Grúa et al., 2018).

All the strains were checked for the presence of *mec*A/*mec*C genes by PCR (***Table 5***), as previously described (Geha et al., 1994; Khairalla et al., 2017). Isolates were classified into MRSA or MSSA according to the presence or absence results of the *mec*A/*mec*C genes obtained by PCR.

2.3.1.3. Detection of *chp*, *sak*, *scn*, *bla*Z, *tst*, *sea*, and PVL-encoding genes, and *agr* typing

An MRSA strain of each ST type was selected from all the towns. These selected MRSA isolates were subjected to a PCR assay (***Table 5***) to detect the *luk*F/S-PV genes encoding the PVL toxin, and genes *sea* and *tst* that encode the SEA and TSST-1 toxin and *agr* typing, as previously described (Viana et al., 2015b). Strains were also checked for the presence of immune-evasion cluster (IEC) genes (*sak*, *chp*, and *scn*) by PCR, as described elsewhere (van Wamel et al., 2006). *bla*Z gene detection was investigated by PCR (Schnellmann et al., 2006).

2.3.1.4. Antibiotic susceptibility testing

The antibiotic susceptibility of the all isolates was determined by the disk diffusion method on Mueller–Hinton agar (Becton–Dickinson, Sparks, MD, USA) according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI). The disk diffusion assay was run with 15 antibiotics: bacitracin (10 U), enrofloxacin (5 µg) (OXOID), streptomycin (10 µg), spiramycin (100 µg), sulfadiazine (25 µg), chloramphenicol (30 µg) (BD), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), neomycin (30 µg), penicillin (10 U), tetracycline (30 µg), cefoxitin (30 µg), trimethoprim/sulfamethoxazole (1.25 µg/23.75 µg, respectively), and vancomycin (30 µg and 5 µg) (BIO-RAD, Hercules, CA, USA). S. aureus strain ATCC 25923 and Enterococcus faecalis strain ATCC 29212 were used as controls in the susceptibility test.

2.3.2. Statistical analysis

In order to know if the number of animals positive for *S. aureus* was statistically representative in relation to the number of studied animals in each district, the following statistical analysis was carried out. Categorical data were compared by the Fisher exact test. All the reported p values were two-tailed and analyses were performed using the GraphPad software. The variables with $p < 0.01$ were considered statistically significant.

Primer		Sequence (5' -3')	References
mecA	*mecA*-1m	GTAGAAATGACTGAACGTCCGATAA	(Geha et al., 1994; Khairalla et al., 2017)
	mecA-2c	CCAATTCCACATTGTTTCGGTCTAA	
mecC	*mecC*-1m	CTACACGTTCCATACCATTAG	(Geha et al., 1994; Khairalla et al., 2017)
	mecC-2c	CGCCTCTATGATAAACAATTGC	
lukF/S-PV	*lukF/S-PV*-1m	ATCATTAGGTAAAATGTCTGGACATGATCCA	(Viana et al., 2015b)
	lukF/S-PV-2c	GCATCAAGTGTATTGGATAGCAAAAGC	
sea	*sea*-1m	AAAGTCCCGATCAATTTATGGCTA	(Viana et al., 2015b)
	sea-2c	GTAATTAACCGAAGGTTCTGTAGA	
tst	*tst*-1m	CTAATCAAATAATCAAAACTGC	(Viana et al., 2015b)
	tst-2c	TTTCCAATAACCACCCGTTT	
agr type I	*agr* type I-1m	GTCACAAGTACTATAAGCTGCGAT	(Viana et al., 2015b)
	agr type I-2c	ATGCACATGGTGCACATGC	
agr type II	*agr* type II-1m	TATTACTAATTGAAAAGTGGCCATAGC	(Viana et al., 2015b)
	agr type II-2c	ATGCACATGGTGCACATGC	
agr type III	*agr* type III-1m	GTAATGTAATAGCTTGTATAATAATACCCAG	(Viana et al., 2015b)
	agr type III-2c	ATGCACATGGTGCACATGC	
agr type IV	*agr* type IV-1m	CGATAATGCCGTAATACCCG	(Viana et al., 2015b)
	agr type IV-2c	ATGCACATGGTGCACATGC	
sak	*sak*-1m	AAGGCGATGACGCGAGTTAT	(vanWamel et al., 2006)
	sak-2c	GCGCTTGGATCTAATTCAAC	
chp	*chp*-1m	TTTACTTTTGAACCGTTTCCTAC	(vanWamel et al., 2006)
	chp-2c	CGTCCTGAATTCTTAGTATGCATATTCATTAG	
scn	*scn*-1m	ACTTTAGCAATCGTTTTAGC	(vanWamel et al., 2006)
	scn-2c	CTGAAATTTTTATAGTTCGC	
blaZ	*blaZ*-1m	CAGTTCACATGCCAAAGAG	(Schnellmann et al., 2006)
	blaZ-2c	TACACTCTTGGCGGTTTC	

Table 5. The primer sequences used in this study.

2.4. Results

2.4.1. Identification of *Staphylococcus aureus*, MRSA and lesions

S. aureus was isolated in 41.3% (150/363) of all the wild rabbits and hares included in this study in at least one of the sampled locations (nasal cavity, ears or perineum). Of the 150 animals positive for *S. aureus*, the *mec*C gene was detected in 94 (62.7% of the positive animals) and the *mec*A gene was observed in one animal (0.7% of the positive animals) (***Table 6***).

Of the 10 hares included in this study, only one from the town of Cheste carried *S. aureus* in its nostrils. The strain isolated from this hare was MSSA,

and it had a different CC (CC121) to the isolated strains of rabbits hunted in the same district (CC5, CC130, CC398 and CC425).

The number of animals carrying *S. aureus* varied among the different sampled towns (***Table 6***). The towns with more animals carrying *S. aureus* were Pedralba (n = 37), Castellón de Rugat (n = 26) and Alfafara (n = 19). In addition, Alfafara, Pedralba and Cheste were the towns where a high percentage of animals with MRSA was detected (100%, 86.5% and 69.2%, respectively) and they all carried the *mec*C gene, except for one from the town of Cheste for which *mec*A was identified.

In all the following were found: 244 *S. aureus* isolates (244/1100; 22.2%) from the swabs taken from the nasal cavity, ears and perineum (n = 1089) in the 363 animals; additional swabs of six rabbits and two hares presenting one lesion or more (n = 11); 57.4% (140/244) of the *S. aureus* isolates were MRSA (***Table 7***). Of the 140 MRSA isolates, *mec*A was detected in only one isolate, and *mec*C was amplified in the 139 remaining isolates.

Id	District	Town	No. of Animals Tested	No. of Animals *S. aureus* Positive [1]	*p* [2]	Animals *mecC* +	Animals *mecA*+	*mec* (%)
1	El Baix Maestrat	Vinaroz	1	1	0.4132	1	0	100
2	La Plana Alta	Cabanes	120	18	**0.0001**	10	0	55.6
3	Camp de Morvedre	Faura	31	9	0.1826	1	0	11.1
4	El Camp de Túria	Llíria	1	0	1	0	0	0
4	El Camp de Túria	Vilamarxant	2	1	1	1	0	100
5	Serranos	Pedralba	39	37	**0.0001**	32	0	86.5
6	La Plana Utiel-Requena	Requena	2	0	0.5137	0	0	0
6	La Plana Utiel-Requena	Utiel	32	6	**0.0077**	0	0	0
7	Hoya de Buñol	Cheste	53	13	**0.0098**	8	1	69.2
7	Hoya de Buñol	Godelleta	15	11	0.0143	4	0	36.4
8	La Costera	Montesa	1	0	1	0	0	0
9	La Vall d'Albaida	Castelló de Rugat	28	26	**0.0001**	15	0	57.7
9	La Vall d'Albaida	Montaverner	18	9	0.4695	3	0	33.3
10	El Comtat	Alfafara	20	19	**0.0001**	19	0	100
	Total		363	150		94	1	63.3

Table 6. Animals positive for *S. aureus* and MRSA in the different studied districts.
[1] *Number of animals positive for S. aureus at one or more of the sampling sites.*
[2] *Values in bold denote the variables with p < 0.01, considered statistically significant.*

Sampling localization	Samples	*S. aureus*	(%)	MRSA	(%)
Nasal cavity	363	70	19.3	34	48.6
Ears	363	121	33.3	80	66.1
Perineum	363	45	12.4	20	44.4
Lesions	11	8	72.7	6	75
Dermatitis	6	5	83.3	3	60
Hepatic abscesses	2	2	100	2	100
Conjunctivitis	3	1	33.3	1	100
Total	1100	244	22.2	140	57.4

Table 7. Positive samples to *S. aureus* and MRSA according to sampling localization (in the different studied locations).

When considering the anatomical location of sampling, ears were by far the place from which more positive samples were isolated and where a higher percentage of MRSA was found. *S. aureus* was isolated in 33.3% (121/363) of the swabs taken from ears, and 66.1% of them were MRSA (80/121). In nasal cavity and perineum, fewer swabs were positive for *S. aureus* (70 and 45, respectively), but MRSA strains were also detected (48.6% and 44.4%, respectively) (***Table 7***). In general, very few lesions were observed after the necropsy of animals, except for traumatic lesions caused by animal hunting with firearms. However, *S. aureus* had evolved in many of them because of the 11 samples taken from lesions (dermatitis, hepatic abscesses, conjunctivitis), eight were positive for S. aureus and six carried the *mec*C gene.

In some animals, *S. aureus* strains were isolated in different locations at the same time. Therefore, the number of animals carrying *S. aureus* and the number of positive samples differed. Eighty-five animals carried *S. aureus* only in one location; in 45, 16 and 4 rabbits, the bacterium was isolated in 2, 3 or 4 different locations, respectively. Another interesting result was that two different *S. aureus* strains were found in the same anatomical location in five animals.

2.4.2. Characterization of *Staphylococcus aureus* isolates

The MLST typing analysis revealed 13 different STs (***Table 8***). The MLST type with the most isolates was ST1945 (n = 177), followed immediately by

ST425 (n = 42) and ST121 (n = 7). Other less frequent STs were ST5821 (n = 4), ST5826 (n = 3), ST398 (n = 3), ST5 (n = 2) and ST5822, ST5823, ST5824, ST5825, ST5844 and ST5845 (n = 1, each).

Most of the strains isolated from animals belonged to CC130 (74.2%; 181/244). The next most prevalent CC was CC425 (19.3%; 47/244). The other found CCs were CC121 (7/244), CC398 (3/244) and CC5 (2/244), but they were infrequently isolated.

The 140 MRSA isolates belonged mostly to ST1945 (97.1%, 136/140). The remaining four isolates corresponded to ST398, ST5822, ST5823 and ST5824. The *mec*A gene was found only in one sample, the only MRSA belonging to ST398 (CC398). The other samples (n = 139) carried the *mec*C gene and were included in CC130, except for one (ST5824), which was a CC that has not yet been described.

MLST	CC	MRSA	MSSA	Total
ST1945	CC130	136	41	177
ST425	CC425	-	42	42
ST121	CC121	-	7	7
ST5821	CC425	-	4	4
ST5826	singleton	-	3	3
ST398	CC398	1	2	3
ST5	CC5	-	2	2
ST5822	CC130	1	-	1
ST5823	CC130	1	-	1
ST5824	singleton	1	-	1
ST5825	CC130	-	1	1
ST5844	CC425	-	1	1
ST5845	CC130	-	1	1
Total		140	104	244

Table 8. Correlation between MLST and the clonal complexes of the studied isolates.

Four rabbits presented lesions and carried *S. aureus* in some anatomical locations simultaneously. The genotypic analysis showed that the *S. aureus* strains isolated from nostrils/ear/perineum and lesions were clonally related in 100% animals. In a single case, corresponding to OC19006, a strain different from those from nostril/perineum was identified in the lesion (***Table 9***).

Lesions	Rabbits	CC Lesion	CC Nostril	CC Ear	CC Perineum
Dermatitis	OC19006	CC130/CC425	CC425	-	CC425
Conjunctivitis	OC19190	CC130	CC130	CC130	CC130
Hepatitis	OC19275	CC130	CC130	CC130	CC130
Hepatitis	OC19276	CC130	CC130	CC130	CC130

Table 9. Relation among the clonal complex (CC) isolates from rabbits with lesion/s and *S. aureus* carriers.

2.4.3. Antibiotic resistance profile

Antibiograms were performed of all the *S. aureus* positive isolates obtained in each studied town (244 isolates: 140 MRSA and 104 MSSA). Penicillin resistance was detected in 28 *mec* negative isolates and, in one case, bacitracin resistance. *mec*A isolate presented resistance to enrofloxacin and tetracycline, and 10 *mec*C isolates also showed bacitracin resistance. Of the 140 MRSA strains, 49 were sensitive to cefoxitin and 34 to penicillin. None of the 104 MSSA strains showed resistance to cefoxitin, but 28 strains resisted penicillin. Eleven isolates were Bacitracin-resistant (10 MRSA and 1 MSSA). The strain isolated with the *mec*A gene was also resistant to enrofloxacin and tetracycline (***Table 10***).

2.4.4. Detection of the IEC cluster (*scn*, *chp*, *sak* and *sea*), *bla*Z, *tst* and the PVL genes among MRSA isolates

The PCR detection of the IEC genes, *bla*Z, *tst*, and the PVL-encoding genes was carried out to select MRSA isolates. None of these isolates were positive for genes *chp*, *sea*, *tst* and PVL. Two ST1945 isolates contained the IEC type E (comprising genes *scn* and *sak*). The *mec*A-isolate was positive for *bla*Z (***Table 10***). Of the 28 MSSA strains displaying resistance to penicillin, 20 carried the *bla*Z gene. ***Figure 5*** shows the positive and negative controls of the genes tested in this study, and the size of the PCR amplified fragments of each gene.

Strain	Town	Anatomical Location	Molecular Typing				SCC*mec*	IEC	Antimicrobial Resistance Phenotype	Antimicrobial Resistance Genes
			coa/spa	**MLST**	**CC**	***agr***				
1439	Alfafara	Ear	B4 I1	ST1945	CC130	III	XI	*scn-sak* (group E)	PEN-FOX	*mec*C-SCC*mec*XI
1490	Cabanes	Dermatitis	B4 I1	ST5822	CC130	III	XI	-	PEN	*mec*C-SCC*mec*XI
1564	Cabanes	Nostril	B4 I1	ST1945	CC130	III	XI	-	Susceptible	*mec*C-SCC*mec*XI
1768	Castelló de Rugat	Ear	B4 I1	ST1945	CC130	III	XI	-	PEN-FOX	*mec*C-SCC*mec*XI
1657	Cheste	Ear	D1 I6	ST398	CC398	I	V	-	PEN-FOX-ENO-TET	*mec*A-*bla*Z-SCC*mec*V
1660	Cheste	Ear	B4 I1	ST5823	CC130	III	XI	-	PEN-FOX	*mec*C-SCC*mec*XI
1651	Cheste	Perineum	B4 I1	ST1945	CC130	III	XI	-	PEN-FOX	*mec*C-SCC*mec*XI
1867	Faura	Ear	B4 II4	ST1945	CC130	III	XI	-	FOX	*mec*C-SCC*mec*XI
1707	Godelleta	Nostril	B4 I1	ST1945	CC130	III	XI	-	PEN-FOX	*mec*C-SCC*mec*XI
1712	Godellerta	Ear	A1 IV1	ST5824	singleton	II	XI	-	PEN-FOX	*mec*C-SCC*mec*XI
1620	Montaverner	Ear	B4 I1	ST1945	CC130	III	XI	-	PEN-FOX	*mec*C-SCC*mec*XI
1468	Pedralba	Perineum	B4 II4	ST1945	CC130	III	XI	-	PEN-FOX	*mec*C-SCC*mec*XI
1802	Vilamarxant	Nostril	B4 I1	ST1954	CC130	III	XI	-	PEN-FOX	*mec*C-SCC*mec*XI
1272	Vinaròs	Dermatitis	B4 I1	ST1945	CC130	III	XI	*scn-sak* (group E)	PEN-FOX	*mec*C-SCC*mec*XI

Table 10. Characteristics of the MRSA isolates recovered in the different studied high-density towns. *PEN, penicillin; FOX, cefoxitin; ENO, enrofloxacin; TET, tetracycline.*

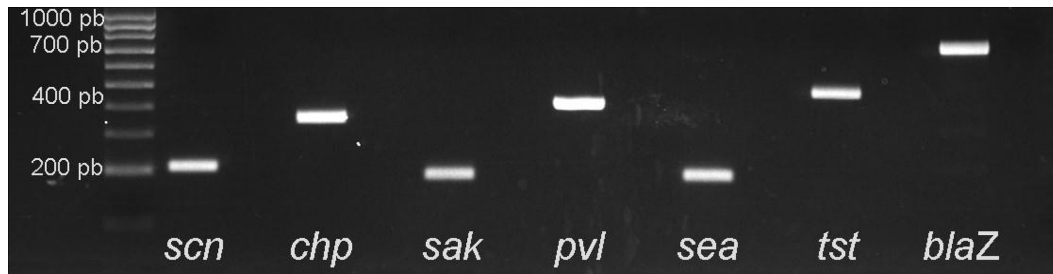

Figure 5. PCR detection of the IEC Cluster (*scn*, *chp*, *sak* and *sea*), *bla*Z, *tst* and the PVL genes among MRSA isolates. The figure shows the positive and negative control of each gene.

2.5. Discussion

In the present study, 244 *S. aureus* isolates obtained from 150 wild rabbits and hares, located in 10 high-density population areas (districts) of the Valencia Region in Spain, were analyzed. As far as the authors are aware, this is the first time that a study with such a large number of animals, conducted in high-density areas of animals, has been carried out. The reason why this study was performed in rabbit high-density areas was because the probability of detecting *S. aureus* carrier animals was believed to be higher in these areas. In total, 41.3% (150 of 363) of the animals carried *S. aureus* and positive animals were detected in all the studied geographical areas. Apparently, there was no clear geographical distribution of towns with more positive animals, although they were mainly located in the center and south (La Vall d'Albaida and El Comtat) of the Valencian region. Further studies in areas with lower rabbit density would be necessary to assess the relationship between *S. aureus* prevalence and rabbit density of animal.

Sixty-five animals carried *S. aureus* in more than one location; and two different *S. aureus* strains were identified in the same anatomical location in five rabbits. This agrees with previous studies which have reported how several different *S. aureus* strains can simultaneously colonize individual rabbits (Hermans et al., 1999). This is an important issue because it could affect the diagnosis of this condition if only one colony is selected during the

microbiological identification of staphylococcal infections or only one anatomical site is selected for sampling.

In humans, the nose is the main ecological niche where *S. aureus* resides (Wertheim et al., 2005). However, in this study, the highest percentage of *S. aureus* was detected in ears (49.6%). This finding has also been observed in commercial rabbitries where the presence of *S. aureus* was isolated from mainly samples taken from the ears and perineum among nine different anatomical locations (Hermans et al., 1999). The percentage of nasal carriers (28.7%) was lower than previously described in farm rabbits with staphylococcal problems (56%) (Selva et al., 2015), but was higher than other wild mammals (22.3%), and was substantially higherthan recently described for wild rabbits (8%) (Ruiz-Ripa et al., 2019a).

In the present study, only eight animals (6 rabbits, 2 hares) showed lesions; *S. aureus* was isolated from the lesions presented by the 6 rabbits. The high percentage of samples (8 of 11) obtained from the animals positive for *S. aureus* (72.7%) was surprising, especially when considering the different characteristics of the observed lesions compared to commercial rabbits (Corpa et al., 2009). Four of the six animals with positive *S. aureus* lesions (66.7%) also carried this bacterium in their noses. In humans, it is reported that nasal carriers of *S. aureus* are at increased risk of acquiring infection with this pathogen (Wertheim et al., 2005). This has also been found in animals, specifically rabbits, where the colonization capacity of this bacterium plays an important role in spreading the disease (Hermans et al., 1999). There are reports that *S. aureus* carriage in rabbits can be a risk for developing clinical infections (Selva et al., 2015). These results agree with this asseveration because the *S. aureus* strains isolated from nostrils/ear/perineum and lesions were clonally related in 100% animals. Only in one case, corresponding to rabbit OC19006, was a strain different from those from nostril/perineum identified in the lesion.

In order to understand the diffusion and possible origin of *S. aureus* (especially MRSA), correctly identifying the involved strain is vital. The most

frequently detected CC herein was CC130, followed by CC425. The ST121 (CC121) lineage has been identified in most nasal carriers (Selva et al., 2015) and chronic staphylococcal infections (Viana et al., 2011; Guerrero et al., 2015; Moreno-Grúa et al., 2018) in commercial rabbits. Therefore, these results indicate that the *S. aureus* strains which affect commercial and wild rabbits are different, which reinforces recently observed findings in wild rabbits in Aragón (a region in north Spain), where three strains were typed as t843 (ascribed to CC130). This finding has also been observed in samples from other wild species, such as reed deer (*Dama dama*) and wild boar (*Sus scrofa*) (Ruiz-Ripa et al., 2019a). Only one hare carried *S. aureus*, isolated from its nostril (MSSA), and it differed (CC121) from the strains isolated in the rabbits from the same district (CC5, CC130, CC398 and CC425). However, this number was not big enough to collect data on possible species specificity.

In the present study, 138 of the 181 CC130 strains carried *mec*C and the rest were methicillin-sensitive. The isolates reported to date carrying *mec*C belong mainly to common lineages in cattle, namely CC130, CC1943, and CC425, which suggests a zoonotic reservoir (Aires-de-Sousa, 2017). Besides cattle, *mec*C has been found in other farm animals, with isolates ST130 in sheep (Ariza-Miguel et al., 2014; Giacinti et al., 2017), ST4774 (a novel *tpi* single locus variant of ST130) in rabbits (Moreno-Grúa et al., 2018) and isolate ST425, which caused highly virulent infection, in one rabbit (Paterson et al., 2012). ST425 is a lineage that has been found in both wild and domestic animals, and has been previously noted in wild boar from Germany (Meemken et al., 2013), red deer from Spain (Porrero et al., 2014c) and also in humans. In this work, all the ST425 strains were methicillin-sensitive. The appearance of ST425-MRSA-XI, from cattle in the UK in 2011 (García-Álvarez et al., 2011), in wildlife (fallow deer, wild boar), and in an environmental sample from Spain (Porrero et al., 2014d), should put us on alert and to follow up these lineages in the future.

One interesting finding was that the only strain to carry the *mec*A gene belonged to the ST398 lineage. The first LA-MRSA case reported in rabbits for meat production belonged to this lineage (Agnoletti et al., 2014). Moreover, the first case of LA-MRSA in rabbits in Spain showed limited genetic diversity (ST2855, ST146, ST398, ST4774), with ST2855 being the predominant clone (Moreno-Grúa et al., 2018). It is notorious that this study found neither this lineage nor ST146 strains. This ST belongs to CC5, which has been previously described in *S. aureus* isolates from rabbit carcasses, but were not resistant to methicillin (Merz et al., 2016).

Of all the strains, 72.5% belonged to the ST1945 lineage, which is included in CC130. ST1945, a single-locus variant of ST130, has been reported as an *mec*C-carrying MRSA in humans in the UK (García-Álvarez et al., 2011), Germany (Cuny et al., 2011), France (Laurent et al., 2012) and Spain (García-Garrote et al., 2014). This lineage has been described in fecal samples taken from free-ranging wild small mammals in southern Spain, specifically in two wood mice. MRSA were detected in 7% of the analyzed wood mice (Gómez et al., 2014). The MRSA percentage herein observed was much higher, as 57.4% of *S. aureus* isolates had MRSA.

Another technique used to characterize the MRSA strains included the identification of the *agr* and staphylococcal cassette chromosome *mec* (SCC*mec*). In our study, all the isolates belonged to *agr* type III, as reported in other series (Cuny et al., 2011), except for isolate ST398, which was *agr*I. CC130 presented SCC*mec*XI and ST398 SCC*mec*V, as previously described (Witte et al., 2007; García-Álvarez et al., 2011; Bardiau et al., 2013).

Two *mec*C-positive MRSA isolates ST1945 carried genes *scn* and *sak* from the IEC system, and were consequently ascribed to IEC-type E. The origin of the *mec*C gene is unclear, but it has been detected in staphylococci from humans and animals (Gómez et al., 2014). Very little research has determined the presence of IEC genes in *mec*C-positive isolates, and all the available research works found isolates lacking genes *sak*, *chp* and *scn*

(Cuny et al., 2011; Paterson et al., 2014b; Monecke et al., 2016), which supports the hypothesis of the possible animal origin of these isolates. The detection of genes *sak* and *scn* in these two strains is relevant and poses questions about the potential origin of these isolates.

The possible origin of the MRSA strains remains an interesting enigma. In Spain, some wild animals (wild boars and fallow deer) located on a game estate had similar *mec*C-MRSA isolates detected in river water. Therefore, it was proposed that water could be a source that disseminates this type of strain in nature (Porrero et al., 2014b). The colonization of wild animals with *S. aureus* lineages from humans has been confirmed in a zoological park (Drougka et al., 2015). The transmission of MRSA strains between animals and persons is relatively easy, because it has been observed how 22% of students who visited pork farms (30% prevalence of MRSA) became MRSA nasal carriers (Frana et al., 2013). It has also demonstrated that the most prevalent *S. aureus* strain in commercial rabbits in Spain (ST121) has a human origin. This host adaptation took place 45 years ago when a single nucleotide mutation was sufficient to convert a human-specific *S. aureus* strain into one that could infect rabbits (Viana et al., 2015a). However, close contact between humans and animals has been assumed in all these cases, and the animals on commercial farms lived in high-density communities with interactions between animals. These conditions are not, a priori, those that take place in wild lagomorphs. However, it is worth considering that the present study entails the peculiarity of being conducted in rabbit high-density areas that could increase the probability of contact between animals and favour *S. aureus* spreading through rabbit populations. Additionally, CC130 (the most isolated CC in this study) is a lineage associated mainly with cattle, with transmission patterns that might be assumed in which wild rabbits could be infected by ingesting contaminated feces from wild ruminants in the same area. Our study did not analyze water or other animals that could interact with wild rabbits (predators, ruminants, etc.). Therefore, it would be interesting to extend this study along this line in the future to know the epidemiological origin of these bacteria.

Most MRSA isolates show resistance to cefoxitin. As previously reported for *mec*C-carrying MRSA, most isolates were susceptible to all the tested antibiotics, except for β-lactams (García-Garrote et al., 2014), and 10 strains were Bacitracin-resistant. However, the ST398-MRSA-*mec*A strain also shows resistance to enrofloxacin and tetracycline, and carried the *bla*Z gene. Resistance to tetracycline has also been associated with livestock-related ST398 (Harrison et al., 2017).

Food-producing animals, both livestock and wildlife, and derived products are considered potential sources of MRSA in humans (Traversa et al., 2015). However in that study, which included 1365 wild animals, *S. aureus* was detected in only 2.0% of wild animal carcasses and in 3.2% of wild boar lymph nodes, and none had MRSA. Therefore, the authors concluded that the risk of transmission to humans was limited. Conversely, in our study, the high presence of *S. aureus* (41.3% of animals), of which 63.3% had MRSA, indicates an alarming situation for wild rabbits in our study area, because these animals can be consumed directly by hunters, or even be donated to charities, without undergoing adequate veterinary inspections.

2.6. Conclusion

In conclusion, the results suggest that wild lagomorphs can constitute a reservoir of MRSA isolates in nature. Our report confirms the high presence of the MRSA CC130 lineage containing the *mec*C gene in wild rabbits and hares in east Spain, which could be transmitted to domestic animals or humans with major public health implications. It would be advisable to conduct similar studies in low-density areas of animals and in other regions of Spain and Europe to determine the degree of dissemination of MRSA isolates in lagomorphs and other wild species.

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2. Phenotypic and genotypic study of the SCC*mec* cassette element in the MRSA and MSSA strains isolated from rabbits

In Works 1 and 2 we observed a wider spread of *S. aureus*, specifically of MRSA strains and multiresistant strains, than expected in both commercial farm and wild rabbits. This confirms that we face a global problem that affects animals and can pose a public health risk. Antibiotic-resistance genes have been described as being found among mobile genetic elements like many virulence genes, but they are not virulence factors. Furthermore, despite the numerous studies on MRSA in many species, information on these strains in *S. aureus* isolated from rabbits is very scarce. This, together with the fact that *S. aureus* is a very versatile bacterium that continuously evolves and new clones with new resistance continually appear due to the horizontal transmission of genetic material, makes the in-depth study of the new strains being isolated necessary to know the mechanisms that make these strains acquire virulence and resistance factors to antibiotics.

In this context, we intended to study in more depth the most representative MRSA and MSSA clones that we previously found, and to evaluate their relation with antibiotic resistance, which is another important point of global concern. To do so, in Work 3 we sequenced a selection of MRSA isolates to study mobile genetic element SCC*mec* and the genes involved in antibiotic resistance. Then we carried out antibiograms of many isolates to evaluate the resistance phenotype.

Work 3: Phenotypic and genotypic study of the SCC*mec* cassette element in MRSA and MSSA strains isolated from rabbits, paying special attention to antibiotic resistance.

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3.1. Summary

The increase in antibiotic resistant bacteria is a problem, and specifically in *Staphylococcus aureus* one of the most important resistances is methicillin resistance. The acquisition of the mobile genetic element SCC*mec* (staphylococcal chromosomal cassette containing the *mec* gene) results in methicillin-resistant *Staphylococcus aureus* (MRSA) strains. In this study, the sequences of a selection of 36 strains representing the most frequent ST types (96, 121, 146, 398, 1945, 2855, 3764, 4774, 4998 and 5001) and main resistance profiles were compared in order to study the characteristics of their SCC*mec* elements and molecular structure. Additionally, the antibiotic resistance profile of 424 MRSA strains isolated from commercial and wild rabbits were carried out. First, after sequencing the MRSA strains, 5 different types of SCC*mec* cassette were obtained and it was observed that each ST was associated with one of the SCC*mec* elements. The SCC*mec* cassette types IVc, V and XI found were similar to those described above. However, we found a new type of cassette harboring *mec*C gene in a ST2855 type strain, and another strain harboring *mec*A and *mec*C genes simultaneously. Second, in the phenotypic resistance profile we found that *mec*A MRSA strains were resistant to more antibiotics than *mec*C MRSA ones, and MRSA strains with *mec*A and *mec*C genes showed similar resistance profile as MRSA strains with only the *mec*A gene.

Key words: *Staphylococcus aureus*, MRSA, staphylococcal chromosomal cassette, SCC*mec*.

3.2. Introduction

Staphylococcus aureus is a bacterium that affects humans and numerous animal species, both domestic and wild, among which one of the most affected is the rabbit. It is a ubiquitous microorganism and difficult to eliminate and it is a very versatile bacterium capable of adapting to highly variable environments. In addition, it is part of the normal microbiota of the skin and mucosa, from where it can colonize other niches and produce various pathogenic processes due to its numerous virulence factors (Simpson et al., 2003; Vancraeynest et al., 2004). In commercial rabbits *S. aureus* is one of the main causes of elimination on farms (Segura et al., 2007; Rosell and de la Fuente, 2009), producing mainly mastitis, pododermatitis, abscesses, pyodermas, otitis and rhinitis (Corpa et al., 2009). One of the most important problems in treating this pathogen is resistance to antibiotics. *S. aureus* has a great capacity to acquire multiple mechanisms of resistance to various antimicrobial agents (Pantosti et al., 2007; Foster, 2017), which limits its therapeutic effectiveness. One of the most important due to its clinical repercussions is methicillin resistance. Recently, many resistant strains obtained from numerous rabbit farms have been described, among which the methicillin-resistant *S. aureus* (MRSA) strains stand out (Moreno-Grúa et al., 2018). Further, antibiotic resistance is present not only in nosocomial bacteria, but also in community environments microorganisms (Pesavento et al., 2007), and in wild animals like wild rabbits and hares (Moreno-Grua et al., 2020).

The *S. aureus* genome is highly clonal and is divided into lineages defined by clonal complexes (CCs) of sequence types (STs) that determine its phylogenetic classification. Within the bacterial chromosome of *S. aureus* there is a part of the genome considered variable, made up of mobile genetic elements, including bacteriophages, pathogenicity islands, plasmids, transposons, integrative conjugative elements, integrons and

staphylococcal chromosomal cassettes (SCC). Antibiotic resistance genes have been described in all mobile genetic elements of *S. aureus*, except in bacteriophages (Haaber et al., 2017), being these elements the main way of acquiring resistance. The SCC*mec* cassette element includes large variability of antibiotic and metal resistance genes in addition to the *mec* gene that confers methicillin resistance (Ito et al., 2004). This means that the transmission of this mobile genetic element does not only affect the transmission of resistance to beta-lactam antibiotics but also the transmission of resistance to many other families of antibiotics. This represents a serious public health problem because bacteria carrying the mobile genetic element SCC*mec* cassette are in many cases multi-resistant (Rahi et al., 2020), which makes the treatment more complicated.

One of the characteristics of the mobile genetic element SCC*mec* is the presence of the *mec* gene complex that contains one of the methicillin resistance genes (*mec*A, *mec*B and *mec*C) together with the genes that control its expression, *mec*R1 (encoding the signal that transduces the MecR1 protein) and *mec*I (encoding the repressor protein MecI) and is a carrier that exchanges genetic information between *Staphylococcus* strains. This *mec* gene complex together with the cassette chromosome recombinase complex (*ccr*) are involved in the specific insertion or split-off of this element in the bacterial genome, form the basis of its classification (IWG-SCC, 2009; Ito et al., 2014) and are essential elements for the functionality of the SCC*mec*. In addition, in this mobile genetic element the so-called non-essential, very heterogeneous J-regions (J1-3) are located, varying in length and composition, where other antibiotics resistance genes are found (Ito et al., 2004; Xue et al., 2015). Traditionally, MRSA strains have been characterized by the presence of the *mec*A gene, however in recent years the prevalence of *mec*C MRSA has increased in humans and in domestic and wild animals (García-Garrote et al., 2014; Paterson et al., 2014a; Moreno-Grúa et al., 2020).

Due to the increasing importance of antibiotic resistances, especially methicillin resistance, and its relationship with mobile genetic elements, the objective of this work was the phenotypical study of the antibiotic resistances presented by the strains MSSA and MRSA, and the genotypic study of the mobile genetic element SCC*mec* in a selection of *S. aureus* strains isolated from rabbits.

3.3. Materials and methods

3.3.1. *Staphylococcus aureus* isolates

In this study, 424 strains of *S. aureus* isolated from commercial and wild rabbits between 2014 and 2019 in the Iberian Peninsula were included. These isolates were seeded in plates of blood-agar (BioMérieux, Marcy l'Etoile, France) and incubated aerobically at 37ºC for 24–48h. *S. aureus* strains were identified based on morphological growth characteristics and hemolytic properties (Devriese et al., 1996).

All the strains were checked for the presence of the *mec*A/*mec*C genes by PCR, as previously described (Geha et al., 1994; Khairalla et al., 2017). To perform PCR, genomic DNA was extracted from each isolate with a Genelute Bacterial Genomic DNA kit (Sigma), according to the manufacturer's protocol, except for bacterial cells, which were lysed by lysostaphin (12.5 mg/ml, Sigma) at 37ºC for 1 h before DNA purification. Furthermore, isolates were genotyped by MLST (Enright et al., 2000).

3.3.2. Sequencing and analysis of mobile genetic elements

A selection of 36 strains representing the most frequently isolated ST types (96, 121, 146, 398, 1945, 2855, 3764, 4774, 4998 and 5001) and main resistance profiles was made, focusing above all on the presence of the *mec* gene. The complete sequencing of the genomes was carried out in Edinburg Genomics (Edinburgh, United Kingdom). The genomic sequences obtained were annotated and compared with other genomes available through the online program RAST (Rapid Annotation using Subsystem

Technology, 2.0 version, http://rast.nmpdr.org) (Glass et al., 2010). The comparison of mobile genetic elements was completed using the programs BLAST (Basic Local Alignment Search Tool, 2.0 version) from the NCBI server (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1997)) and the program SnapGene (GSL Biotech LLC, 3.2.1 version, www.snapgene.com).

3.3.3. Antibiotic susceptibility test

The antibiotic susceptibility of the *S. aureus* isolates was determined by the disk diffusion method on Mueller-Hinton agar (MHA, CONDA, Spain), in accordance with the recommendations of the Institute of Clinical and Laboratory Standards (CLSI). *S. aureus* strain ATCC 25923 and *Enterococcus faecalis* strain ATCC 29212 were used as controls in the susceptibility test. The disk diffusion assay was done with 15 antibiotics: amoxicillin-clavulanic acid (20 µg /10 µg, respectively), bacitracin (10 U), enrofloxacin (5 µg) (OXOID), streptomycin (10 µg), spiramycin (100 µg), chloramphenicol (30 µg) (BD), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), neomycin (30 µg), penicillin (10 U), tetracycline (30 µg), cefoxitin (30 µg), and trimethoprim/sulfamethoxazole (1.25 µg /23.75 µg, respectively) and vancomycin (30 µg) (BIO-RAD). The selection of antibiotics was carried out focusing on the antibiotics that are most used in rabbit farming and it was tried that all the families of antibiotics were represented. ***Table 11*** represents the families of antibiotics that we have included in this study.

Resistance to antibiotics was evaluated in two ways. First analysis aimed to evaluate the number of antibiotics to which each group of strains methicillin sensitive and methicillin resistant was resistant. To test it, a chi-squared test was performed. The second way aimed to evaluate mean resistance to 15 antibiotics depending on the group of strains methicillin sensitive and methicillin resistant and the antibiotic resistance profile of each group. To test it, we used a generalized linear mixed model, with a binomial probability distribution for the response and a logit transformation $[\ln(\mu/(1-\mu))]$ as the

link function (Proc GLIMMIX of SAS). Strains presenting Resistance to a given antibiotic were coded as 1; Strains not presenting resistance were coded as 0 and unclear strains presenting intermediate resistance were coded as 0.5. The model included group of strains methicillin sensitive and methicillin resistant (4 levels) and the interaction between group and antibiotic type (60 levels). *P*-values for the differences of LS-means were obtained using a Tuckey multiple comparison adjustment. However, for the interaction, we only performed comparisons within a given antibiotic or within a given group. These comparisons were performed using the slicediff option of Proc GLIMMIX (SAS). It enables us to perform multiple comparisons among the levels of one effect at a fixed level of the other effect. The Tuckey multiple comparison adjustment was based on the number of comparisons within each level.

Group	**Subgroup**	**Antibiotic**
Beta-lactams	Penicillins	Penicillin
		Amoxicillin + Clavulanic acid
		Methicillin
	2nd generation cephalosporins	Cefoxitin
Macrolides		Erythromycin
		Spiramycin
Tetracyclines		Doxycycline
		Tetracycline
Aminoglycosides		Streptomycin
		Gentamicin
		Neomycin
Peptides	Polypeptides	Bacitracin
	Glycopeptides	Vancomycin
	Sulphonamides	Sulfamethoxazole
	Diamino pyridines	Trimethoprim
		Chloramphenicol
Quinolones	Fluoroquinolones	Enrofloxacin

Table 11. Antibiotic groups and subgroups involved in this study.

3.4. Results

3.4.1. Genotyping by MLST and description of the mobile genetic element SCC*mec*

Sequenced strains ST96, ST121, ST3764 and only one ST2855 were methicillin sensitive *S. aureus* (MSSA). The *mec*A gene was found in ST146 and ST398 strains, and the *mec*C gene in ST1945, ST2855 and ST4774 strains. The simultaneous presence of both genes was found in ST2855, ST4998 and ST5001 strains (***Table 13***).

After studying the complete sequences of the SCC*mec* element in each of the selected strains, 5 different types of this mobile genetic element were found:

3.4.1.1. SCC*mec* composed of Type III SCC*mec* and the new SCC*mec*.

The SCCmec type III strains were ST2855, ST4998 and ST5001. The cassette found in these strains contained an incomplete fragment of the described type III SCC*mec*, which was inserted into another SCCmec cassette with the *mec*C gene specifically between the J1 zones and the cassette chromosome recombinas (*ccr*) complex (***Figure 6***). This *mec*C-SCC*mec* was the same found in one ST2855-type strain described later in section 1.5 as a new type of SCC*mec* cassette; the combination of these two cassettes: incomplete *mec*A-SCC*mec* type III and new mecC-SCCmec, made these strains simultaneously contain the gene *mec*A and *mec*C within the same SCC*mec* cassette. Therefore, this cassette is the combination of two different cassettes by insertion of the incomplete *mec*A-SCC*mec* type III into the other unknown *mec*C-SCC*mec*. This mobile genetic element also had a cadmium resistance gene (Cd) and the chromosome cassette recombinases *ccr*A and *ccr*B. The two ST5001 strains sequenced with this type of SCC*mec* had it excised from the bacterial chromosome, that is, this SCC*mec* cassette in these bacteria is not inserted in the bacterial chromosome and is part of the non-genomic DNA.

3.4.1.2. Type IVc SCC*mec*.

SCC*mec* type IVc was found in both ST146 evaluated samples and these strains had the *mec*A gene. We also found a bleomycin resistance gene (BRP), replication proteins (RP), and the cassette chromosome recombinase *ccr*A and *ccr*B.

3.4.1.3. Type V SCC*mec*.

SCC*mec* type V was found in all the ST398 evaluated samples and these strains had the *mec*A gene. We also found the cassette chromosome recombinase *ccr*B and *ccr*C.

3.4.1.4. Type XI SCC*mec*.

SCC*mec* type XI with the *mec*C gene were found in all ST1945 and ST4774 strains. The PBP2a homolog *mec*C found in this type of SCC*mec* cassette shares only 70% similarity to *mec*A PBP2a. However, the SCC*mec* of ST4774 strains isolated from farm rabbits shared 99.9% similarity to ST1945 strains isolated from wild rabbits. We also found the *bla*Z resistance gene and the cassette chromosome recombinases *ccr*A and *ccr*B in these strains.

3.4.1.5. New type of SCC*mec* element.

In one of the ST2855 sequenced strains, the SCC*mec* cassette did not resemble those described above, but the *mec*C gene contained in this element was class E. This gene shared 96% similarity with the *mec*C gene of the strains with SCC*mec* type XI, however the complete SCC*mec* cassette only had 44% similarity with the SCC*mec* cassette type XI. As we have mentioned previously, the sequence of this new SCC*mec* cassette was equal to a part of that found in the isolates of the new type III cassette described in section 1.1. of our study. This new element found differs from those described previously in that the J1 region was inserted after the *orf*X, instead of the J3 region, and the complete structure of the SCC*mec* was

located inversely. This causes that the *mec* gene complex is located at the end of the mobile genetic element, just before the J3 region. Despite this, the *mec* gene complex and the *ccr* complex were in the same direction as in the other SCC*mec* cassette. Furthermore, this new type of SCC*mec* cassette presented the *bla*Z resistance gene and the cassette chromosome recombinases *ccr*A and *ccr*B as do the type XI SCC*mec* cassette.

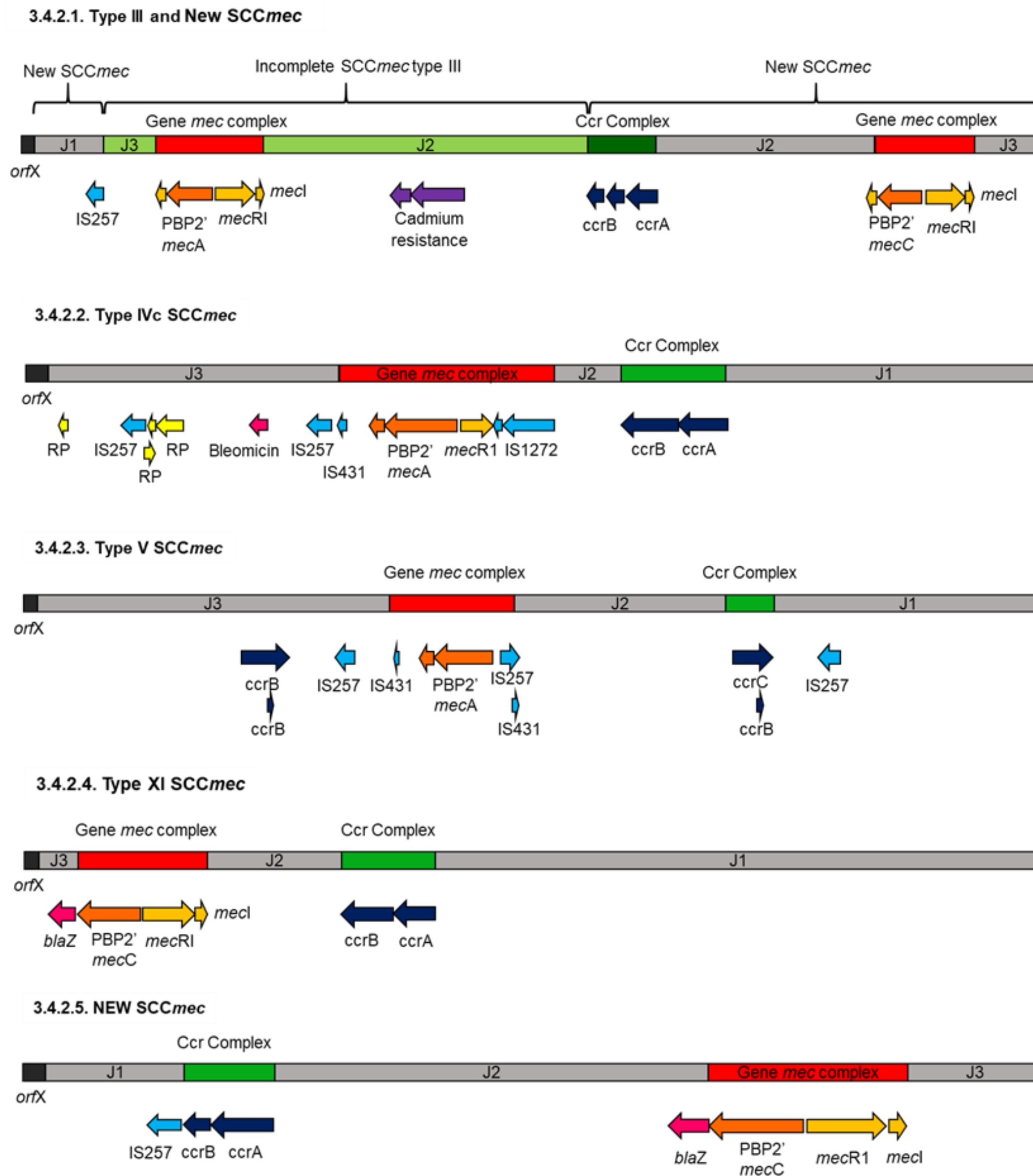

Figure 7. Different types of SCC*mec* found in the selection of sequenced strains. *Ccr: cassette chromosome recombinase; PBP2': Penicillin-binding protein 2; RP: Replication protein.*

3.4.2. Antibiotic resistance profile

Table 12 shows the mean resistance to 15 antibiotics (Main effect of group) and the antibiotic resistance profile (Interaction between group and antibiotic) of the four groups of strains methicillin sensitive and methicillin resistant. Regarding to the main effect, strains from the *mec*A-MRSA and *mec*A-*mec*C-MRSA groups presented higher average value than MSSA (+30 and +40 percentage points respectively; *P*<0.05). Moreover, *mec*C-MRSA presented lower average value than MSSA (-5 percentage points; P<0.05). However, antibiotic resistance profile was different among groups.

It is evident that MSSA strains generally present less resistance to antibiotics than MRSA strains, but they presented similar resistance to gentamicin than *mec*A-MRSA and *mec*A-*mec*C-MRSA strains (-2 and -3 percentage points respectively; *P*<0.05) and higher resistance to enrofloxacin (+23 percentage points; *P*<0.05), erythromycin (+34 percentage points; *P*<0.05), spiramycin (+34 percentage points; *P*<0.05), streptomycin (+15 percentage points; *P*<0.05), gentamycin (+26 percentage points; *P*<0.05) and tetracycline (+37 percentage points; *P*<0.05) compared to *mec*C-MRSA strains.

MRSA strains had an antibiotic resistant profile depending on whether they were *mec*A-MRSA, *mec*A-*mec*C-MRSA or *mec*C-MRSA. The antibiotic resistant profile of *mec*A-MRSA strains was very similar to *mec*A-*mec*C-MRSA strains and both different from *mec*C-MRSA antibiotic resistant profile. Strains with the *mec*A gene and *mec*A-*mec*C genes had a higher percentage of resistance to the antibiotics in general and specifically to amoxicillin-clavulanic acid (+10 and +18 percentage points respectively; *P*<0.05), doxycycline (+22 and +50 percentage points respectively; *P*<0.05), enrofloxacin (+79 and +84 percentage points respectively; *P*<0.05), erythromycin (+51 and +72 percentage points respectively; *P*<0.05), spiramycin (+51 and +78 percentage points respectively; *P*<0.05), streptomycin (+56 and +69 percentage points respectively; *P*<0.05), gentamicin (+28 and +29 percentage points respectively; *P*<0.05),

neomycin (+65 and +21 percentage points respectively; *P*<0.05) and tetracycline (+81 and +83 percentage points respectively; *P*<0.05) compared to *mec*C-MRSA strains. However, in the case of cefoxitin and penicillin, the three groups of MRSA strains show similar resistance percentages (except if we compare the *mec*A-MRSA strains with the *mec*C-

	MSSA	***mec*A-MRSA**	***mec*C-MRSA**	***mec*A-*mec*C-MRSA**
Main effect of group (P<0.001)				
average value	8 B	38 C	3 A	48 C
Interaction between group and antibiotic (P<0.001)				
Antibiotic:				
Amox./Clav.	1 a A	10 a B	0 a A	18 ab B
Bacitracin	1 a	1 a	7 a	3 a
Cefoxitin	1 a A	97 b C	69 b B	97 b BC
Chloramphenicol	1 a	3 a	1 a	3 a
Doxycycline	11 a AB	22 a BC	0 a A	50 ab C
Enrofloxacin	28 b B	84 b C	5 a A	89 b C
Erythromycin	38 c B	55 b BC	4 a A	76 ab C
Spiramycin	38 c B	55 b BC	4 a A	82 ab C
Streptomycin	20 ab B	61 b C	5 a A	74 ab C
Gentamicin	26 bc B	28 a B	0 a A	29 ab B
Neomycin	5 a A	65 b C	0 a A	21 ab B
Penicillin	29 b A	99 b B	78 b B	97 b B
Sulfa./Trim.	7 a AB	4 a AB	0 a A	18 ab B
Tetracycline	41 c B	85 b C	4 a A	87 b C
Vancomycin	0 a	1 a	0 a	3 a

Table 12. Resistance to antibiotics for the effect of group of strains methicillin sensitive and methicillin resistant and the interaction between group and antibiotic (LSMeans of percentage of resistance). *Amox,/Clav: Amoxicillin and Clavulanic acid; Sulfa. Trim.: Sulfamethoxazole Trimethoprim.[A, B, C] Means in the same row not sharing capital letter differed statistically at P<0.05. [a, b, c] For a given group in the interaction, means in the same column not sharing lower-case letter differed statistically at P<0.05*

MRSA strains in the case of cefoxitin, the latter have a lower percentage of resistance (-28 percentage points; *P*<0.05). It is remarkable that, although the *mec*A-*mec*C-MRSA strains present a percentage of resistance to antibiotics similar to that of *mec*A-MRSA, in the case of neomycin, having

the two *mec* resistance genes reduces the resistance to this antibiotic (-41 percentage points; P<0.05).

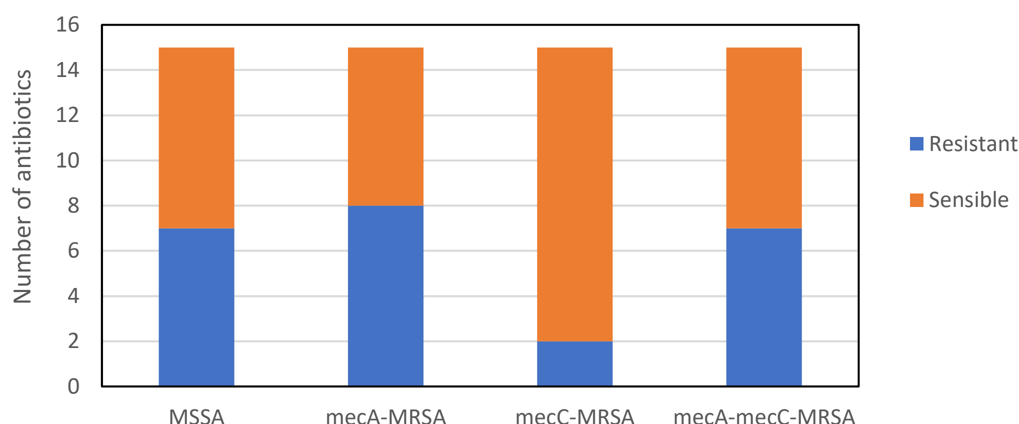

Figure 6. Number of antibiotics to which each group is resistant or sensitive.

Figure 6 shows the number of antibiotics to which each group is resistant or sensitive. In the strains from the MSSA group, seven antibiotics presented resistance (*P*<0.05). In the strains from the *mec*A-MRSA group, eight antibiotics presented resistance (*P*<0.05). In the strains from the *mec*C-MRSA group, only two antibiotics presented resistance (*P*<0.05). In

ST	CC	Number of samples	MRSA	***mec*** **gene**	**SCC*mec* type**	***ccr***	**Lenght (kb)**	**Resistant genes**
96	96	4	0	-	-	-	-	-
121	121	5	0	-	-	-	-	-
146	5	2	2	*mecA*	IVc	A, B	28,66	Bleomicin
398	398	5	5	*mecA*	Vc	B, C	34,11	-
1945	130	2	2	*mecC*	XI	A, B	32,56	*blaZ*
2855	96	3	0	-	-	-	-	-
2855	96	1	1	*mecC*	New	A, B	21,89	*blaZ*
2855	96	5	5	*mecA* y *mecC**	III and New	A, B	43,72	*blaZ* y Cd
3764	121	8	8	-	-	-	-	-
4774	130	2	2	*mecC*	XI	A, B	32,56	*blaZ*
4998	96	1	1	*mecA* y *mecC**	III and New	A, B	43,72	*blaZ* y Cd
5001	96	2	2	*mecA* y *mecC**	III and New	A, B	43,72	*blaZ* y Cd

Table 13. Relationship of the mobile genetic element SCC*mec* cassette with the different clonal complexes and ST types. *CC: Clonal Complex; Cd: Cadmium; blaZ: gene that confers resistance to beta-lactams. * MRSA strains that simultaneously presented the mecA and mecC genes.*

the strains from the *mec*A- *mec*C-MRSA group, four antibiotics presented resistance (*P*<0.05).

Moreover, comparing resistance to specific antibiotics between strains from *mec*A- MRSA group and the *mec*A- *mec*C-MRSA *mec*A- *mec*C-MRSA group presented lower resistance to neomycin than *mec*A- MRSA group (-44 percentage points; *P*<0.05), not existing statistical differences for the rest of antibiotics.

3.5. Discussion

All ST96, ST121, ST3774 (clonal complex CC121) strains were found to be methicillin sensitive (MSSA), and the *mec*A and *mec*C genes were found in ST146, ST398, ST2855 and ST4774 strains, which was previously described in the study of Moreno-Grua et al., 2018. It seems that each type of SCC*mec* cassette is always associated with one type of ST, this demonstrates a common origin of these MRSA strains and leads to think that horizontal transmission between strains of this mobile genetic element is not usual. Nevertheless, recent studies indicated that horizontal spread of SCC*mec* is more frequent than was believed before (Robinson and Enright, 2003; Hanssen and Ericson Sollid, 2006).

This study describes a new type of SCC*mec* cassette that contains the *mec*C gene. This element maintains the structure of the SCC*mec* cassette, but is located in the opposite way to those described above, which means that the *mec* gene complex is located at the end of the mobile genetic element before the J3 region and that the J1 region is inserted in *orf*X and not the J3 region as occurs in the other SCC*mec* cassettes. This finding corroborates the versatility and variability of this mobile genetic element (Becker et al., 2014). Despite this, the new type *mec*C-SCC*mec* had the same resistance genes (*bla*Z) as the type XI SCC*mec* and the structure of the *mec* gene and *ccr* complexes is maintained in both strains. This reflects the complexity of transmission of this mobile genetic element between the different bacterial strains.

A new type of SCC*mec* cassette formed by the insertion of part of a *mec*A-SCC*mec* type III in the new *mec*C-SCC*mec* was found in this study, which results in another new SCC*mec* cassette formed by recombination of both and simultaneously containing the genes *mec*A and *mec*C. The presence of both genes in the same strain has recently been described in *S. aureus* isolated from cows (Aklilu and Chia, 2020) but not in isolated strains of rabbits. The transmission mechanisms of the mobile genetic element SCC*mec* cassette are very complex and despite these findings close to knowing more about the transmission mechanisms, more studies are necessary to understand them completely.

The SCC*mec* cassette of ST4774 strains isolated from farm rabbits showed a 99.9% similarity to ST1945 strains isolated from wild rabbits. This suggests that this mobile genetic element has a common origin in bacteria isolated from farm animals and in those isolated from wild animals. The presence of *mec*C-MRSA isolates in river water has been described (Porrero, et al., 2014b). This may explain the fact that wild and farm animals have strains with the same phylogenetic origin and that the isolates of wild and farm animals share mobile genetic elements due to water that would act as a reservoir for environmental dissemination but more studies are necessary to confirm this hypothesis. Equally, more studies are needed to explain the reason for the emerging spread of *mec*C-MRSA strains isolated from wild animals (Moreno-Grúa et al., 2020), in order to demonstrate the benefit that these strains obtain carrying this gene when they colonize wild animals, theoretically not subjected to antibiotic pressure.

The *bla*Z resistance gene was detected within SCC*mec* that were type XI and the new *mec*C type, this had been previously described for CC130 strains (Shore et al., 2011a), but not for strains of the clonal complex CC96 belonging to ST1945 as we have found in this study. The *bla*Z gene encodes a beta-lactamase and is generally found as part of the *bla* operon (*bla*I, *bla*R1 y *bla*Z), which is widespread among gram-positive bacteria. The only previous evidence of a *mec* complex with *bla*Z was from *M. caseolyticus*

(Shore et al., 2011a). It has been speculated that the *mec* gene complex was generated after integration of *mec*A into the *bla* operon and that the *bla*Z-*mec*A-*mec*R1-*mec*I complex in *M. caseolyticus* may represent the ancestral form of the *mec* gene complex in staphylococci (Baba et al., 2009; Tsubakishita et al., 2010). The *mec* complex of SCC*mec* type XI may represent a supposed complex of ancestral *mec* genes in MRSA from which the new type of SCC*mec* found derives.

Strains with ST5001 belong to CC96 clonal complex and have recently been isolated by our research group. The peculiarity that we find in the SCC*mec* of these strains is that it was excised from the chromosome instead of being inserted in *orf*X. This had not been previously described. Excision and integration of SCC*mec* elements are mediated by cassette chromosome recombinases (*ccr*), which are large serine recombinases of the resolvase/invertase family (Wang and Archer, 2010). *Ccr*AB or *Ccr*C1 can carry out both excision and integrative recombination. Moreover, a *Ccr*A protein has little or no recombination activity on its own and requires a *Ccr*B protein for its functions (Misiura et al., 2013). In the excised *mec*A-*mec*C-SCC*mec* cassette described in this study, we only found *Ccr*A and *Ccr*B, so they may be responsible for the excision of the SCC*mec* element from the bacterial chromosome. In addition, the fact of being free outside the bacterial chromosome may represent a greater advantage because it can be transmitted horizontally to other strains more easily, and because it may present a greater phenotype of resistance to antibiotics due to in general more copies of the excised genetic material are produced than of the genetic material that is inserted in the bacterial chromosome.

Previous studies reveal that MRSA strains generally have resistance to other families of antibiotics (macrolides, lincosamides, tetracyclines, phenicols, aminoglycosides and even quinolones) (Ma et al., 2002; Rahi et al., 2020), since in *S. aureus*, the SCC*mec* element can variably carry genes for resistance to other antibiotics and metals in the J-regions (J1-3) by integrating into the bacterial chromosome. It may be because they have

acquired resistance determinants from other staphylococcal species, including coagulase negative ones (Ito et al., 2004). In our study we have observed that the *mec*A-MRSA strains presented a specific antibiotic resistance profile similar to others described for *mec*A-MRSA strains (Obaidat et al., 2018) but different from the resistance profile that *mec*C-MRSA strains had in our study. This would suggest a less close phylogenetic relationship between the *mec*A-MRSA and *mec*C-MRSA strains than the strains that have the different variants of the *mec*A SCC*mec* cassette and would demonstrate the great complexity and diversity of the SCC*mec* element (Becker et al., 2014). In case of *mec*C-MRSA strains, we have observed that the *mec*C gene confers resistance to beta-lactam antibiotics, as do the homologue *mec*A gene as already described (Ballhausen et al., 2014); this is due to the fact that the PBP2a protein in the *mec*C-MRSA strains is equally functional despite sharing only 70% homology with the PBP2a of the *mec*A-MRSA strains. However, in this study the *mec*C-MRSA strains did not show resistance to other antibiocic families apart from resistance to β-lactam antibiotics. This may be because the *mec*C-SCC*mec* cassette types (type XI and new) contain fewer antibiotic resistance genes than the *mec*A-SCC*mec* cassette types (type IVc and V). However, in work 1 we found that the *mec*C-MRSA strains isolated from farms presented resistance to other families of antibiotics, this suggests that in its origin the *mec*C-SCCmec did not have resistance to antibiotics but in farms where there is antibiotic pressure these strains have been acquiring resistance to other antibiotics in addition to β-lactam antibiotics. The strains that simultaneously carried the genes *mec*A and *mec*C presented a resistance profile similar to the strains that only had the gene *mec*A and different to the strains with *mec*C. It is probably due because the new SCC*mec* cassette is composed by the recombination of two cassettes and the antibiotic resistance genes are provided by the *mec*A-SCC*mec* cassette and not by the *mec*C-SCC*mec* cassette. The *mec*A-MRSA strains seem to have advantages over the *mec*C-MRSA because they present resistance to a greater number of antibiotics and metals.

However, the fact that a new SCC*mec* cassette is formed including the *mec*A and *mec*C genes simultaneously suggests that the combination of both SCC*mec* cassette provides benefits to bacteria versus those with only a simple SCC*mec* cassette, but despite this, in our study we have observed that *mec*A-*mec*C-SCC*mec* does not provide more resistance than *mec*A-SCC*mec* and, in fact, in some antibiotic such as neomycin, the number of resistant strains is less. This means that phenotypically, having both genes at the same time does not provide real advantages. More studies are needed to know the reason for the recent expansion of *mec*C-MRSA strains and *mec*A-*mec*C-SCC*mec* (Paterson et al., 2014a; Moreno-Grúa et al., 2020).

Because the SCC*mec* element plays a central role in the antimicrobial resistance characteristics, molecular epidemiology, and evolution of MRSA, a comprehensive study and understanding of prevalence and structural characteristics of the mobile genetic element SCC*mec* cassette can help in vigilance, implementation and research on MRSA isolates, as well as further development of preventive and therapeutic approaches.

3.6. Conclusion

Two new types of SCC*mec* cassette have been described one containing the *mec*C gene and another containing simultaneously *mec*A and *mec*C genes, two ST5001 strains harboured the new SCC*mec* containing *mec*A and *mec*C genes excised from de genome. Regarding to the resistance phenotype, *mec*A-MRSA strains presented a specific antibiotic resistance profile different from the one that *mec*C-MRSA strains had; on the contrary, *mec*A-*mec*C MRSA strains presented an antibiotic resistance profile similar to *mec*A MRSA strains.

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4.Effect of selection by growth rate and vitrification of embryos on the rabbit (*Oryctolagus cuniculus*) immune system and its response after a *Staphylococcus aureus* experimental infection

When a pathogen colonises a host, many circumstances determine resolution infection. These circumstances depend on the pathogen, the host, and the interaction between both. All factors are important in determining the success or failure of infection. Therefore, it is important to consider all these factors to study the pathogenesis of *S. aureus* infections.

The rabbit is a common host for *S. aureus*, and it is a very useful model for studying infections with this bacterium, as previous studies carried out by our research group have demonstrated. On rabbit farms, this bacterium is a serious problem that causes significant economic losses. Productive pressure and the selection of certain characters to which these animals are subjected make pathologies worsen, possibly because these circumstances cause the immune system to alter. For this reason, it is important to study the host's extrinsic and intrinsic conditions (immune system, animal genetics, phase of the productive cycle, etc.) as a basis to improve the problem. One of the conditions that can affect animals' immune system state is genetic selection because, by selecting certain characters, others may be indirectly improved or worsening. For this reason, when selecting animals it is very important to control other aspects that are relevant to maintain their health and productivity.

After studying pathogen *S. aureus* in Works 1, 2 and 3, in Work 4 we propose, in the specific case of a paternal line of rabbits selected for average daily gain, to study the host's immune system in the basal state, as well as the pathogen-host interaction when the latter faces an infectious challenge. This work evaluated if the immune system of the R line of rabbits was affected by the genetic selection for daily weight gain. To do so, we found that employing the skin infection model of the rabbit with *S. aureus* very useful To obtain animals from previous generations, it was necessary to use vitrified embryos. The vitrification technique may affect several

parameters, including those related to the immune system. To know these effects and to take them into account, in this study two groups of animals belonging to the current generation were used: a group from the vitrified embryos and another obtained by conventional insemination.

Work 4: **Effect of selection by growth rate and vitrification of embryos on the rabbit (*Oryctolagus cuniculus*) immune system and its response after a Staphylococcus aureus experimental infection**

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4.1. Summary

The immune system constitutes the defence system of animals against any pathogenic infection or disease. For animals is important to maintain the immune competence under conventional conditions and to have the ability to mount the immune response after challenges. We hypothesised that the selection criteria (e.g., growth rate) or the process of vitrification and restoration of embryos may affect the immune system. To achieve these aims, two experiments were performed. First experiment aimed to evaluate the effect of selection by average daily gain on the ability of females to maintain the immune competence under conventional farm conditions. This experiment involved 80 rabbit females controlled at five different physiological states: First artificial insemination (1IA), first parturition (1P), first weaning (1W), second parturition (2P) and second weaning (2W). Two different genetic types were evaluated: VR18 was vitrified generation 18 (n=43); VR36 was vitrified generation 36 (n=37). Immunophenotypic characterization of peripheral blood and hematology studies were performed to evaluate the immune system. The effect of genetic type nor its interaction with physiological state were significant for any trait. Second experiment aimed to evaluate the effect of selection and vitrification on the

immune response to an infection inoculating *S. aureus* as immunological challenge. Three different genetic types were evaluated: VR18 was vitrified generation 18 (n=39); VR36 was vitrified generation 36 (n=34); R36 was non-vitrified generation 36 18 (n=24). The ability to mount the immune response was evaluated each one of the seven days after inoculation by the characterization of gross lesions and the use blood samples (immunophenotypic characterization of peripheral blood and hematology studies). Rabbit females of VR36 presented lower counts for total lymphocytes, $CD5^+$, $CD4^+$, $CD8^+$, $CD25^+$, monocytes and platelets than those of VR18 (-16, -23, -26, -16, -31, -20 and -10%, respectively; $P<0.05$). VR36 had lower presence of erythema (-9 percentage points; $P<0.05$), nodule (-10 percentage points; $P<0.05$) and size of nodule (-1.05 cm^3 and -0.50 cm^3 at day seven post-inoculation; $P<0.05$) compared to VR18. Rabbit females from the vitrified group (VR36) presented lower values for total lymphocytes, $CD5^+$, $CD4^+$, monocytes, granulocytes, $CD4^+$/CD8 ratio and white blood cells than animals from the non-vitrified group (R36) (-16, -15, -18, -25, -22, -20, -20%, respectively; $P<0.05$). Focusing on nodules, VR36 had lower presence (-17.0 percentage points; $P<0.05$) and they were smaller (-0.86 cm^3 at day seven post-inoculation; $P<0.05$) compared R36. Therefore, our study indicates that genetic selection by average daily gain does not seem to have affected negatively to the immune system at both levels (the ability of rabbit females to maintain a competent immune system under conventional conditions and the ability to mount an immune response). Finally, when we are interested to compare different generations of the same line, restoration of cryopreserved populations must be done in both generations, not only in the ancient one.

4.2. Introduction

During the last 50 years, the productivity of animals has increased considerably due to genetic selection (Hill, 2008). However, this selection has sometimes been accompanied by undesired side effects (Rauw et al., 1998). To give some examples, ascites syndrome of chickens has usually

been associated with genetic progress (Wideman et al., 2013). In pigs, the ovulation rate is negatively correlated with prenatal survival (Blasco et al., 1993). In dairy cattle, selection to increase milk usually is associated with a decrease in health, fertility and functional longevity (Veerkamp et al., 2009). In rabbits, some examples of these side effects have been reported from paternal lines selected by growth rate. Females from these types of lines present poorer reproductive performance (Baselga et al., 2003) and stayability on farms (Arnau-Bonachera et al., 2018) than females from maternal lines. Moreover, young rabbits from these lines are more sensitive to digestive disorders when antimicrobials are not used in the feedstuff (García-Quirós et al., 2014). However, with current information, it is not clear whether these problems are the effect of genetic selection criteria or they are intrinsic to the breeds as a consequence of animals used at the foundation. To elucidate between both options, it is necessary to perform selection experiments to evaluate the indirect effects of selection. In rabbits, four alternatives have been described (Khalil and Al-Saef, 2008): (i) comparing a selected population with a non-selected-control population; (ii) selecting populations in two directions, one to increase the trait and the other to decrease; (iii) estimating genetic trends with statistical methods such as mixed model theory or Bayesian approaches and (iv) comparing the contemporaries of two different generations by using frozen embryos of the same line. The last method presents the advantages of using a control population, avoiding problems of the chosen statistical model or the genetic drift in long-term experiments (Piles and Blasco, 2003). However, some authors have shown that the process of vitrification could have long-term effects that should be considered when comparing vitrified and non-vitrified populations (Dulioust et al., 1995).

On the other hand, the immune system constitutes the defence system of animals against any pathogenic infection or disease. In agreement with that stated previously, selection could impair immunity of animals (Rauw, 2012) as prioritizing resources towards selected productive functions may decrease resources for other functions (Pascual et al., 2013), increasing the

sensitivity of animals to environmental factors (Kolmodin et al., 2002). The consequences of selection could act at two levels (Lochmiller and Deerenberg, 2000): (i) affecting the way animals maintain a competent immune system; (ii) affecting the way animals mount the immune response. In the evaluation of the first one, it seems that the basal level of cells from the immune system evolve with age and physiological state of animals (Wells et al., 1999; Guerrero et al., 2011; Ferrian et al., 2012). As a consequence, maintaining a competent immune system at some key points of the life trajectory is highly relevant. In this sense, weaning of young rabbits and first parturitions of females have been reported as the most relevant (Jeklova et al., 2007a, 2009; Penadés et al., 2017). To evaluate the way animals mount the immune response, an immunological challenge using one pathogen under experimentally controlled conditions must be performed. In this sense, *Staphylococcus aureus* present interesting features. The pathogen is the causal agent of several pathological processes in growing rabbits and especially in rabbit females. It has been associated with mastitis of lactating does (Corpa et al., 2009), which is one of the main reasons of culling females (Segura et al., 2007; Rosell and de la Fuente, 2009). Consequently, the study of *S. aureus* infections is relevant for rabbit farming. Moreover, it is possible to infect animals with this pathogen under controlled and standardized conditions (Viana et al., 2015b; Muñoz-Silvestre et al., 2020; Penadés et al., 2020) and there is literature about this kind of infections with different strains of the bacteria, at different age of animals, etc. The use of flow cytometry for the evaluation of the immune system of rabbits is much less widespread than in other species due to the limitation in the existing monoclonal antibodies (mAbs) for this species. However, more and more studies show its usefulness for the use of the rabbit as a research animal (Davis and Hamilton, 2008), for the study of diseases that affect rabbits (Guerrero et al., 2011) and for the comparison of different lines of selected rabbits based on different productive parameters (Ferrian et al., 2012). In this context, we have the tools to assess whether selection for growth rate is really affecting the immune status of

animals, although we should know the possible effect of cryopreservation itself on populations and their own immune status.

Therefore, the aims of the present work were: (i) to evaluate the effect of selection by growth rate on the immune system (both maintaining immune competence under conventional conditions and mounting immune response under an immunological challenge with *S. aureus*) and (ii) to evaluate the effect of vitrification of embryos when mounting the immune response under an immunological challenge with *S. aureus*.

4.3. Materials and methods

In this study two different experiments were carried out. Experiment 1 aimed to evaluate the effect of selection on the immune system under commercial farm conditions. Experiment 2 aimed to evaluate the effect of selection and vitrification on the immune response to an immunological challenge. The following experimental protocols were approved by the Animal Welfare Ethics Committee of the Universitat Politècnica de València (authorization code: 2018/VSC/PEA/0116) and the ethical committee of the Universidad CEU Cardenal Herrera and by the Conselleria d'Agricultura, Pesca i Alimentació, Generalitat Valenciana (permit numbers 2011/010 and 2017/VSC/PEA/00192; date of approval: 20 January 2011). All animals were handled according to the principles of animal care published by Spanish Royal Decree 1201/2005 (BOE, 2005; BOE = Official Spanish State Gazette).

4.2.1. Experiment 1

4.2.1.1. Animals and experimental procedure

The first experiment involved a total of 80 rabbit females and their litters (*Oryctolagus cuniculus*), which were evaluated from the first artificial insemination of females until their second weaning. Rabbit females came from a line long-term selected for average daily gain (ADG) during the growing period (between the 4th and 9th weeks of life) (line R; Estany et al.,

1992), but the two experimental genetic types differed in the degree of selection. Fourty-three females (VR18) belonged to generation 18 of this line, whereas thirty-seven females (VR36) belonged to generation 36. To prevent any possible effect of vitrification of former generation, all the animals from both genetic types were obtained after restoring completely both populations from vitrified embryos, having only used their progeny to avoid the direct effects of cryopreservation. Females were housed in individual cages (700 x 500 x 320 mm) and raised under conventional environmental conditions, alternating 16h of light and 8h of darkness. At 19 weeks of age, all the females were inseminated for the first time with pooled semen from males belonging to their respective generation. At day 28 of gestation, cages were provided with a nest for litters. At parturition, litters were standarized to 5 - 8 kits per litter (on av. 5.4 for the first reproductive cycle and 6.9 for the second). Litters were weanned at day 28 of lactation. Females were inseminated again 11 days after parturition. When females did not get pregnant they were re-inseminated every 21 days until a maximum of three consequtive times when they were culled for reproductive failure. During the whole experiment all the animals had free access to water and to a commercial diet for reproductive rabbit females. Blood samples were taken from three random kits per litter at weaning in both reproductive cycles of the female (blood from the three kits per litter was mixed and processed as a unique sample) and from females at five different physiological states: First artificial insemination (1IA), first parturition (1P), first weaning (1W), second parturition (2P) and second weaning (2W). To prevent from diurnal variations in haematological parameters, blood samples were collected aproximatelly at the same time (9:00 am to 10:00 am). Three mililiters of blood were extracted from the central artery of the ear in two tubes of 1 mL with EDTA as anticoagulant (AQUISEL Tube EDTA K3).

4.2.1.2. Immunophenotypic characterization of peripheral blood by flow cytometry and hematology studies

One milliliter of each blood sample was used to carry out the immunophenotypic characterization by flow cytometry, a second other milliliter was used to carry out hematology studies, and a third milliliter, centrifugated at 2500 g to obtain serum, was used to determine haptoglobin concentration. The milliliter of whole blood of each sample was lysed by adding 45 milliliters of ammonium chloride lysing solution. After spinning, the pellet of leukocytes was resuspended in 1 milliliter of Dulbecco's Phosphate-Buffered Saline suspension (Sigma-Aldrich®) (DPBS) and this cell suspension was divided into 6 sample tubes for each animal. Primary monoclonal antibodies (***Table 14***) were added to the cell suspension following the manufacturer's technical specifications and incubated for 25 minutes at room temperature. After that, samples were washed and secondary antibodies (Rat anti-mouse IgG2ak or IgG2bk Phycoerythrin [Nordic-MUbio] and Goat anti-mouse IgM: R-Phycoerythrin [Biorad]) were added following the manufacturer's technical specifications and incubated 25 minutes at room temperature. Then, samples were washed again and 1 milliliter of DPBS was added before analyzing samples on the flow cytometer. The outcome leukocyte (WBC) suspensions were analysed in a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA). The common leucocyte antigen CD14 and CD45 expression was used for the "lymphogate" setup as previously described (Jeklova et al., 2007b; Guerrero et al., 2011) Total lymphocyte count was calculated from WBC count and lymphocyte percentage, and lymphocyte subset counts as described by Guerrero *et al.* (2011).

The following blood parameters were evaluated using a hematological counter (MEK-6410, Nihon Kohden, Japan): leukocytes (WBC), hematocrit (HCT), hemoglobin (HGB), platelets (PLT) and red blood cell count (RBC). In addition, serum from the rabbits was sent to a reference laboratory for haptoglobin determination by a colorimetric assay (Phase Range; Tridelta

Developments Ltd, Maynooth, Ireland) according to manufacturer's protocol.

Monoclonal antibodies	Isotype	Specificity	Cell labeling	Clone	References	Company
Mouse anti-rabbit T lymphocytes: FITC[a]	IgG1	CD5	T cell	KEN-5	Kotani et al., (1993a)	Abd Serotec
Mouse anti-rabbit α-pan B	IgM	IgM	B cell	MRB143A	Davis and Hamilton, (2008)	VMRD Inc.
Mouse anti-rabbit CD4	IgG2a	CD4	T cell subset	KEN-4	Kotani et al. (1993a)	Abd Serotec
Mouse anti-rabbit α-CD8	IgG2a	CD8	T cell subset	ISC27A	Davis and Hamilton (2008)	VMRD, Inc.
Mouse anti-rabbit CD25	IgG2b	CD25	Activated T cells	KEI-ALPHA1	Kotani et al., (1993b)	Abd Serotec
Mouse anti-human CD14: FITC	IgG2a	CD14	Monocytes and granulocytes	TÜK 4	Jacobsen et al., (1993)	Abd Serotec
Mouse anti-rabbit α-CD45	IgM	CD45	All leukocytes	ISC76A	Davis and Hamilton (2008)	VMRD Inc.

Table 14. Monoclonal antibodies used in this study. [a] *Clon KEN-5 recognises rabbit T lymphocytes and immunoprecipitates. This antibody recognises rabbit CD5, but does not bind to rabbit CD5 transfectants. Known rabbit CD5 antibodies also show binding to most B lymphocytes, which are not labelled by this clone (information obtained from datasheet).*

4.2.1.3 Statistical analysis

Previous to statistical analysis, variables were submitted to a preliminary analysis to detect outliers and asymmetrical distributions. When asymmetrical distributions were found, original data was transformed using

a logarithmic transformation. Data from all the variables was analyzed using linear mixed models (Proc MIXED; SAS, 2009). For parameters from blood of females the model included the genetic type (2 levels; VR18, VR36), the physiological state (5 levels; 1IA, 1P, 1W, 2P, 2W) and their interaction as fixed effects. For parameters from blood of litters the model included the genetic type (2 levels; VR18, VR36), the reproductive cycle (RC) of the females when kits were born (2 levels; 1st RC, 2nd RC) and their interaction as fixed effects. As random effects, all the analysis included de permanent effect of the female [81 levels ~ *N* (0; s^2_p)] and error term [315 levels ~ *N* (0; s^2_e)].

4.2.2. Experiment 2

4.2.2.1. Animals and experimental procedure

This experiment involved 97 rabbit females of 19-weeks-old from a paternal line selected by average daily gain during the growing period (between the 4th and 9th weeks of life), over 36 generations (line R; Estany et al., 1992). To evaluate the effect of selection and vitrification on the immune response to an immunological challenge, three different genetic types were evaluated: VR18 was vitrified generation 18 (n=39); VR36 was vitrified generation 36 (n=34); R36 was non-vitrified generation 36 18 (n=24). To restore vitrified populations, embryos were transferred to females. After one generation without selection, 42 rabbit females were selected to obtain the 97 young rabbits. Animals from vitrified and non-vitrified populations were housed under conventional environmental conditions with free access to water and to a commercial diet. To perform infections, animals were sedated with a combination of ketamine (Imalgene®, 100 mg/mL, Merial, Barcelona, Spain) and xylazine (Xilagesic, 200 mg/mL, Calier, Barcelona, Spain) and a 10x10 cm area of the dorsal-lumbar region was shaved and disinfected with chlorhexidine. Later, rabbits were intradermally-inoculated in their backs with 300 *S. aureus* colony forming units (CFU) of two rabbit strains of different virulence, Jwt (high virulence) and Jrot$^+$ (low virulence) (Viana et al., 2015b), suspended in 0.1 mL of phosphate-buffered saline (PBS). Each

rabbit was infected at four points (2 per strain). After inoculation, characteristics of the skin gross lesions (presence of erythema, nodules, dermo-necrosis and ulceration) were daily recorded for seven days. Erythema and abscess dimensions were daily measured with a caliper [length (L) and width (W)]. These values were used to calculate area of the erythema ($A = \pi \cdot \frac{L}{2} \cdot \frac{W}{2}$) and the volume of the nodule ($V = \frac{4\pi}{3} \cdot \left(\frac{L}{2}\right)^2 \cdot \frac{W}{2}$) as suggested by Muñoz-Silvestre et al., (2020).

4.2.2.2. Immunophenotypic characterization of peripheral blood by flow cytometry and hematology studies

Two ml of blood were extracted from the central artery of the ear in two tubes of 1 ml with EDTA as anticoagulant (AQUISEL Tube EDTA K3) at days 0, 1, 3 and 7. One ml of blood was used to carry out the immunophenotypic characterization by flow cytometry and the other ml was used to carry out hematology studies. Both analyses were carried out in the same way as explained in point 4.2.1.2. of the Experiment 1.

4.2.2.3. Study of phagocytosis of polymorphonuclear leukocytes and macrophages measured by flow cytometry

The week before the start of the infection (week 19 of age) blood samples were taken from each animal involved in the study to purify polymorphonuclear leukocytes and monocytes separately in different days. An amount of 16 ml of blood was taken from each one of the rabbits to carry out this experiment, 8 ml for the purification of polymorphonuclear leukocytes and 8 ml for the purification of macrophages. The blood samples were kept into heparin tubes of 10 ml (BD Vacutainer® Heparin Tubes).

Purification of polymorphonuclear leukocytes (PMN) and macrophages: PMN and macrophage purification were carried out according to previously described protocols (Siemsen et al., 2014; Yamane and Leung, 2016) and in the same way that Penadés et al. (2020) describes.

Phagocytosis of PMN: Fluorescent yellow-green latex beds in aqueous suspension of 2.0 µm mean particle size (Sigma-Aldrich®) were used to measure phagocytosis. The inoculum of beads for the cells was made by adding 10 times more latex beds than cells (10^6 cells/ml). For each animal, three replicates and a negative control (without beds) were made. The phagocytosis was remained for 30 minutes at 37ºC and was stopped with ice. The cells were labelled with the monoclonal antibody CD11b-PE (Thermo Fisher®) following the manufacturer's technical specifications and the cells were fixed with paraformaldehyde (4%). Finally, the cells were resuspended in 1 millilitre of DPBS and transferred to cytometer tubes. Phagocytosis was measured on an FC500 cytometer (Beckman Coulter). All the cells labelled with the CD11b-PE antibody were taken as the granulocyte population. It was considered that the cells that had phagocytosed were the cells that showed FITC fluorescence of the latex beads, and those that had not phagocytosed were the cells that did not present this fluorescence.

Phagocytosis of macrophages: Fluorescent yellow-green latex beds in aqueous suspension of 2.0 µm mean particle size (Sigma-Aldrich®) were used to measure phagocytosis. The animals with less than 5% of confluence of cells in the wells were discarded. For each animal, three replicates and a negative control (without beds) were made. The phagocytosis was remained for 30 minutes at 37ºC and was stopped with ice. The cells were collected from the wells to be placed in cytometer tubes using Trypsin solution from porcine pancreas (1x) (Sigma-Aldrich®). Then, the cells were labelled with the monoclonal antibody CD11b-PE (Thermo Fisher®) following the manufacturer's technical specifications. The phagocytosis data were obtained by measuring the phagocytosis of the cells in a flow cytometer FC500 (Beckman Coulter). All the cells labelled with the CD11b-PE antibody were taken as the macrophages population. It was considered that the cells that had phagocytosed were the cells that showed FITC fluorescence of the latex beads, and those that had not phagocytosed were the cells that did not present this fluorescence.

4.2.2.4 Statistical analysis

The six evaluated traits in the second experiment were analysed using two different models. Both models included the effect of genetic type (3 levels: VR18, VR36, R36), Strain of *S. aureus* (2 levels: Jwt, J*rot*$^{+}$), day post-infection (7 levels: 1, 2, 3, 4, 5, 6, 7) and their interactions as fixed effects. For presence of erythema, nodule, dermo-necrosis and ulceration (dichotomic traits) a generalized linear model was used (proc GENMOD, SAS) after considering that the response variable followed a binomial distribution and by using a logistic transformation [ln(m / (1-m))] as a link function. For area of erythema and volume of nodule a linear mixed model was performed (proc MIXED, SAS) including the permanent effects of animal [97 levels; N ~ (0, s_p)] and infection [388 levels; N ~ (0, s_i)], and residuals [2716 levels; N ~ (0, s_e)] as random effects. Assuming that measures of the same infection close in time are more correlated than far in time, residuals were considered to be correlated in a decreasing way as increasing the lag.

4.3. Results

4.3.1. Experiment 1

Results from immunological blood parameters of rabbit females breed under commercial farm conditions are reported in ***Table 15***. The genetic type had not any effect on any parameter, and its interaction with physiological state either. On the contrary, the effect of physiological state was significant for almost any parameter. The total number of lymphocytes decreased at both weaning respect to AI and parturition counts (on av. -21%; P<0.05). A similar pattern was observed for main of the lymphocyte sub-populations ($CD5^{+}$, $CD4^{+}$, $CD8^{+}$ and $CD25^{+}$), that showed a progressive decrease from the first AI to the first weaning, a recovery at the second parturition and a new decrease at the second weaning. The number of lymphocytes B significantly decreased 75% throughout the first reproductive cycle (-45% from AI to parturition, -55% from parturition to weaning; P<0.05), and any

variation was not observed from this point on. The number of monocytes increased progressively from first AI to second weaning (+199%; $P<0.05$). Similarly, the number of granulocytes increased from first AI to second weaning (+95%; $P<0.05$), but within a reduction from parturition to weaning in the first cycle (-13%; $P<0.05$) not observed on the second one. The ratio $CD4^+/CD8^+$ increased during the gestation (+49%; $P<0.05$), not existing significant differences from this point on. Granulocytes to total lymphocytes ratio increased progressively from first AI to second weaning (+137%; $P<0.05$). White blood cells count showed an overall increase of 32% ($P<0.05$) from first AI and second weaning, but within a significant reduction of 16% ($P<0.05$) from first parturition to first weaning. Red blood cells, haematocrit and haemoglobin followed a similar pattern during the experiment. In all three cases, there was a progressive overall decreased from first AI to second weaning (-12% for red blood cells, -14% for haematocrit and -17% for haemoglobin; $P<0.05$). On the contrary, there was an overall increase of platelets during the experiment (+10% from first AI to second weaning; $P<0.05$). No differences were observed for serum haptoglobin content throughout the study.

Results from immunological-blood parameters of young rabbits, obtained at weaning when breed under commercial-farm conditions, are reported in ***Table 16***. The effect of group was significant in four of the ten evaluated parameters. The number of granulocytes from VR36 animals was 41.25% ($P<0.05$) higher compared to animals from VR18. The ratio between granulocytes and total lymphocytes from VR36 animals was also 55.28% ($P<0.05$) higher compared to animals from VR18. On the contrary, values of both haematocrit and haemoglobin were lower in VR36 weaned rabbits compared to those from VR18 (-5 and -7% respectively; $P<0.05$). On the other hand, total lymphocytes, $CD5^+$, $CD4^+$, $CD8^+$ and monocytes counts in weaned rabbits were higher at second weaning (+17, +43, +46, +51 and +100%, respectively; $P<0.05$).

	Group (G)[1]			State (S)[2]						Contrasts[3]		*P values*		
	VR18	VR36	SEM	1AI	1P	1W	2P	2W	SEM	RC	WP	G	S	GxS
Leukocyte counts ($\log_{10}$ 10^6/l)														
Total lymphocytes (L)	3.391	3.371	0.019	3.441^{b}	3.395^{b}	3.309^{a}	3.434^{b}	3.328^{a}	0.021	0.029	-0.096***	0.453	<0.001	0.139
Lymphocytes B	0.406	0.508	0.070	0.847^{c}	0.586^{b}	0.238^{a}	0.289^{a}	0.324^{a}	0.074	-0.106	-0.157**	0.319	<0.001	0.434
Lymphocytes T $CD5^+$	3.069	3.046	0.020	3.191^{c}	3.062^{b}	2.971^{a}	3.096^{b}	2.966^{a}	0.026	0.014	-0.11***	0.426	<0.001	0.432
$CD4^+$	2.827	2.815	0.020	2.894^{d}	2.829^{bc}	2.742^{a}	2.872^{cd}	2.768^{b}	0.025	0.034	-0.096***	0.681	<0.001	0.483
$CD8^+$	2.352	2.291	0.033	2.519^{c}	2.280^{ab}	2.234^{a}	2.342^{b}	2.232^{a}	0.035	0.03	-0.077*	0.190	<0.001	0.927
$CD25^+$	1.298	1.221	0.059	1.277^{ab}	1.198^{a}	1.169^{a}	1.369^{b}	1.285^{ab}	0.068	0.144*	-0.057	0.359	0.166	0.266
Monocytes	2.487	2.500	0.044	2.228^{a}	2.501^{b}	2.465^{b}	2.570^{bc}	2.704^{c}	0.063	0.153*	0.049	0.837	<0.001	0.889
Granulocytes (G)	3.621	3.626	0.017	3.445^{a}	3.658^{c}	3.598^{b}	3.684^{cd}	3.734^{d}	0.020	0.081***	-0.005	0.822	<0.001	0.912
$CD4^+$/$CD8^+$[4]	3.310	4.019	0.280	2.814^{a}	4.181^{b}	3.806^{b}	3.830^{b}	3.692^{b}	0.316	-0.233	-0.257	0.078	0.004	0.228
G/L[4]	2.073	2.200	0.138	1.213^{a}	2.187^{b}	2.156^{b}	2.249^{b}	2.878^{c}	0.162	0.393*	0.299*	0.516	<0.001	0.158
White blood cells (10^3/µl)	0.969	0.959	0.010	0.900^{a}	0.993^{bc}	0.915^{a}	1.019^{c}	0.994^{c}	0.011	0.052***	-0.052***	0.465	<0.001	0.797
Red blood cells (10^6/µl)	5.230	5.312	0.059	5.740^{c}	5.241^{b}	5.334^{b}	4.983^{a}	5.055^{a}	0.070	-0.269***	0.083	0.328	<0.001	0.521
Haematocrit (%)	34.61	34.37	0.373	37.91^{d}	34.46^{b}	35.82^{c}	31.55^{a}	32.70^{a}	0.446	-3.015***	1.26***	0.646	<0.001	0.099
Haemoglobin (g/l)	11.41	11.27	0.149	12.42^{c}	11.50^{b}	11.79^{b}	10.36^{a}	10.61^{a}	0.175	-1.156***	0.271	0.504	<0.001	0.298
Platelets (10^3/µl)	293.1	282.6	9.233	278.4^{a}	280.9^{a}	286.7^{ab}	285.6^{ab}	307.5^{b}	9.999	12.745	13.825	0.420	0.158	0.956
Haptoglobine (g/l)	-0.443	-0.467	0.025	-0.491	-0.471	-0.456	-0.386	-0.471	0.044	0.035	-0.034	0.507	0.507	0.719

Table 15. Effect of group and physiological state on immunological-blood parameters of rabbit females breed under commercial-farm conditions ^{a,b,c,d} *Means in a row within an effect not sharing superscript were significantly different (P<0.05); * Significantly differed from 0 (P<0.05) .** (P<0.01). *** (P<0.001); SEM: Pooled standard error of means;[1] VR18: vitrified R line at 18th generation, VR36: vitrified R line at 36th generation. [2] 1AI: first artificial insemination, 1P: first parturition, 1W: fist weaning, 2P: second parturition, 2W: second weaning. [3] RC= (2P+2W)/2-(1P+1W)/2. WP= (1W+2W)/2-(1P+2P)/2. [4] ratios were directly obtained from the counts without logarithmic transformation.*

	Group (G)[1]			Reproductive cycle (RC)[2]			*P values*		
	VR18	**VR36**	**SEM**	**1W**	**2W**	**SEM**	**G**	**RC**	**GxRC**
Leukocyte counts (log10 106/l)									
Total lymphocytes (L)	3.306	3.281	0.023	3.259^{a}	3.328^{b}	0.022	0.451	0.026	0.167
Lymphocytes B	0.141	0.186	0.124	0.230	0.097	0.105	0.796	0.259	0.456
Lymphocytes T $CD5^{+}$	2.992	2.995	0.026	2.916^{a}	3.071^{b}	0.024	0.950	0.001	0.686
$CD4^{+}$	2.795	2.753	0.024	2.692^{a}	2.857^{b}	0.022	0.227	0.001	0.571
$CD8^{+}$	2.462	2.470	0.033	2.376^{a}	2.555^{b}	0.031	0.863	0.001	0.388
$CD25^{+}$	1.243	1.129	0.068	1.219	1.153	0.065	0.242	0.443	0.876
Monocytes	2.466	2.332	0.067	2.249^{a}	2.549^{b}	0.065	0.167	0.002	0.067
Granulocytes (G)	3.189^{a}	3.339^{b}	0.036	3.269	3.259	0.036	0.005	0.834	0.742
$CD4^{+}$/$CD8^{+}$[3]	2.105	1.911	0.072	2.085	1.930	0.069	0.063	0.097	0.384
G/L[3]	0.890^{a}	1.382^{b}	0.101	1.250	1.022	0.101	0.001	0.117	0.390
White blood cells (10^{6}/l)	0.760	0.809	0.024	0.772	0.797	0.022	0.155	0.358	0.344
Red blood cells (10^{9}/l)	4.326	4.389	0.067	4.311	4.404	0.062	0.511	0.243	0.940
Haematocrit (%)	30.89^{b}	29.35^{a}	0.52	29.95	30.29	0.46	0.041	0.549	0.723
Haemoglobin (g/l)	9.485^{b}	8.853^{a}	0.197	9.106	9.231	0.177	0.027	0.568	0.693
Plateletes (10^{6}/l)	407.0	405.8	20.5	401.6	411.2	17.3	0.966	0.613	0.856

Table 16. Effect of group and reproductive cycle on immunological-blood parameters of young rabbits at weaning and breed under commercial-farm conditions. [a,b] *Means in a row within an effect not sharing superscript were significantly different (P<0.05); SEM: Pooled standard error of means;* [1] *VR18: vitrified R line at 18th generation, VR36: vitrified R line at 36th generation.* [2] *Reproductive cycle of the young-rabbits' mother; 1W: fist weaning, 2W: second weaning.* [3] *ratios were directly obtained from the counts without logarithmic transformation.*

4.3.2. Experiment 2

Results from leukocyte populations and haematological parameters in peripheral blood of 19-weeks-old rabbit females after intradermal inoculation with *S. aureus* are reported in ***Table 17***. To evaluate the effect of genetic type two contrast were performed, one to evaluate the effect of selection level (VR36-VR18) and the other to evaluate the effect of cryopreservation by vitrification (VR36-R36). Rabbit females of VR36 presented lower counts for total lymphocytes, $CD5^+$, $CD4^+$, $CD8^+$, $CD25^+$, monocytes and platelets than those of VR18 (-16, -23, -26, -16, -31, -20 and -10%, respectively; P<0.05). On the contrary, lymphocytes B and red blood cells were higher in VR36 animals (+38 and +4%, respectively; P<0.05). Rabbit females from the vitrified group (VR36) presented lower values for total lymphocytes, $CD5^+$, $CD4^+$, monocytes, granulocytes, $CD4^+$/CD8 ratio and white blood cells than animals from the non-vitrified group (R36) (-16, -15, -18, -25, -22, -20, -20%, respectively; P<0.05). On the contrary, red blood cells, haematocrit and haemoglobin were higher in VR36 animals (+5, +4 and +5%, respectively; P<0.05). No significant effects of genetic type were observed for phagocytosis of macrophages and heterophiles.

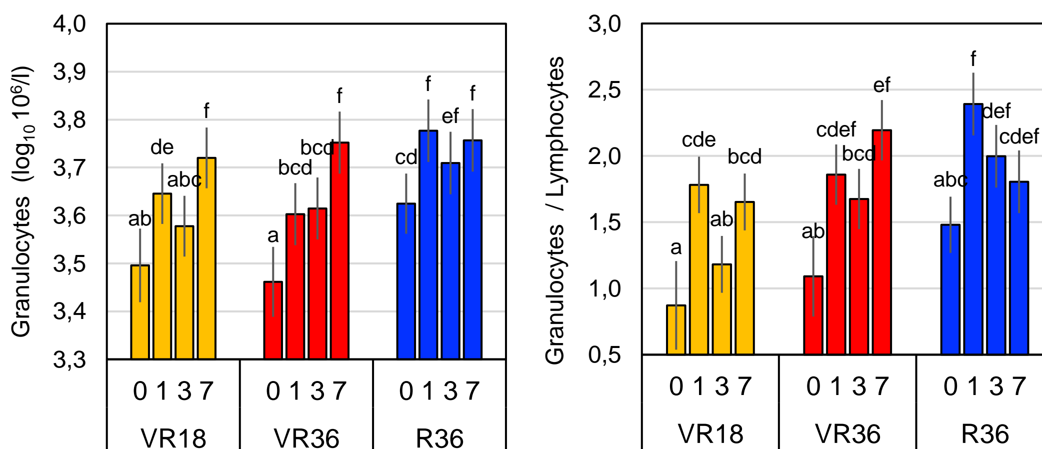

Figure 8. Effect of group (VR18: vitrified R line at 18th generation, VR36: vitrified R line at 36th generation, R36: non-vitrified R line at 36th generation) depending on the day post infection (0, 1, 3, 7) on granulocytes (A) and granulocytes / lymphocytes ratio (B). LS-means and standard errors. [a-f] Means not sharing letter differed significantly (*P*<0.05)

The effect of the day post-infection was significant in almost every parameter (***Table 17***). Total lymphocytes, lymphocytes B, $CD25^{+}$, monocyte, granulocyte, ratio between granulocytes and total lymphocytes, white blood cells and platelets presented an overall increase of values during the experiment (+22, +179, +42, +134, +64, +64, +43 and +17%, respectively; P<0.05). The evolution of granulocytes and granulocytes to total lymphocytes ratio diverged slightly from this pattern, due granulocytes evolution in function of the genetic type (***Figure 8***). Compared to VR18 and VR36, R36 females presented more granulocytes at the day of infection, but this difference was not evident at day seven post-infection. Granulocyte to lymphocyte ratio increased the day one (+75%; P<0.05), decrease at day three (-19.5%; P<0.05) and shows a subsequent recovery at day seven post-infection (+16%; P<0.05). On the other hand, CD4+, CD4+/CD8+, red blood cells, haematocrit and haemoglobin values decreased the first days post-infection (-13, -17, -4, -4 and –5%, respectively; P<0.05) and showed a partial recovery afterwards. In no case, final values were significantly lower than the initial ones.

Results from the macroscopic lesions of female rabbits after intradermal inoculation with two strains of *S. aureus* are reported in ***Table 18***. More selected animals (VR36) had lower presence of erythema (-9 percentage points; P<0.05) and nodule (-10 percentage points; P<0.05) compared to less selected animals (VR18). Besides, animals from the vitrified group (VR36) had lower presence nodule (-17.0 percentage points; *P*<0.05) compared to the non-vitrified group (R36). Lesions from inoculations with Jwt presented erythema more frequently and bigger (+6 percentage points and +0.65cm^2 respectively; *P*<0.05), nodules more frequently and bigger (+49 percentage points and +1.39cm^2 respectively; *P*<0.05) and dermo-necrosis and ulceration more frequently (+7 percentage points and +3.9cm^2 respectively; *P*<0.05) than with J*rot*+ (***Figure 9.1 and 9.2***).

The effect of time post-infection was significant in all the parameters recorded to evaluate macroscopic lesions. The presence of erythema was

maximum at day one post-infection. It was present in nearly three of four inoculations. Afterwards, the presence of erythema decreased progressively until it almost disappeared at the end of the experiment (-74 percentage points; $P<0.05$). Similarly, the area of the erythema decreased throughout the experiment (-0.8 cm^2; $P<0.05$), but the maximum size was observed at day two post-infection. Presence and volume of nodule increased throughout the experiment. The presence of nodule increased between day one and three post-infection (+24 percentage points; $P<0.05$), not existing differences from this point on. The volume of nodule got bigger between day one and six (+1.39 cm^3; $P<0.05$), not existing significant differences between the last two days. However, the volume of nodule evolved differently depending on genetic type and strain (***Figure 10***). Nodules from inoculations with Jrot+ were small and not different among genetic types or days post-infection. Nodules from inoculations with Jwt were bigger and significantly increased over time. Moreover, at day one post-infection there were no differences among groups, but nodules from VR36 females did not reach a size as large as nodules from VR18 and R36 (-1.05 cm3 and -0.86 cm3 at day seven post-inoculation with Jwt; P<0.05). Finally, dermo-necrosis and ulceration were almost no present in lesions for the first three days post-infection (<0.1 percentage points). Later, the presence of both increased progressively between day three and seven (+8.1 percentage points for dermo-necrosis and +7.5 percentage points for ulceration; P<0.05).

	Group (G)[1]				Days post infection (t)					Contrasts[2]		*P values*		
	VR18	**VR36**	**R36**	**SEM**	**0**	**1**	**3**	**7**	**SEM**	**Gen**	**Vit**	**G**	**t**	**Gxt**
Leukocyte counts (log_{10} 10^6/l)														
Total lymphocytes (L)	3.569^{b}	3.492^{a}	3.570^{b}	0.031	3.496^{a}	3.545^{a}	3.552^{b}	3.582^{b}	0.029	-0.077^{**}	-0.078^{*}	0.016	<0.001	0.931
Lymphocytes B	1.266^{a}	1.406^{b}	1.468^{b}	0.085	1.137^{a}	1.391^{b}	1.410^{b}	1.582^{c}	0.082	$+0.141^{*}$	-0.062	0.018	<0.001	0.774
Lymphocytes T $CD5^+$	3.301^{b}	3.188^{a}	3.260^{b}	0.030	3.259	3.277	3.227	3.237	0.028	-0.113^{***}	-0.072^{*}	<0.001	0.206	0.659
$CD4^+$	3.115^{b}	2.984^{a}	3.068^{b}	0.029	3.090^{b}	3.030^{a}	3.033^{ab}	3.069^{b}	0.027	-0.131^{***}	-0.084^{**}	<0.001	0.056	0.733
$CD8^+$	2.739^{b}	2.661^{a}	2.696^{ab}	0.035	2.678	2.703	2.705	2.710	0.032	-0.077^{*}	-0.035	0.098	0.468	0.383
$CD25^+$	1.642^{b}	1.481^{a}	1.542^{ab}	0.092	1.499^{a}	1.548^{a}	1.522^{a}	1.652^{b}	0.095	-0.161^{**}	-0.061	0.021	0.046	0.060
Monocytes	2.553^{b}	2.458^{a}	2.585^{b}	0.067	2.377^{a}	2.443^{a}	2.561^{b}	2.746^{c}	0.066	-0.095^{**}	-0.127^{*}	0.016	<0.001	0.335
Granulocytes (G)	3.610^{a}	3.608^{a}	3.717^{b}	0.061	3.527^{a}	3.675^{c}	3.634^{b}	3.743^{d}	0.060	-0.002	-0.109^{***}	0.004	<0.001	0.012
$CD4^+/CD8^{+3}$	2.445^{ab}	2.160^{a}	2.599^{b}	0.131	2.625^{c}	2.371^{b}	2.189^{a}	2.420^{bc}	0.103	-0.285	-0.441^{*}	0.048	<0.001	0.080
G/L[3]	1.372^{a}	1.705^{ab}	1.919^{b}	0.187	1.148^{a}	2.011^{c}	1.618^{b}	1.884^{c}	0.177	+0.333	-0.214	0.017	<0.001	0.039
White blood cells (10^6/l)	1.018^{a}	0.980^{a}	1.079^{b}	0.024	0.950^{a}	1.021^{b}	1.027^{b}	1.105^{c}	0.022	-0.037	-0.099^{***}	<0.001	<0.001	0.160
Red blood cells (10^9/l)	5.577^{a}	5.802^{b}	5.519^{a}	0.120	5.748^{b}	5.708^{b}	5.510^{a}	5.566^{ab}	0.123	$+0.226^{**}$	$+0.283^{**}$	0.008	0.031	0.458
Haematocrit (%)	38.51^{b}	39.23^{b}	37.63^{a}	0.837	39.24^{b}	38.83^{b}	37.67^{a}	38.10^{ab}	0.871	+0.721	$+1.608^{**}$	0.015	0.054	0.775
Haemoglobin (g/l)	120.3^{b}	122.7^{b}	116.6^{a}	2.873	122.7^{b}	121.3^{b}	116.7^{a}	118.7^{ab}	2.975	+2.350	$+6.124^{**}$	0.007	0.019	0.532
Platelets (10^6/l)	322.4^{b}	290.6^{a}	287.3^{a}	10.67	281.7^{a}	284.0^{a}	304.4^{b}	330.3^{c}	9.178	-31.83^{*}	+3.272	0.007	<0.001	0.205
Phagocytosis (%)														
Macrophages	44.69	41.60	41.42	7.794						-3.085	+0.185	0.403	-	-
Heterophiles	45.58	45.15	43.22	4.425						-0.429	+1.923	0.651	-	-

Table 17. Effect of group and time post infection on leukocyte populations and haematological parameters in peripheral blood of young rabbits after intradermal inoculation with *S.aureus*. [a,b,c,d] *Means in a row within an effect not sharing superscript were significantly different (P<0.05); * Significantly differed from 0 (P<0.05) .** (P<0.01). *** (P<0.001); SEM: Pooled standard error of means;* [1] *VR18: vitrified R line at 18th generation, VR36: vitrified R line at 36th generation, R36: non-vitrified R line at 36th generation.* [2] *Gen=VR36-VR18. Vit=VR36-R36.* [3] *ratios were directly obtained from the counts without logarithmic transformation.*

	Group[1]				Strain			Days post infection							
	VR18	**VR36**	**R36**	**SEM**	**Jwt**	**J*rot*+**	**SEM**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**SEM**
Erythema															
Presence (%)	28.1^{b}	19.1^{a}	13.5^{a}	-	22.6^{b}	16.9^{a}	-	76.9^{f}	58.2^{e}	30.9^{d}	17.4^{c}	8.9^{b}	4.4^{ab}	2.5^{a}	-
Area (cm2)	0.511	0.466	0.591	0.137	0.847^{b}	0.198^{a}	0.124	0.836^{d}	1.017^{e}	0.487^{c}	0.462^{c}	0.620^{c}	0.197^{b}	0.036^{a}	0.130
Nodule															
Presence (%)	31.1^{b}	20.7^{a}	37.7^{c}	-	59.1^{b}	10.6^{a}	-	10.1^{a}	23.7^{b}	33.6^{c}	36.7^{c}	36.7^{c}	37.2^{c}	37.6^{c}	-
Volume (cm3)	0.937	0.594	0.912	0.210	1.495^{b}	0.103^{a}	0.186	0.071^{a}	0.212^{b}	0.467^{c}	0.693^{d}	1.210^{e}	1.461^{f}	1.480^{f}	0.183
Presence of dermo-necrosis (%)	2.8	2.4	2.5	-	7.7^{b}	0.8^{a}	-	0.8^{a}	0.8^{a}	1.0^{ab}	3.2^{bc}	5.2^{c}	7.1^{c}	8.9^{c}	-
Presence of ulceration (%)	2.1	2.0	2.3	-	4.8^{b}	0.9^{a}	-	0.9^{a}	0.9^{a}	0.9^{a}	1.6^{ab}	3.4^{bc}	5.9^{c}	8.4^{c}	-

Table 18. Effect of group, strain and time post infection on parameters from macroscopic lesions of young rabbits after intradermal inoculation with two strains of *S. aureus*. ^{a,b,c,d,e,f} Means in a row within an effect not sharing superscript were significantly different (P<0.05). SEM: Pooled standard error of means. [1] VR18: vitrified R line at 18th generation, VR36: vitrified R line at 36th generation, R36: non-vitrified R line at 36th generation.

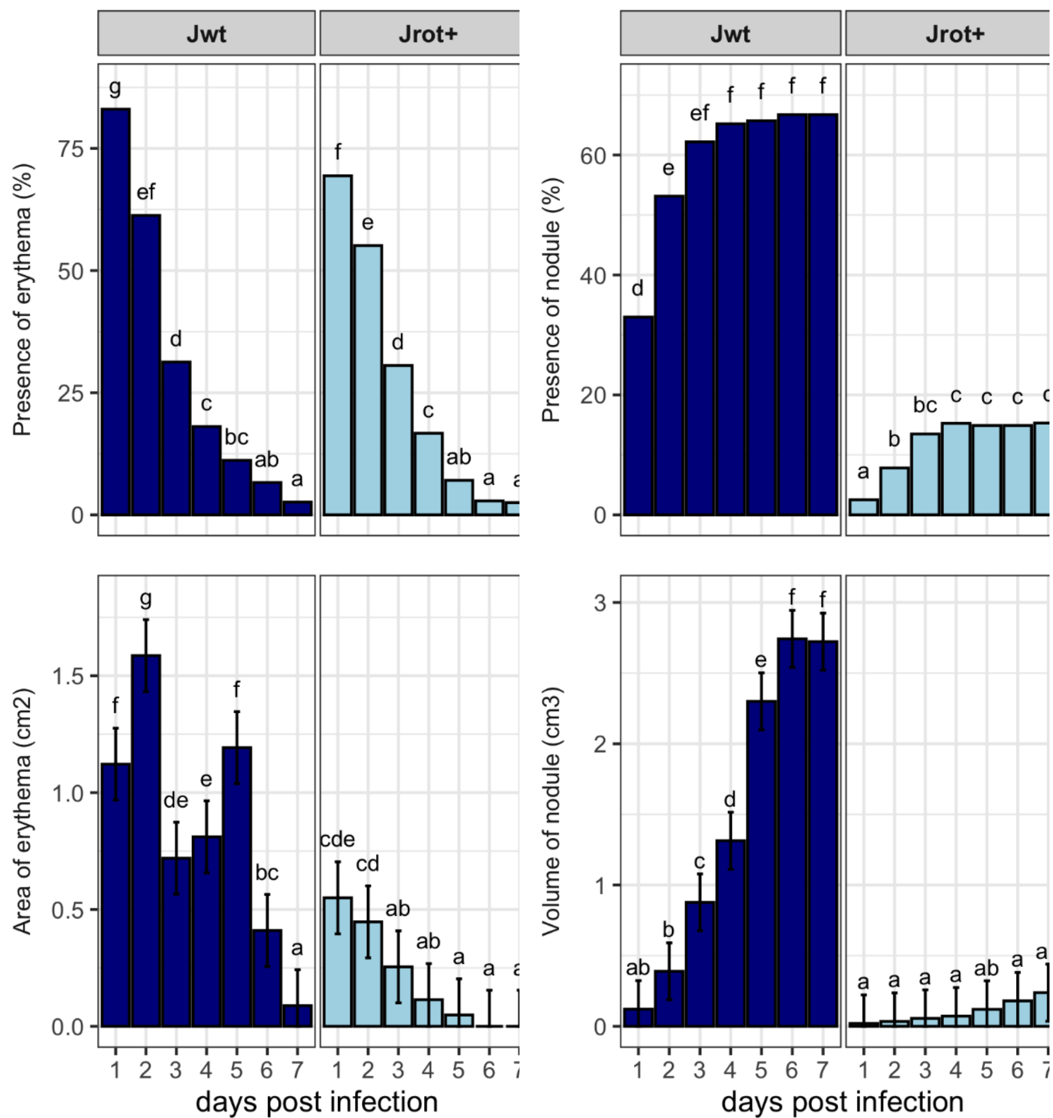

Figure 9. Effect of strain (Jwt, J*rot*+) and day post infection (1-7) on presence and volume of erythema. *LS-means and standard errors.* [a-f] *Means not sharing letter differed significantly (P<0.05).*

Figure 9.2. Effect of strain (Jwt, J*rot*+) and day post infection (1-7) on presence and volume of nodule. *LS-means and standard errors.* [a-f] *Means not sharing letter differed significantly (P<0.05).*

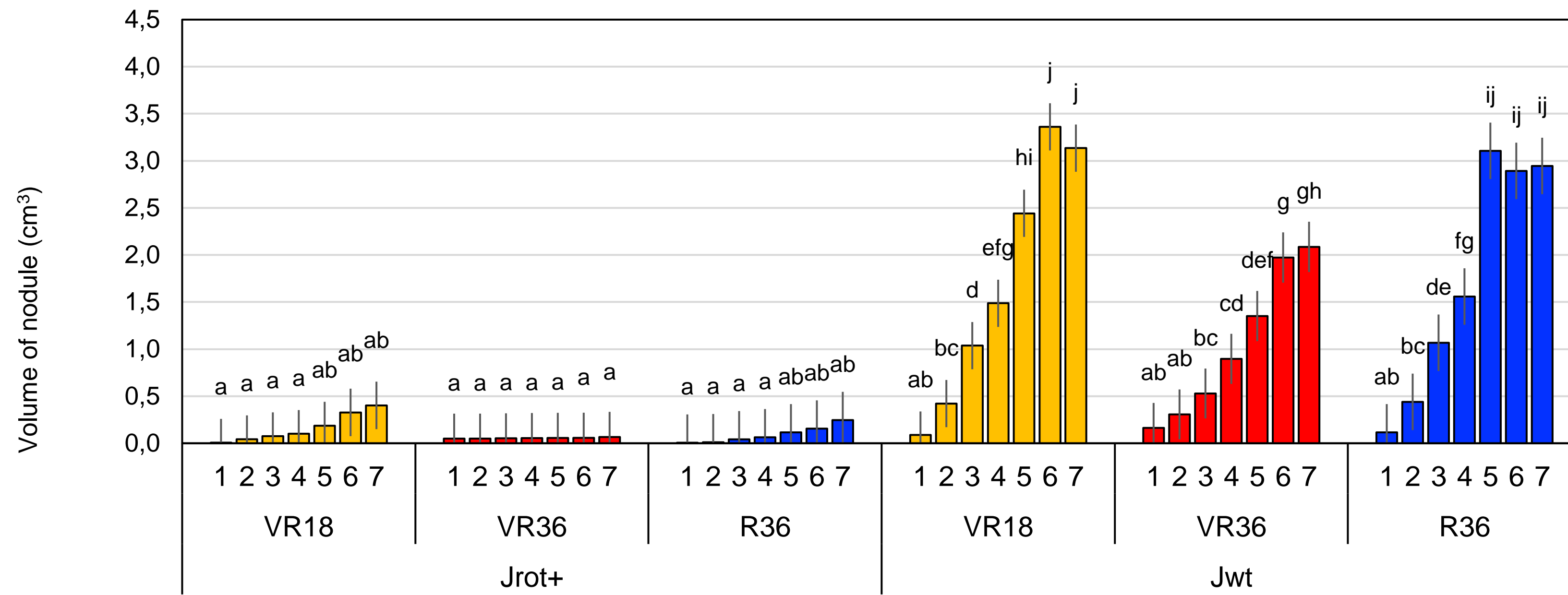

Figure 10. Effect of group (VR18: vitrified R line at 18th generation, VR36: vitrified R line at 36th generation, R36: non-vitrified R line at 36th generation) and strain (Jwt, Jrot+) depending on the day post infection (0, 1, 3, 7) on Volume of nodule. *LS-means and standard errors.* [a-j] *Means not sharing letter differed significantly (P<0.05).*

4.4. Discussion

In many species of animals, including the rabbit, the study of the different leukocyte subpopulations and other hematological parameters are indicative of the health status of the animals and also if their immune system is competent (Jeklova et al., 2009; Juul-Madsen et al., 2010; Guerrero et al., 2011). Regarding the leukocyte subpopulations, smaller counts of white blood cell do not always indicate a good condition of the immune system, nevertheless very high counts suggest pathological problems. Therefore, is worth discussing in order to evaluate the results obtained. On the one hand, a low white blood cell count indicates that the animal's immune system has not been activated due to an infectious or other type of agent, or if it has been activated, a slight reaction of the immune system has been enough to avoid infection. However, simultaneously a low leukocyte count may indicate immunosuppression by some intrinsic or extrinsic factors of the animal and may be the cause that the immune system is not able to face an infectious challenge effectively and result in disease. As an example of this, it has been described that before parturition in some animals a physiological immunosuppression occurs (Madsen et al., 2002; Sordillo et al., 2009; Ingvartsen and Moyes, 2013), which makes it a critical time for females, however in this study we have found the highest total lymphocyte counts at the time of parturition when compared to the subsequent weanings. In the previous study carried by Penadés et al., (2017), no notable differences were observed in the leukocyte count in the first weaning and the second parturition in the case of the paternal line. Therefore, in this study we consider that low leukocyte counts are due to a healthy state of the animal since there are no obvious reasons that they are due to immunosuppression, and high counts are due to infection or disease and therefore worse health status of the females. To support this, it is useful to collect macroscopic data from the lesions during infection in Experiment 2 to interpret them together with the data obtained by the hematological counter and the immunophenotype evaluated by flow cytometry.

During the productive cycle of rabbits, two of the most decisive points for physiology and health of the animals, for both females and litters, are the parturition and weaning of the kits. First, the moment of first parturition is a crucial point for the females and their health status will affect both, females and litters, and second, the state of rabbits at weaning age is decisive in their future health during the fattening stage (Rashwan and Marai, 2000; Bivolarski and Vachkova, 2014). Besides, the first mating is the best moment to evaluate the immune system of rabbits in relation to their genetics because later it can be affected by the environment, infections and other factors (Pascual et al., 2013), and therefore is an important point in the rabbit cycle on farms. For this reason, in this study the moments of first insemination, first parturition and first weaning in the first cycle of the females have been sampled.

4.4.1 Experiment 1:

Effect of selection by growth rate on the maintenance of immune competence under conventional conditions. In this study, no significant differences were found in any of the parameters studied between the two genetic types of rabbit females breed under commercial farm conditions separated by 18 generations of selection. In a study comparing the different blood leukocyte populations of rabbits at weaning of three rabbit lines selected by different parameters, the parental line R showed lower counts of total lymphocytes, B lymphocytes, CD5 + T lymphocytes, CD4 + T lymphocytes, CD8 + T lymphocytes, CD25 + T lymphocytes, CD4 + / CD8 + ratio, monocytes and granulocytes (García-Quirós et al., 2014). The previous study seems to indicate that the R line presents lower white blood cell counts than other genetic lines selected based on other parameters. However, the fact of not having found differences between the females of the genetic types separated 18 generations of selection and having found only differences in granulocytes in the case of weaning rabbits, shows that genetic selection has not been the cause. On the contrary, in a previous study carried out by our research group comparing different generations of

rabbits belonging to line V selected for litter size at weaning, it was shown that selection by reproductive parameters can affect the blood lymphocyte populations (Ferrian et al., 2012). Therefore, it seems that selection by productive parameters can affect the immunological status of breeding females, but in the specific case of genetic selection by average daily gain, it is not.

Interestingly, values from some parameters reported in the present experiment were slightly different from those reported by Penadés et al. (2017) for R females (lymphocytes B: 1.31 vs. 0,51 $\log_{10} 10^6$/L; G/L: 1.69 vs. 2.20). These differences could be associated with the experimental procedure, either because females nursed 2-3 kits more than in the present experiment (which imposed a greater reproductive effort to females (Elmaghraby et al., 2004)), or because in that experiment females did not come from the restoration of a vitrified population; which can have long-term effects on litters (Dulioust et al., 1995). No difference in the presence of haptoglobin in plasma was observed at any time. Haptoglobin is a plasma protein that can be increased in inflammatory processes, but it can be increased or decreased for other reasons such as pregnancy or intravascular haemolysis, so it is quite nonspecific. However, in other studies, an increase in this protein has been observed by inducing an acute phase response in the immune system (Ferrian et al., 2013). This may be because in the study by Ferrian et al. (2013) an acute phase immune response was induced, and in this work the immune system is in a basal state and since there is no inflammatory process, the parameter of haptoglobin is not affected.

On the other hand, except for lymphocytes B, results from the young rabbits were similar to those reported by García-Quirós et al. (2014) for R animals. However, in the present experiment we also compared the first and second weaning of the litters. In this comparison, we found higher total lymphocyte, CD5 + T lymphocyte, CD4 + T lymphocyte, CD8 + T lymphocyte, and monocytes counts in the second weaning compared to first weaning. This

may be due to a more maturation of the females' immune system at the second weaning than at the first weaning when they are not fully adult (affecting to the transmission of immunity towards young rabbits throughout placenta before birth and milk after birth). Regarding to the effect of selection on blood parameters, the main difference was observed in granulocytes. Selected animals presented 41.25% more granulocytes than non-selected animals. García-Quirós et al. (2014) reported that groups with higher counts of granulocytes presented also lower mortality the growing period. In the present experiment, mortality during the growing period was very high in both groups (61.9% in VR18 and 53.1% in VR36, P=0.06; Unpublished results). However, results are not consistent enough to establish any relation between immune system, genetic selection and mortality.

4.4.2. Experiment 2:

In this experiment macroscopic lesions from inoculations with both strains of *S. aureus* evolved similarly to those reported by Muñoz-Silvestre et al. (2020). Consequently, discussion will be focused exclusively on the objectives of the work.

Effect of selection by growth rate on mounting immune response under an immunological challenge with S. aureus. In rabbit production, breeding programmes may have affected the capacity of rabbits to respond to immune challenges (Ferrian et al., 2012). This has also been previously reported in other species where there are evidences about that the immunological capability may differ depending on the genetic origin of the animals (Rauw et al., 1998; Siegel and Honaker, 2009). In Experiment 2, we induced an acute infectious challenge over the immune system of rabbit females inoculating the pathogen *Staphylococcus aureus* and the lesions developed by the rabbits and their immune response were simultaneously evaluated. Analysing the data obtained by flow cytometry during the infection of females at age of first insemination, the genetic type of VR18 rabbits presented counts of total lymphocytes, T lymphocytes, CD25 +

lymphocytes, CD4 + and CD8 +, significantly higher than those of the VR36 genetic type of rabbits. However, B lymphocyte values were higher in the VR36 genetic type than in the VR18 genetic type. Additionally, more selected animals (VR36) showed lower presence of erythema and nodules compared to less selected animals (VR18). These data indicate that the females of the VR18 genetic type developed a higher inflammatory response based in the cellular counts and more severe lesions observed than the females of the VR36 genetic type when an acute inflammatory response is triggered. This is interesting because traditionally it has assumed that a T cell-mediated immunity is better than a humoral response to confer protection against a staphylococcal infections (Armentrout et al., 2020). As we have said before, previous studies (García-Quirós et al., 2014) show that the R line generally has lower white blood cell counts, and probably a lower immune status, than other lines when the immune system is in a basal state (without triggering an immune response). However, when the immune system is challenged with acute infection (as in Experiment 2 of this work), higher white blood cell counts are not always indicative of a better immune response, they can sometimes indicate an inability of the immune system to cope with and overcome an infection. In fact, it has been reported the ability of superantigenic toxins secreted by *S. aureus* to activate T-cells (Islander et al., 2010). In our study, it is observed that the rabbits of the most selected genetic type (VR36) develop fewer lesions and with lower leukocyte counts and therefore show a greater capacity to face an infectious challenge than the less selected rabbits (VR18). That shows that the immune system in a situation of acute response to an infection has not been negatively affected by ADG selection. In other words, our study suggests that the higher incidence of diseases observed in this genetic line compared to others selected by other parameters, is not due to the strategy used during the selection of these animals. Furthermore, the fact that more selected females develop a lower lesion count when faced with an infectious challenge suggests that selection for ADG has favored the immune system and its ability to cope with this kind of infections. We hypothesized that it

could be due to an indirect selection on the breeding program as only animals reaching to adulthood are selected for the next generation, which may improve the ability of animals to cope with immunological challenges consequently.

Eff*ect of vitrification of embryos when mounting the immune response under an immunological challenge with S. aureus.* Vitrification is a resource that allows obtaining animals from previous generations of selection and compare them with current generations coetaneously to see the effects of selection on desired traits. However, the vitrification process can affect several aspects of the animals recovered and their progeny (Marco-Jiménez et al., 2013; Lavara et al., 2015). Therefore, it is important to evaluate the real effect of the vitrification on the immunological traits for our aim, by comparing VR36 and R36. In this sense, many of the parameters evaluated by flow cytometry (counts of total lymphocytes, T lymphocytes, CD4 + lymphocytes, Granulocytes, Monocytes and the CD4 + / CD8 + ratio) were significantly higher in the R36 genetic type than VR36. On the contrary, for many of the blood parameters measured with a haematological analyser, the leukocyte counts were significantly lower in the R36 genetic type compared to VR36. These results joined with those reported for macroscopic lesions (higher presence of nodules a bigger size of nodules for R36 animals) indicate a clear long-term effect of vitrification on the immune response to an infection with *S. aureus*. In this sense, it seems that animals coming from the restoration of a vitrificated population presented a better performance during the infection. Our hypothesis for this effect is that many embryos are lost during the vitrification process, eliminating worse embryos and selecting embryos with a better immunological competence. Moreover, it is important to highlight that differences between Non-Selected-Vitrified animals (VR18) and Selected-Non-Vitrified animals (R36) were small in haematological parameters and macroscopic lesions. Therefore, if the present experiment would have involved only VR18 and R36, as many works from genetic selection do, no effect of selection would have been reported.

4.5. Conclusions

Genetic selection by average daily gain does not seem to have affected the ability of rabbit females to maintain a competent immune system under conventional conditions. However, it is not so clear for young rabbits. On the other hand, it seems clear that selection by average daily gain has not influenced negatively the ability to mount an immune response. In fact, the data obtained indicates that this breeding program has favored the ability of the immune system to undergo an infectious challenge with *S. aureus*. Finally, when we are interested to compare different generations of the same line, restoration of cryopreserved populations must be done in both generations.

GENERAL DISCUSSION

GENERAL DISCUSSION

During infection, many factors determine its development. These factors may depend on the pathogen, the host, the relation between both, and the environment. For this reason, in these four works we related the study of both the pathogen and host by focusing on each relevant aspect, especially in the rabbit farming field.

Pathogen *S. aureus* is an extremely versatile bacterium with the capacity to adapt to different environments thanks to its variety of virulence factors (Viana et al., 2015b). It is very well able to develop resistance to antibiotics (Lyon and Skurray, 1987). In recent years, the emerging increase in antibiotic-resistant bacteria has raised global concern because clones of bacteria resistant to all antibiotics have already appeared, which makes it impossible to treat the infections caused by these bacteria (Olsen et al., 2006; Chambers and DeLeo, 2009). This is why we focused the present study on the antibiotic resistance of *S. aureus* by paying special attention to clones resistant to methicillin for their great importance for public and animal health. First we sampled animals from rabbit farms throughout the Iberian Peninsula and found an unexpected large number of MRSA strains from infrequent lesions in *S. aureus* infections. This finding suggests that the pathogenesis of MRSA strains differs from that of MSSA strains. Most *S. aureus* lesions in rabbits are produced by ST121-type strains, which are usually MSSA (Rao et al., 2015). This is the first time that *mec*C-MRSA strains isolated from rabbit farms are described. The MRSA strains isolated from rabbit farms showed limited genetic diversity and corresponded to ST2855, ST146, ST398, and ST4774 in prevalence order. This is important because previously only ST398 MRSA strains have been described on rabbit farms (Agnoletti et al., 2014). The majority of the tested MRSA isolates were multidrug-resistant and ST2855 strains showed resistance to a larger number of antibiotic groups than other strains, as well as a higher frequency of resistance to macrolides. The importance of these findings lies

in the role of rabbits acting as a reservoir to transmit dangerous *S. aureus* clones to humans and other animals.

In addition to what we observed on farms, MRSA strains have been isolated from wildlife and the environment (Porrero et al., 2014b, 2014c). In this scenario, wild rabbits and hares hunted in high density areas in east Spain were sampled in nose, ear, perineum and lesions, which were not related to hunting, to study if the observed problem extended to wild fauna. A high percentage of *S. aureus* carriers was detected, where the ear was the main ecological niche from which *S. aureus* was isolated. The presence of ST1945 MRSA of CC130 containing the *mec*C gene in these animals was marked. These strains harboured mobile genetic element SCC*mec* type XI. Compared to the *S. aureus* isolates from rabbit farms, the *S. aureus* strains isolated from commercial and wild rabbits differed with CC130, followed by CC425, being the most frequently clonal complexes detected in wild rabbits. Clone CC130 is a ruminant-associated clone that has been isolated from wild animals (Ruiz-Ripa et al., 2019a, 2019b), but we herein noticed that many isolates obtained from rabbit farms came from injuries, and most of the isolates from wild rabbits came from healthy carrier animals. However, a clonal relation has been observed between strains isolated from lesions and from the nasal cavity (Selva et al., 2015). Differences in resistance to antibiotics were also observed. On rabbit farms, *mec*C-MRSA strains showed resistance to β-lactams, enrofloxacin, tetracycline and macrolides. However, most *mec*C-MRSA wild rabbit strains were susceptible to all the tested antibiotics, except for β-lactams. This suggests that *mec*C-MRSA strains in origin had no resistance to more antibiotics than to β-lactams, but the strains from the farms subjected to antibiotic pressure had acquired these resistances. Therefore, we observed in this study that, like farm rabbits, wild rabbits can act as a reservoir to transmit MRSA strains in nature and are, therefore, less controlled.

In order to finalise the study of the pathogen, specifically of MRSA strains, we described the genotype of mobile genetic element SCC*mec* in-depth

because this element carries many antibiotic resistance genes (Murugesan et al., 2015). We also studied the antibiotic resistance phenotype of *S. aureus* strains. The phenotypic and genotypic study of antibiotic resistant strains helps to understand the resistance mechanisms and the reason why the dissemination of certain clones have advantages over others (Oliveira et al., 2001; Glaser et al., 2016). In the genotypic study of SCC*mec* mobile genetic element, we described two new types of SCC*mec* cassette: one in which its sequence has not been previously described and contains the *mec*C gene; another was made up of a fragment of the SCC*mec* type III cassette and the new SCC*mec* type to result in an SCC*mec* simultaneously with genes *mec*A and *mec*C . Furthermore, two of these new *mec*A-*mec*C-MRSA strains had the SCC*mec* excised from the genome. The presence of both genes in the same rabbit strain is remarkable because it has been recently described in *S. aureus* isolated from cows (Aklilu and Chia, 2020), but not in *S. aureus* isolated from rabbits. During the comparison of the phenotypic resistance profile, strains *mec*A-MRSA showed resistance to much more antibiotics than strains *mec*C-MRSA. Nevertheless, carrying the two genes simultaneously did not present more advantages in the resistances to the tested antibiotics than having only the *mec*A gene. We also observed that each SCC*mec* type was associated with certain STs. This shows that although the different SCC*mec* cassette types are conserved in each ST, recombinations sometimes occur and give rise to new SCC*mec* with different characteristics (Hill-Cawthorne et al., 2014). For this reason, it is important to continue studying this mobile genetic element and its transmission between strains because it is related to the appearance of multiresistant strains to antibiotics.

Finally to study the host, we selected rabbit because is one of the hosts, after humans, to which *S. aureus* best adapts and is, therefore, a very useful model of the pathogenesis of this bacterium (Muñoz-Silvestre et al., 2020). In addition, *S. aureus* is one of the pathogens that poses major problems on rabbit farms (Segura et al., 2007; Rosell and de la Fuente, 2009). Moreover, the selection made in paternal rabbit lines can affect other

important characters for maintaining the productive level, such as reproductive characters (Baselga et al., 2003). For this reason, other traits like susceptibility to disease could also have been affected. In the R line, which is a paternal rabbit line selected by average daily gain, a higher incidence of diseases was observed, especially in digestion terms. The last work aims to study if the selection for average daily gain may affect the immune system and animals' susceptibility to suffer diseases. After evaluating the immune system by flow cytometry at main or more susceptible time points of the rabbit productive cycle, the genetic selection by average daily gain did not affect rabbit females' ability to mount an immune response. Moreover, after evaluating lesions and immune system evolution during an experimental infection by *S. aureus* in skin, the results evidenced that this breeding programme favoured the immune system's capability to undergo an infectious challenge with *S. aureus*. To carry out this work, it was necessary to use vitrified embryos to obtain animals from previous generations of selection. This technique is very useful for conserving populations, and evaluating the effects of selection, but it may entail effects on the individuals obtained by this technique that can affect the results (Lavara et al., 2015). Therefore to evaluate these effects, a group of animals obtained from vitrified embryos was compared to another group obtained by routine artificial insemination. A long-term effect of vitrification on the immune response to infection with *S. aureus* was observed insofar as the animals from the restoration of a vitrificated population presented better performance during infection.

In conclusion, while studying the pathogen we observed an incidence of MRSA strains in commercial and wild rabbits, which can act as a transmission reservoir. The study into the host, genetic selection by mean daily gain did not affect these animals' ability to successfully overcome infectious challenges, but actually seemed to improve it.

CONCLUSIONS

CONCLUSIONS

1. An unexpected large number of MRSA strains obtained from rabbit farms in the Iberian Peninsula were isolated from infrequent lesions in *S. aureus* infections.

2. It is the first time that *mec*C MRSA is described in rabbit samples from farms of the Iberian Peninsula.

3. MRSA isolates from rabbit farms showed limited genetic diversity that corresponded to ST2855, ST146, ST398, and ST4774 in prevalence order, except for ST398. This is the first time that these MRSA lineages associated with livestock have been described on rabbit farms of the Iberian Peninsula.

4. The majority of the tested MRSA isolates were multidrug-resistant and strains ST2855 showed resistance to many antibiotic groups than other strains, along with a higher resistance frequency to macrolides.

5. A high percentage of *S. aureus* carriers in wild rabbits and hares in high density areas of east Spain was detected, and the ear was the main ecological niche from which *S. aureus* was isolated.

6. The presence of ST1945 MRSA of the CC130 lineage containing the *mec*C gene and mobile genetic element SCC*mec* type XI in wild rabbits and hares in high density areas of east Spain was marked.

7. On rabbit farm, isolates *mec*C strains showed resistance to β-lactams, enrofloxacin, tetracycline and macrolides, but most *mec*C MRSA wild rabbit isolates were susceptible to all the tested antibiotics, except for β-lactams.

8. Two new SCC*mec* cassette types are described in rabbit samples of *S. aureus*: one containing the *mec*C gene and another simultaneously containing genes *mec*A and *mec*C.

9. Two *mec*A-*mec*C-MRSA ST5001-type strains harboured the SCC*mec* excised from the genome.

10. Strains *mec*A-MRSA presented resistance to more families of antibiotics than strains *mec*C-MRSA.

11. Genetic selection by average daily gain did not affect the ability of rabbit females to maintain a competent immune system under conventional conditions.

12. Genetic selection by average daily gain did not negatively influence the ability to mount an immune response. In fact, this breeding programme favoured the immune system's ability to undergo an infectious challenge with *S. aureus*.

13. Vitrification has a long-term effect on the immune response to infection with *S. aureus* insofar as animals from the restoration of a vitrificated population presented better performance during infection.

REFERENCES

REFERENCES

Agnoletti, F., Mazzolini, E., Bacchin, C., Bano, L., Berto, G., Rigoli, R., Muffato, G., Coato, P., Tonon, E., and Drigo, I. (2014). First reporting of methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 in an industrial rabbit holding and in farm-related people. *Veterinary Microbiology*, 170(1–2), 172–177.

Aires-de-Sousa, M. (2017). Methicillin-resistant *Staphylococcus aureus* among animals: current overview. *Clinical Microbiology and Infection*, 23(6), 373–380.

Aklilu, E., and Chia, H. Y. (2020). First *mec*C and *mec*A positive livestock-associated methicillin resistant *Staphylococcus aureus* (*mec*C MRSA/LA-MRSA) from dairy cattle in Malaysia. *Microorganisms*, 8(2), 147.

Alexander, Z., and Klaus, L. (2012). Neutrophil adhesion and activation under flow. *Microcirculation*, 40(6), 1301–1315.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25(17), 3389–3402.

Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D., and Zychlinsky, A. (2012). Neutrophil function: From mechanisms to disease. *Annual Review of Immunology*, 30, 459–489.

Angen, Ø., Feld, L., Larsen, J., Rostgaard, K., Skov, R., Madsen, A. M., and Larsen, A. R. (2017). Transmission of methicillin-resistant *Staphylococcus aureus* to human volunteers visiting a swine farm. *Applied and Environmental Microbiology*, 83(23), e01489-17.

Appelbaum, P. C., and Bozdogan, B. (2004). Vancomycin resistance in *Staphylococcus aureus*. *Clinics in Laboratory Medicine*, 24(2), 381–402.

Arciola, C. R., Baldassarri, L., and Montanaro, L. (2001). Presence of *ica*A and *ica*D genes and slime production in a collection of Staphylococcal strains from catheter-associated infections. *Journal of Clinical Microbiology*, 39(6), 2151–2156.

Arciola, C. R., Campoccia, D., Ravaioli, S., and Montanaro, L. (2015). Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. *Cellular and Infection Microbiology*, 5(7), 1–10.

Ariza-Miguel, J., Hernández, M., Fernández-Natal, I., and Rodríguez-Lázaro, D. (2014). Methicillin-resistant *Staphylococcus aureus*

harbouring *mec*C in livestock in Spain. *Journal of Clinical Microbiology*, 52(11), 4067–4069.

Armand-Lefevre, L., Ruimy, R., and Andremont, A. (2005). Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. *Emerging Infectious Diseases*, 11(5), 711–714.

Armbrust, T. A., and Eisen, E. J. (1994). Effect of cryoprotectant and genetic selection for body fat content on embryonic cryosurvival in mice. *Theoretical and Applied Genetics*, 88(3–4), 479–485.

Armentrout, E. I., Liu, G. Y., and Martins, G. A. (2020). T cell immunity and the quest for protective vaccines against *Staphylococcus aureus* infection. *Microorganisms*, 8(12), 1–20.

Arnau-Bonachera, A., Cervera, C., Blas, E., Larsen, T., Martínez-Paredes, E., Ródenas, L., and Pascual, J. J. (2018). Long-term implications of feed energy source in different genetic types of reproductive rabbit females: I. Resource acquisition and allocation. *Animal*, 12(9), 1867–1876.

Azevedo, E. C., Rios, E. M., Fukushima, K., and Campos-Takaki, G. M. (1993). Bacitracin production by a new strain of *Bacillus subtilis*. *Applied Biochemistry and Biotechnotogy*, 42(1), 1–7.

Baba, T., Bae, T., Schneewind, O., Takeuchi, F., and Hiramatsu, K. (2008). Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: Polymorphism and evolution of two major pathogenicity islands. *Journal of Bacteriology*, 190(1), 300–310.

Baba, T., Kuwahara-Arai, K., Uchiyama, I., Takeuchi, F., Ito, T., and Hiramatsu, K. (2009). Complete genome sequence of *Macrococcus caseolyticus* strain JSCS5402, reflecting the ancestral genome of the human-pathogenic staphylococci. *Journal of Bacteriology*, 191(4), 1180–1190.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K. I., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K., and Hiramatsu, K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *The Lancet*, 359, 1819–1827.

Balaban, N., and Rasooly, A. (2000). Staphylococcal enterotoxins. *International Journal of Food Microbiology*, 61, 1–10.

Ballhausen, B., Kriegeskorte, A., Schleimer, N., Peters, G., and Becker, K. (2014). The *mec*A homolog *mec*C confers resistance against β-

lactams in *Staphylococcus aureus* irrespective of the genetic strain background. *Antimicrobial Agents and Chemotherapy*, 58(7), 3791–3798.

Bardiau, M., Yamazaki, K., Duprez, J. N., Taminiau, B., Mainil, J. G., and Ote, I. (2013). Genotypic and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk of bovine mastitis. *Letters in Applied Microbiology*, 57(3), 181–186.

Baselga, M, García, M., Sánchez, J., Vicente, J., and Lavara, R. (2003). Analysis of reproductive traits in crosses among maternal lines of rabbits. *Animal Research, EDP Sciences*, 52(5), 473–479.

Baselga, Manuel, García, M. L., Gómez, E. A., Rafel, O., and Ramón, J. (1999). Selection, diffusion and performances of six spanish lines of meat rabbit. In Testik A. (ed.), Baselga M. (ed.). 2. International Conference on Rabbit Production in Hot Climates. Zaragoza: CIHEAM: Vol. Cahiers Op (pp. 147–152).

Becker, K., Ballhausen, B., Köck, R., and Kriegeskorte, A. (2014). Methicillin resistance *in Staphylococcus* isolates: The "*mec* alphabet" with specific consideration of *mec*C, a *mec* homolog associated with zoonotic *S. aureus* lineages. *International Journal of Medical Microbiology*, 304(7), 794–804.

Benito, D., Gómez, P., Aspiroz, C., Zarazaga, M., Lozano, C., and Torres, C. (2016). Molecular characterization of *Staphylococcus aureus* isolated from humans related to a livestock farm in Spain, with detection of MRSA-CC130 carrying *mec*C gene: A zoonotic case? *Enfermedades Infecciosas y Microbiologia Clinica*, 34(5), 280–285.

Bergmann S. and Hammerschmidt S. (2007). Fibrinolysis and host response in bacterial infections. *Thrombosis and Haemostasis*, 98(3), 512–520.

Bhakdi, S., Muhly, M., and Füssle, R. (1984). Correlation between toxin binding and hemolytic activity in membrane damage by Staphylococcal alpha-toxin. *Infection and Immunity*, 46(2), 318–323.

Bivolarski, B. L., and Vachkova, E. G. (2014). Morphological and functional events associated to weaning in rabbits. *Journal of Animal Physiology and Animal Nutrition*, 98(1), 9–18.

Bjerketorp, J., Nilsson, M., Ljungh, A., Flock, J., Jacobsson, K., and Frykberg, L. (2002). A novel von Willebrand factor binding protein expressed by *Staphylococcus aureus. Microbiology*, 148, 2037–2044.

Blasco, A., Bidanel, J. P., Bolet, G., Haley, C. S., and Santacreu, M. A. (1993). The genetics of prenatal survival of pigs and rabbits: a review. *Livestock Production Science*, 37(1–2), 1–21.

Bohach, G. A., Fast, D. J., Nelson, R. D., and Schlievert, P. M. (1990). Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. *Critical Reviews in Microbiology*, 17(4), 251–272.

Boyle-Vavra, S., and Daum, R. S. (2007). Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton – Valentine leukocidin. *Laboratory Investigation*, 87, 3–9.

Brandt, S. L., Putnam, N. E., Cassat, J. E., and Henrique, C. (2018). Innate immunity to *S. aureus*: evolving paradigms in soft tissue and invasive infections. *Journal of Immunology*, 200(12), 3871–3880.

Branson, D. (1968). Identification of Micrococcaceae in clinical bacteriology. *Applied Microbiology*, 16(6), 906–911.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science*, 303(5663), 1532–1535.

Brodersen, R., Bijlsma, F., Gori, K., Jensen, K. T., Chen, W., Dominguez, J., Haverson, K., Moore, P. F., Saalmüller, A., Sachs, D., Slierendrecht, W. J., Stokes, C., Vainio, O., Zuckermann, F., and Aasted, B. (1998). Analysis of the immunological cross reactivities of 213 well characterized monoclonal antibodies with specificities against various leucocyte surface antigens of human and 11 animal species. *Veterinary Immunology and Immunopathology*, 64(1), 1–13.

Burdett, V. (1991). Purification and characterization of Tet (M), a protein that renders ribosomes resistant to tetracycline. *Journal of Biological Chemistry*, 266(5), 2872–2877.

Burdett, V. (1996). Tet (M)-promoted release of tetracycline from ribosomes is GTP dependent. *Journal of Bacteriology*, 178(11), 3246–3251.

Burton, S., Reid-Smith, R., McClure, J. T., and Weese, J. S. (2008). *Staphylococcus aureus* colonization in healthy horses in Atlantic Canada. *Canadian Veterinary Journal*, 49(8), 797–799.

Casewell, M. W., and Hill, R. L. R. (1986). The carrier state: methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, 18(Suppl. A), 1–12.

Cauwelier, B., Gordts, B., Descheemaecker, P., and Van Landuyt, H. (2004). Evaluation of a disk diffusion method with cefoxitin (30 µg) for detection of methicillin-resistant *Staphylococcus aureus*. *European Journal of Clinical Microbiology and Infectious Diseases*, 23(5), 389–392.

Centers for Disease Control and Prevention. (1997). *Staphylococcus aureus* with reduced susceptibility to vancomycin–United States. *Morbidity and Mortality Weekly Report (MMWR)*, 46, 765–766.

Chabbert, Y. (1956). Antagonism in vitro between Erythromycin and Spiramycin. *Annales de l'Institut Pasteur*, 90(6), 787–790.

Chalker, A. F., Ingraham, K. A., Lunsford, R. D., Bryant, A. P., Bryant, J., Wallis, N. G., Broskey, J. P., Pearson, S. C., and Holmes, D. J. (2000). The *bac*A gene, which determines bacitracin susceptibility in *Streptococcus pneumoniae* and *Staphylococcus aureus*, is also required for virulence. *Microbiology*, 146(7), 1547–1553.

Chambers, H. F. (2005). Community-associated MRSA - Resistance and virulence converge. *The New England Journal of Medicine*, 352(14), 1485–1487.

Chambers, H. F., and DeLeo, F. R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews Microbiology*, 7(9), 629–641.

Chan, L. C., Chaili, S., Filler, S. G., Miller, L. S., Solis, N. V, Wang, H., Johnson, C. W., Lee, H. K., Diaz, L. F., and Yeaman, M. R. (2017). Innate immune memory contributes to host defense against recurrent skin and skin structure infections caused by methicillin-resistant *Staphylococcus aureus*. *Infection and Immunity*, 85(2), 1-19.

Chaplin, D. D. (2010). Overview of the immune response. *Journal of Allergy and Clinical Immunology*, 125(2), S3-23.

Chatterjee, S. S., and Otto, M. (2013). Improved understanding of factors driving methicillin-resistant *Staphylococcus aureus* epidemic waves. *Clinical Epidemiology*, 5, 205-217.

Chen, J., Quiles-Puchalt, N., Chiang, Y. N., Bacigalupe, R., Fillol-Salom, A., Chee, M. S. J., Fitzgerald, J. R., and Penadés, J. R. (2018). Genome hypermobility by lateral transduction. *Science*, 362, 207-212.

Cheung, A. L., Eberhardt, K. J., Chung, E., Yeaman, M. R., Sullam, P. M., Ramos, M., and Bayer A. S. (1994). Diminished virulence of a *sar*

lagr mutant of *Staphylococcus aureus* in the rabbit model of endocarditis. *Journal of Clinical Investigation*, 94(5), 1815-1822.

Conceicão, T., De Lencastre, H., and Aires-de-sousa, M. (2017). Frequent isolation of methicillin resistant *Staphylococcus aureus* (MRSA) ST398 among healthy pigs in Portugal. *PLoS ONE*, 12(4), e0175340.

Corpa, J. M., Hermans, K., and Haesebrouck, F. (2009). Main pathologies associated with *Staphylococcus aureus* infections in rabbits: a review. *World Rabbit Science*, 17(3), 115–125.

Cosmi, L., Maggi, L., Santarlasci, V., Liotta, F., and Annunziato, F. (2014). T helper cells plasticity in inflammation. *Cytometry Part A*, 85(1), 36-42.

Cuny, C., Layer, F., Strommenger, B., and Witte, W. (2011). Rare occurrence of methicillin-resistant *Staphylococcus aureus* CC130 with a novel *mec*A homologue in humans in Germany. *PLoS ONE*, 6(9), e24360.

Cuny, C., Wieler, L. H., and Witte, W. (2015). Livestock-Associated MRSA: The impact on humans. *Antibiotics*, 4(4), 521–543.

Davis, W. C., Davis, J. E., and Hamilton, M. J. (1995). Use of monoclonal antibodies and flow cytometry to cluster and analyze leukocyte differentiation molecules. *Methods in Molecular Biology (Clifton, N.J.)*, 45, 149-167.

Davis, William C., and Hamilton, M. J. (2008). Use of flow cytometry to develop and characterize a set of monoclonal antibodies specific for rabbit leukocyte differentiation molecules. *Journal of Veterinary Science*, 9(1), 51–66.

De Haas, C. J. C., Veldkamp, K. E., Peschel, A., Weerkamp, F., Van Wamel, W. J. B., Heezius, E. C. J. M., Poppelier, M. J. J. G., Van Kessel, K. P. M., and Van Strijp, J. A. G. (2004). Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *Journal of Experimental Medicine*, 199(5), 687–695.

De Rosa, M., Verdino, A., Soriente, A., and Marabotti, A. (2021). The odd couple (s): An overview of beta-lactam antibiotics bearing more than one pharmacophoric group. *International Journal of Molecular Sciences*, 22, 617.

De Rosa, S. C., and Roederer, M. (2001). Eleven-color flow cytometry. A powerful tool for elucidation of the complex immune system. *Clinics in Laboratory Medicine*, 21(4), 697–712.

Del'Alamo, L., D'Azevedo, P. A., Strob, A. J., Rodríguez-Lopez, D. V, Monteiro, J., Andrade, S. S., Pignatari, A. C. C., and Gales, A. C. (2007). An outbreak of catalase-negative meticillin-resistant *Staphylococcus aureus*. *Journal of Hospital Infection*, 65(3), 226-230.

DeLeo, F. R., Diep, B. A., and Otto, M. (2009). Host defense and pathogenesis in *Staphylococcus aureus* infections. *Infectious Disease Clinics of North America*, 23, 17–34.

DeLeo, F. R., Renee, J., McCormick, S., Nakamura, M., Apicella, M., Weiss, J. P., and Nauseef, W. M. (1998). Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. *Journal of Clinical Investigation*, 101(2), 455–463.

Deurenberg, R. H., Vink, C., Kalenic, S., Friedrich, A. W., Bruggeman, C. A., and Stobberingh, E. E. (2007). The molecular evolution of methicillin-resistant *Staphylococcus aureus*. *Clinical Microbiology and Infection*, 13(3), 222–235.

Devriese, L. A., Hendrickx, W., Godard, C., Okerman, L., and Haesebrouck, F. (1996). A new pathogenic *Staphylococcus aureus* type in commercial rabbits. *Journal of Veterinary Medicine*, *Series B*, 43(5), 313-315.

Devriese, Luc A., Vancanneyt, M., Baele, M., Vaneechoutte, M., De Graef, E., Snauwaert, C., Cleenwerck, I., Dawyndt, P., Swings, J., Decostere, A., and Haesebrouck, F. (2005). *Staphylococcus pseudintermedius sp*. nov., a coagulase-positive species from animals. *International Journal of Systematic and Evolutionary Microbiology*, 55(4), 1569-1573.

Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., Lin, F., Lin, J., Carleton, H. A., Mongodin, E. F., Sensabaugh, G. F., and Perdreau-Remington, F. (2006). Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin -resistant *Staphylococcus aureus*. *The Lancet*, 367, 731–739.

Dinges, M. M., Orwin, P. M., and Schlievert, P. M. (2000). Exotoxins of *Staphylococcus aureus*. *Clinical Microbiology Reviews*, 13(1), 16–34.

Dobrindt, U., Hochhut, B., Hentschel, U., and Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. *Nature Reviews Microbiology*, 2, 414-424.

Dolejska, M., Cizek, A., and Literak, I. (2007). High prevalence of antimicrobial-resistant genes and integrons in *Escherichia coli*

isolates from black-headed gulls in the Czech Republic. *Journal of Applied Microbiology*, 103(1), 11–19.

Dourmad, J. Y., Etienne, M., Prunier, A., and Noblet, J. (1994). The effect of energy and protein intake of sows on their longevity: a review. *Livestock Production Science*, 40(2), 87–97.

Drouet-Viard, F., and Fortun-Lamothe, L. (2002). Review: I -The organisation and functioning of the immune system: particular features of the rabbit. *World Rabbit Science*, 10(1), 15-23.

Drougka, E., Foka, A., Posantzis, D., Giormezis, N., Anastassiou, E. D., Petinaki, E., and Spiliopoulou, I. (2015). Human *Staphylococcus aureus* lineages among zoological park residents in Greece. *Open Veterinary Journal*, 5(2), 148-153.

Dulioust, E., Toyama, K., Busnel, M. C., Moutier, R., Carlier, M., Marchaland, C., Ducot, B., Roubertoux, P., and Auroux, M. (1995). Long-term effects of embryo freezing in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 92(2), 589–593.

El-baz, R., Rizk, D. E., Barwa, R., and Hassan, R. (2017). Virulence characteristics and molecular relatedness of methicillin resistant *Staphylococcus aureus* harboring different staphylococcal cassette chromosome *mec*. *Microbial Pathogenesis*, 113, 385-395.

Elmaghraby, M., Helal, M. A., and El-Sheikh, A. I. (2004). Maximum number of kits a rabbit doe should nurse for optimum litter performance up to weaning. *Alexandria Journal of Veterinary Sciences*, 21(2), 2-4.

Enright, M. C., Day, N. P. J., Davies, C. E., Peacock, S. J., and Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *Journal of Clinical Microbiology*, 38(3), 1008-1015.

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H., and Spratt, B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proceedings of the National Academy of Sciences of the United States of America*, 99(11), 7687–7692.

Estany, J., Camacho, J., Baselga, M., and Blasco, A. (1992). Selection response of growth rate in rabbits for meat production. *Genetics Selection Evolution*, 24(6), 527–537.

European Food Safety Authority (EFSA). (2009). Analysis of the baseline survey on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in holdings with breeding pigs, in the EU, 2008 - Part A: MRSA prevalence estimates. *EFSA Journal*, 7(11), 1376.

Feil, E. J., Cooper, J. E., Grundmann, H., Robinson, D. A., Enright, M. C., Berendt, T., Peacock, S. J., Smith, J. M., Murphy, M., Spratt, B. G., Moore, C. E., and Day, N. P. J. (2003). How Clonal Is *Staphylococcus aureus*? Journal of Bacteriology, 185(11), 3307–3316.

Ferrian, S., Blas, E., Larsen, T., Sánchez, J. P., Friggens, N. C., Corpa, J. M., Baselga, M., and Pascual, J. J. (2013). Comparison of immune response to lipopolysaccharide of rabbit does selected for litter size at weaning or founded for reproductive longevity. *Research in Veterinary Science*, 94(3), 518–525.

Ferrian, S., Guerrero, I., Blas, E., García-Diego, F. J., Viana, D., Pascual, J. J., and Corpa, J. M. (2012). How selection for reproduction or foundation for longevity could have affected blood lymphocyte populations of rabbit does under conventional and heat stress conditions. *Veterinary Immunology and Immunopathology*, 150(1–2), 53–60.

Fitzgerald, J. R. (2012). Livestock-associated *Staphylococcus aureus*: Origin, evolution and public health threat. *Trends in Microbiology*, 20(4), 192–198.

Fitzgerald, J. R., Reid, S. D., Ruotsalainen, E., Tripp, T. J., Liu, M., Cole, R., Kuusela, P., Schlievert, P. M., Járvinen, A., and Musser, J. M. (2003). Genome Diversification in *Staphylococcus aureus*: molecular evolution of a highly variable chromosomal region encoding the staphylococcal exotoxin-like family of proteins. *Infection and Immunity*, 71(5), 2827–2838.

Fluit, A. C. (2012). Livestock-associated *Staphylococcus aureus*. *Clinical Microbiology and Infection*, 18(8), 735–744.

Forsgren, A., and Nordström, K. (1962). Protein A from *Staphylococcus aureus*: the biological significance of its reaction with IgG. *Annals of the New York Academmy of Sciences*, 236, 252–266.

Foster, T. J. (2017). Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. *FEMS Microbiology Reviews*, 41(3), 430–449.

Foster, T. J., Geoghegan, J. A., Ganesh, V. K., and Höök, M. (2014). Adhesion, invasion and evasion: the many functions of the surface

proteins of *Staphylococcus aureus*. *Nature Reviews Microbiology*, 12(1), 49–62.

Frana, T. S., Beahm, A. R., Hanson, B. M., Kinyon, J. M., Layman, L. L., Karriker, L. A., Ramirez, A., and Smith, T. C. (2013). Isolation and characterization of methicillin-resistant *Staphylococcus aureus* from pork farms and visiting veterinary students. *PLoS ONE*, 8(1), e53738.

Francois, P., Huyghe, A., Charbonnier, Y., Bento, M., Herzig, S., Topolski, I., Fleury, B., Lew, D., Vaudaux, P., Harbarth, S., Van Leeuwen, W., Van Belkum, A., Blanc, D. S., Pittet, D., and Schrenzel, J. (2005). Use of an automated multiple-locus, variable-number tandem repeat-based method for rapid and high-throughput genotyping of *Staphylococcus aureus* isolates. *Journal of Clinical Microbiology*, 43(7), 3346–3355.

Freer, J. H., and Birkbeck, T. H. (1982). Possible conformation of delta-lysin, a membrane-damaging peptide of *Staphylococcus aureus*. *Journal of Theoretical Biology*, 94, 535–540.

Frère, J. M., Nguyen-Distèche, M., Coyette, J., and Joris, B. (1992). Mode of action: interaction with the penicillin binding proteins. In: The Chemistry of β-Lactams (pp. 148–197).

Fridkin, S. K., Hageman, J. C., Morrison, M., Sanza, L. T., Como-Sabetti, K., Jernigan, J. A., Harriman, K., Harrison, L. H., Lynfield, R., and Farley, M. M. (2005). Methicillin-resistant *Staphylococcus aureus* disease in three communities. *New England Journal of Medicine*, 352(14), 1436–1444.

Fridkin, S. K., Hill, H. A., Volkova, N. V., Edwards, J. R., Lawton, R. M., Gaynes, R. P., McGowan, J. E., Care, I., and Hospitals, A. R. E. (ICARE) P. (2002). Temporal changes in prevalence of antimicrobial resistance in 23 U.S. hospitals. *Emerging Infectious Diseases*, 8(7), 697–701.

Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V., and Zychlinsky, A. (2007). Novel cell death program leads to neutrophil extracellular traps. *Journal of Cell Biology*, 176(2), 231–241.

García-Álvarez, L., Holden, M. T. G., Lindsay, H., Webb, C. R., Brown, D. F. J., Curran, M. D., Walpole, E., Brooks, K., Pickard, D. J., Teale, C., Parkhill, J., Bentley, S. D., Edwards, G. F., Girvan, E. K., Kearns, A. M., Pichon, B., Hill, R. L. R., Larsen, A. R., Skov, R. L., Peacock, S. J., Maskell, D. J., Holmes, M. A. (2011). Meticillin-resistant *Staphylococcus aureus* with a novel *mec*A homologue in

human and bovine populations in the UK and Denmark: a descriptive study. *The Lancet Infectious Diseases*, 11, 595–603.

García-Garrote, F., Cercenado, E., MarÍn, M., Bal, M., Trincado, P., Corredoira, J., Ballesteros, C., Pita, J., Alonso, P., and Vindel, A. (2014). Methicillin-resistant *Staphylococcus aureus* carrying the *mec*C gene: Emergence in Spain and report of a fatal case of bacteraemia. *Journal of Antimicrobial Chemotherapy*, 69(1), 45–50.

García-Quirós, A., Arnau-Bonachera, A., Penadés, M., Cervera, C., Martínez-Paredes, E., Ródenas, L., Selva, L., Viana, D., Corpa, J. M., and Pascual, J. J. (2014). A robust rabbit line increases leucocyte counts at weaning and reduces mortality by digestive disorder during fattening. *Veterinary Immunology and Immunopathology*, 161(3–4), 123–131.

García, M. L., and Baselga, M. (2002a). Estimation of genetic response to selection in litter size of rabbits using a cryopreserved control population. *Livestock Production Science*, 74(1), 45–53.

García, M. L., and Baselga, M. (2002b). Genetic response to selection for reproductive performance in a maternal line of rabbits. *World Rabbit Science*, 10(2), 71–76.

Geha, D. J., Uhl, J. R., Gustaferro, C. A., and Persing, D. H. (1994). Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. *Journal of Clinical Microbiology*, 32(7), 1768–1772.

Giacinti, G., Carfora, V., Caprioli, A., Sagrafoli, D., Marri, N., Giangolini, G., Amoruso, R., Iurescia, M., Stravino, F., Dottarelli, S., Feltrin, F., Franco, A., Amatiste, S., and Battisti, A. (2017). Prevalence and characterization of methicillin-resistant *Staphylococcus aureus* carrying *mec*A or *mec*C and methicillin-susceptible *Staphylococcus aureus* in dairy sheep farms in central Italy. *Journal of Dairy Science*, 100(10), 7857–7863.

Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., DeBoy, R. T., Ravel, J., Paulsen, I. T., Kolonay, J. F., Brinkac, L., Beanan, M., Dodson, R. J., Daugherty, S. C., Madupu, R., Angiuoli, S. V, Durkin, A. S., Haft, D. H., Vamathevan, J., Khouri, H., Utterback, T., Lee, C., Dimitrov, G., Jiang, L., Quin, H., Weidman, J., Tran, K., Kang, K., Hance, I. R., Nelson, K. E., Fraser, C. M. (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus epidermidis* strain. *Journal of Bacteriology*, 187(7), 2426–2438.

Gillet, Y., Issartel, B., Vanhems, P., Fournet, J. C., Lina, G., Bes, M., Vandenesch, F., Piémont, Y., Brousse, N., Floret, D., and Etienne, J. (2002). Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *The Lancet*, 359, 753–759.

Glaser, P., Martins-Simões, P., Villain, A., Barbier, M., Tristan, A., Bouchier, C., Ma, L., Bes, M., Laurent, F., Guillemot, D., Wirth, T., Vandenesch, F. (2016). Demography and intercontinental spread of the USA300 community-acquired methicillin-resistant *Staphylococcus aureus* lineage. *MBio*, 7(1), e02183-15.

Glass, E. M., Wilkening, J., Wilke, A., Antonopoulos, D., and Meyer, F. (2010). Using the metagenomics RAST server (MG-RAST) for analyzing shotgun metagenomes. *Cold Spring Harbor Protocols*, 2010(1), pdb prot5368.

Goering, R. V. (1993). Molecular epidemiology of nosocomial infection: analysis of chromosomal restriction fragment patterns by pulsed-field gel electrophoresis. *Infection Control and Hospital Epidemiology*, 14(10), 595–600.

Goerke, C., Wirtz, C., Flückiger, U., and Wolz, C. (2006). Extensive phage dynamics in *Staphylococcus aureus* contributes to adaptation to the human host during infection. *Molecular Microbiology*, 61(6), 1673–1685.

Gómez, E., Rafel, O., Ramon, J., and Baselga, M. (1996). A genetic study of a line selected on litter size at weaning. *Proceedings of the 6th World Rabbit Congress, Toulouse, France*, 2, 289–292.

Gómez, M. I., Lee, A., Reddy, B., Muir, A., Soong, G., Pitt, A., Cheung, A., and Prince, A. (2004). *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nature Medicine*, 10(8), 842–848.

Gómez, P., González-Barrio, D., Benito, D., García, J. T., Viñuela, J., Zarazaga, M., Ruiz-Fons, F., and Torres, C. (2014). Detection of methicillin-resistant *Staphylococcus aureus* (MRSA) carrying the *mec*C gene in wild small mammals in Spain. *Journal of Antimicrobial Chemotherapy*, 69(8), 2061–2064.

Gordon, S., Plüddemann, A., and Martinez Estrada, F. (2014). Macrophage heterogeneity in tissues: Phenotypic diversity and functions. *Immunological Reviews*, 262(1), 36–55.

Goswitz, J. J., Willard, K. E., Fasching, C. E., and Petersont, L. R. (1992). Detection of *gyr*A gene mutations associated with ciprofloxacin

resistance in methicillin-resistant *Staphylococcus aureus*: Analysis by polymerase chain reaction and automated direct DNA sequencing. *Antimicrobial Agents and Chemotherapy*, 36(5), 1166–1169.

Graveland, H., Duim, B., Duijkeren, E. Van, Heederik, D., and Wagenaar, J. A. (2011). Livestock-associated methicillin-resistant *Staphylococcus aureus* in animals and humans. *International Journal of Medical Microbiology*, 301, 630–634.

Grim, S. A., Rapp, R. P., Martin, C. A., and Evans, M. E. (2005). Trimethoprim-Sulfamethoxazole as a viable treatment option for infections caused by methicillin-resistant *Staphylococcus aureus*. *Pharmacotherapy*, 25(2), 253–264.

Grüner, B. M., Han, S. R., Meyer, H. G., Wulf, U., Bhakdi, S., and Siegel, E. K. (2007). Characterization of a catalase-negative methicillin-resistant *Staphylococcus aureus* strain. *Journal of Clinical Microbiology*, 45(8), 2684–2685.

Guardabassi, L., Stegger, M., and Skov, R. (2007). Retrospective detection of methicillin resistant and susceptible *Staphylococcus aureus* ST398 in Danish slaughter pigs. *Veterinary Microbiology*, 122, 384–386.

Guerrero, I., Ferrian, S., Blas, E., Pascual, J. J., Cano, J. L., and Corpa, J. M. (2011). Evolution of the peripheral blood lymphocyte populations in multiparous rabbit does with two reproductive management rhythms. *Veterinary Immunology and Immunopathology*, 140(1–2), 75–81.

Guerrero, I., Ferrian, S., Penadés, M., García-Quirós, A., Pascual, J. J., Selva, L., Viana, D., and Corpa, J. M. (2015). Host responses associated with chronic staphylococcal mastitis in rabbits. *Veterinary Journal*, 204(3), 338–344.

Haaber, J., Penadés, J. R., and Ingmer, H. (2017). Transfer of antibiotic resistance in *Staphylococcus aureus*. *Trends in Microbiology*, 25(11), 893–905.

Hackbarth, C. J., and Chambers, H. F. (1993). blaI and blaR1 regulate beta-lactamase and PBP 2a production in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 37(5), 1144–1149.

Hanselman, B. A., Kruth, S. A., Rousseau, J., Low, D. E., Willey, B. M., McGeer, A., and Weese, J. S. (2006). Methicillin-resistant *Staphylococcus aureus* colonization in veterinary personnel. *Emerging Infectious Diseases*, 12(12), 1933–1938.

Hanssen, A. M., and Ericson Sollid, J. U. (2006). SCC*mec* in staphylococci: Genes on the move. *FEMS Immunology and Medical Microbiology*, 46(1), 8–20.

Harmsen, D., Claus, H., Witte, W., Rothgänger, J., Claus, H., Turnwald, D., and Vogel, U. (2003). Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. *Journal of Clinical Microbiology*, 41(12), 5442–5448.

Harrison, E. M., Coll, F., Toleman, M. S., Blane, B., Brown, N. M., Török, M. E., Parkhill, J., and Peacock, S. J. (2017). Genomic surveillance reveals low prevalence of livestock-associated methicillin-resistant *Staphylococcus aureus* in the East of England. *Scientific Reports*, 7(1), 7406–7407.

Harrison, E. M., Paterson, G. K., Holden, M. T. G., Larsen, J., Stegger, M., Larsen, A. R., Petersen, A., Skov, R. L., Christensen, J. M., Zeuthen, A. B., Heltberg, O., Harris, S. R., Zadoks, R. N., Parkhill, J., Peacock, S. J., and Holmes, M. A. (2013). Whole genome sequencing identifies zoonotic transmission of MRSA isolates with the novel *mec*A homologue *mec*C. *EMBO Molecular Medicine*, 5, 1–7.

Hartman, B. J., and Tomasz, A. (1984). Low-affinity penicillin-binding protein associated with β-lactam resistance in *Staphylococcus aureus*. *Journal of Bacteriology*, 158(2), 513–516.

Hayashi, F., Means, T. K., and Luster, A. D. (2003). Toll-like receptors stimulate human neutrophil function. *Blood*, 102(7), 2660–2669.

Heilmann, C., Heilmann, C., Schweitzer, O., Schweitzer, O., Mack, D., Mack, D., Gotz, F., and Götz, F. (1996). Molecular basis of intercellular adhesion in the biofilm-forming. *Molecular Microbiology*, 20(5), 1083–1091.

Hermans, K., De Herdt, P., Devriese, L. A., Godard, C., and Haesebrouck, F. (2000). Colonisation of rabbits with *Staphylococcus aureus* after experimental infection with high and low virulence strains. *Veterinary Microbiology*, 72(3–4), 277–284.

Hermans, K., De Herdt, P., Devriese, L. A., Hendrickx, W., Godard, C., and Haesebrouck, F. (1999). Colonization of rabbits with *Staphylococcus aureus* in flocks with and without chronic staphylococcosis. *Veterinary Microbiology*, 67(1), 37–46.

Herron-Olson, L., Fitzgerald, J. R., Musser, J. M., and Kapur, V. (2007). Molecular correlates of host specialization in *Staphylococcus aureus*. *PLoS ONE*, 2(10), e1120.

Higgins, J., Loughman, A., van Kessel, K. P. M., van Strijp, J. A. G., and Foster, T. J. (2006). Clumping factor A of *Staphylococcus aureus* inhibits phagocytosis by human polymorphonuclear leucocytes. *FEMS Microbiology Letters*, 258, 290–296.

Hill, W. (2008). Estimation, effectiveness and opportunities of long term genetic improvement in animals and maize. *Lohmann Information*, 43(1), 3–20.

Hill-Cawthorne, G. A., Hudson, L. O., El Ghany, M. F. A, Piepenburg, O., Nair, M., Dodgson, A., Forrest, M. S., Clark, T. G., Pain, A. (2014). Recombinations in staphylococcal cassette chromosome *mec* elements compromise the molecular detection of methicillin resistance in Staphylococcus aureus. *PLoS ONE,* 9(6), e101419.

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and Tenover, F. C. (1997). Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *Journal of Antimicrobial Chemotherapy*, 40(1), 135–146.

Holden, M. T. G., Feil, E. J., Lindsay, J. A., Peacock, S. J., Day, N. P. J., Enright, M. C., Foster, T. J., Moore, C. E., Hurst, L., Atkin, R., Barron, A., Bason, N., Bentley, S. D., Chillingworth, C., Chillingworth, T., Churcher, C., Clark, L., Corton, C., Cronin, A., Doggett, J., Dowd, L., Feltwell, T., Hance, Z., Harris, B., Hauser, H., Holroyd, S., Jagels, K., James, K. D., Lennard, N., Line, A., Mayes, R., Moule, S., Mungall, K., Ormond, D., Quail, M. A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Sharp, S., Simmonds, M., Stevens, K., Whitehead, S., Barrell, B. G., Spratt, B. G., Parkhill, J. (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: Evidence for the evolution of virulence and drug resistance. *Proceedings of the National Academy of Sciences of the United States of America*, 101(26), 9786–9791.

Hua, Z., and Hou, B. (2013). TLR signaling in B-cell development and activation. *Cellular and Molecular Immunology*, 10(2), 103–106.

Iandolo, J. J., Worrell, V., Groicher, K. H., Qian, Y., Tian, R., Kenton, S., Dorman, A., Ji, H., Lin, S., Loh, P., Qi, S., Zhu, H., and Roe, B. A. (2002). Comparative analysis of the genomes of the temperate bacteriophages φ11, φ12 and φ13 of *Staphylococcus aureus* 8325. Gene, 289(1–2), 109–118.

Ingvartsen, K. L., and Moyes, K. (2013). Nutrition, immune function and health of dairy cattle. *Animal*, 7(SUPPL.1), 112–122.

International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). (2009). Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): Guidelines for reporting novel SCC*mec* elements. *Antimicrobial Agents and Chemotherapy*, 53(12), 4961–4967.

Islander, U., Andersson, A., Lindberg, E., Adlerberth, I., Wold, A. E., and Rudin, A. (2010). Superantigenic *Staphylococcus aureus* stimulates production of interleukin-17 from memory but not naive T cells. *Infection and Immunity*, 78(1), 381–386.

Ito, T., Kuwahara-Arai, K., Katayama, Y., Uehara, Y., Han, X., Kondo, Y., and Hiramatsu, K. (2014). Staphylococcal cassette chromosome *mec* (SCC*mec*) analysis of MRSA. *Methods in Molecular Biology*, 1085, 131–148.

Ito, T., Ma, X. X., Takeuchi, F., Okuma, K., Yuzawa, H., and Hiramatsu, K. (2004). Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccr*C. *Antimicrobial Agents and Chemotherapy*, 48(7), 2637–2651.

Ito, T., Okuma, K., Ma, X. X., Yuzawa, H., and Hiramatsu, K. (2003). Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. *Drug Resistance Updates*, 6, 41–52.

Jacobsen, C. N., Aasted, B., Broe, M. K., and Petersen, J. L. (1993). Reactivities of 20 anti-human monoclonal antibodies with leucocytes from ten different animal species. *Veterinary Immunology and Immunopathology*, 39(4), 461–466.

Jeklova, E., Leva, L., Knotigova, P., and Faldyna, M. (2009). Age-related changes in selected haematology parameters in rabbits. *Research in Veterinary Science*, 86(3), 525–528.

Jeklova, E., Leva, L., Kudlackova, H., and Faldyna, M. (2007a). Functional development of immune response in rabbits. *Veterinary Immunology and Immunopathology*, 118(3–4), 221–228.

Jeklova, Edita, Leva, L., and Faldyna, M. (2007b). Lymphoid organ development in rabbits: Major lymphocyte subsets. *Developmental and Comparative Immunology*, 31(6), 632–644.

Jevons, M. (1961). “Celbenin”—resistant staphylococci. *British Medical Journal*, 124, 124–125.

Johnson, B. A., Anker, H., and Meleney, F. L. (1945). Bacitracin: A new antibiotic produced by a member of the B. subtilis group. *Science*, 102(2650), 376–377.

Jones, R. N. (2003). Global epidemiology of antimicrobial resistance among community-acquired and nosocomial pathogens: A five-year summary from the SENTRY Antimicrobial Surveillance Program (1997-2001). *Seminars in Respiratory and Critical Care Medicine*, 24(1), 121–133.

Jorasch, P., Warnecke, D. C., Lindner, B., Zähringer, U., and Heinz, E. (2000). Novel processive and nonprocessive glycosyltransferases from *Staphylococcus aureus* and *Arabidopsis thaliana* synthesize glycoglycerolipids, glycophospholipids, glycosphingolipids and glycosylsterols. *European Journal of Biochemistry*, 267(12), 3770–3783.

Josefowicz, S. Z., Lu, L.-F., and Rudensky, A. Y. (2012). Regulatory T cells: Mechanisms of differentiation and function. *Annual Review of Immunology*, 30, 531–564.

Josefsson, E., Mccrea, K. W., Eidhin, D. N., O'Connell, D., Cox, J., Höök, M., and Foster, T. J. (1998). Three new members of the serine-aspartate repeat protein multigene family of *Staphylococcus aureus*. *Microbiology*, 144, 3387–3395.

Juul-Madsen, H. R., Jensen, K. H., Nielsen, J., and Damgaard, B. M. (2010). Ontogeny and characterization of blood leukocyte subsets and serum proteins in piglets before and after weaning. *Veterinary Immunology and Immunopathology*, 133(2–4), 95–108.

Kaatz, G. W., and Seo, S. M. (1995). Inducible *nor*A-mediated multidrug resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 39(12), 2650–2655.

Katayama, Y., Ito, T., and Hiramatsu, K. (2000). A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy*, 44(6), 1549–1555.

Katayama, Yuki, Ito, T., and Hiramatsu, K. (2001). Genetic organization of the chromosome region surrounding *mec*A in clinical staphylococcal strains: role of IS431-mediated *mec*I deletion in expression of resistance in *mec*A-carrying, low-level meticillin-resistant *Staphylococcus haemolyticus*. *Antimicrobial Agents and Chemotherapy*, 45(7), 1955–1963.

Kennedy, A. D., and DeLeo, F. R. (2009). Neutrophil apoptosis and the resolution of infection. *Immunologic Research*, 43(1–3), 25–61.

Kessler, C. M., Nussbaum, E., and Tuazon, C. U. (1991). Disseminated intravascular coagulation associated with *Staphylococcus aureus*

septicemia is mediated by peptidoglycan-induced platelet aggregation. *Journal of Infectious Diseases*, 164(1), 101–107.

Khairalla, A. S., Wasfi, R., and Ashour, H. M. (2017). Carriage frequency, phenotypic, and genotypic characteristics of methicillin-resistant *Staphylococcus aureus* isolated from dental health-care personnel, patients, and environment. *Scientific Reports*, 7(1), 7390.

Khalil, M. H., and Al-Saef, A. M. (2008). Methods, criteria, techniques and genetic responses for rabbit selection: A review. *9th World Rabbit Congress*, 16, 3–34.

Kin, N. W., and Sanders, V. M. (2006). It takes nerve to tell T and B cells what to do. *Journal of Leukocyte Biology*, 79(6), 1093–1104.

Klevens, R. M., Edwards, J. R., Tenover, F. C., McDonald, L. C., Horan, T., Gaynes, R., and National Nosocomial Infections Surveillance System. (2006). Changes in the epidemiology of methicillin-resistant *Staphylococcus aureus* in intensive care units in US hospitals, 1992-2003. Clinical Infectious Diseases, 42(3), 389–391.

Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L. H., Lynfield, R., Dumyati, G., Townes, J. M., Craig, A. S., Zell, E. R., Fosheim, G. E., McDougal, L. K., Carey, R. B., and Fridkin, S. K. (2007). Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *Journal of the American Medical Association*, 298(15), 1763–1771.

Kloos, W. E. (1980). Natural populations of the genus Staphylococcus. *Annual Reviews of Microbiology*, 34, 559–592.

Kluytmans, J. A. J. W., and Wertheim, H. F. L. (2005). Nasal carriage of *Staphylococcus aureus* and prevention of nosocomial infections. Infection, 33(1), 3–8.

Kobayashi, S. D., Braughton, K. R., Whitney, A. R., Voyich, J. M., Schwan, T. G., Musser, J. M., and DeLeo, F. R. (2003). Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. *Proceedings of the National Academy of Sciences of the United States of America*, 100(19), 10948–10953.

Kobayashi, S. D., Malachowa, N., and DeLeo, F. R. (2015). Pathogenesis of *Staphylococcus aureus* abscesses. *American Journal of Pathology*, 185(6), 1518–1527.

Kobayashi, S. D., Voyich, J. M., Buhl, C. L., Stahl, R. M., and DeLeo, F. R. (2002). Global changes in gene expression by human polymorphonuclear leukocytes during receptor-mediated phagocytosis: Cell fate is regulated at the level of gene expression.

Proceedings of the National Academy of Sciences of the United States of America, 99(10), 6901–6906.

Kobayashi, S. D., Voyich, J. M., Burlak, C., and DeLeo, F. R. (2005). Neutrophils in the innate immune response. *Archivum Immunologiae et Therapiae Experimentalis*, 53(6), 505–517.

Kolaczkowska, E., and Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology*, 13(3), 159–175.

Kolmodin, R., Strandberg, E., Madsen, P., Jensen, J., and Jorjani, H. (2002). Genotype by environment interaction in Nordic dairy cattle studied using reaction norms. *Acta Agriculturae Scandinavica - Section A: Animal Science*, 52(1), 11–24.

Kondo, Y., Ito, T., Ma, X. X., Watanabe, S., Kreiswirth, B. N., Etienne, J., and Hiramatsu, K. (2007). Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: Rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. *Antimicrobial Agents and Chemotherapy*, 51(1), 264–274.

Koreen, L., Graviss, S. V., Ramaswamy, E. A., Naidich, S., Musser, J. M., and Kreiswirth, B. N. (2004). *spa* typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro and macrovariation. *Journal of Clinical Microbiology*, 42(2), 792–799.

Koreen, L., Ramaswamy, S. V., Naidich, S., Koreen, I. V., Graff, G. R., Graviss, E. A., and Kreiswirth, B. N. (2005). Comparative sequencing of the serine-aspartate repeat-encoding region of the clumping factor B gene (*clf*B) for resolution within clonal groups of *Staphylococcus aureus*. *Journal of Clinical Microbiology*, 43(8), 3985–3994.

Kotani, M., Yamamura, Y., Tamatani, T., Kitamura, F., and Miyasaka, M. (1993a). Generation and characterization of monoclonal antibodies against rabbit CD4, CD5 and CD11a antigens. *Journal of Immunological Methods*, 157(1–2), 241–252.

Kotani, M., Yamamura, Y., Tsudo, M., Tamatani, T., Kitamura, F., and Miyasaka, M. (1993b). Generation of monoclonal antibodies to the rabbit nterleukin-2 receptor alpha chain (CD25) and its distribution in HTLV-1-transformed rabbit T cells. *Japanese Journal of Cancer Research*, 87(7), 770–775.

Kraushaar, B., Hammerl, J. A., Kienöl, M., Heinig, M. L., Sperling, N., Thanh, M. Di., Reetz, J., Jäckel, C., Fetsch, A., and Hertwig, S. (2017). Acquisition of virulence factors in livestock-associated

MRSA: Lysogenic conversion of CC398 strains by virulence gene-containing phages. *Scientific Reports*, 7(1), 2004.

Krupa, P., Bystron, J., Bania, J., Podkowik, M., Empel, J., and Mroczkowska, A. (2014). Genotypes and oxacillin resistance of *Staphylococcus aureus* from chicken and chicken meat in Poland. *Poultry Science*, 93(12), 3179–3186.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Kobayashi, N., Cui, L., Oguchi, A., Aoki, K. I., Nagai, Y., Lian, J. Q., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N. K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., Hiramatsu, K. (2001). Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *The Lancet*, 357, 1225–1240.

Kurt, K., Rasigade, J., Laurent, F., Goering, R. V, Helena, Z., Machova, I., Struelens, M. J., Zautner, A. E., Holtfreter, S., Bröker, B., Ritchie, S., Reaksmey, S., Limmathurotsakul, D., Peacock, S. J., Cuny, C., Layer, F., Witte, W., and Nübel, U. (2013). Subpopulations of *Staphylococcus aureus* clonal complex 121 are associated with distinct clinical entities. *PLoS ONE*, 8(3), e58155.

Kwan, T., Liu, J., DuBow, M., Gros, P., and Pelletier, J. (2005). The complete genomes and proteomes of 27 *Staphylococcus aureus* bacteriophages. *Proceedings of the National Academy of Sciences of the United States of America*, 102(14), 5174–5179.

Kwiecinski, J., Peetermans, M., Liesenborghs, L., Na, M., Björnsdottir, H., Zhu, X., Jacobsson, G., Johansson, B. R., Geoghegan, J. A., Foster, T. J., Josefsson, E., Bylund, J., Peter Verhamme, P., Jin, T. (2016). Staphylokinase control of *Staphylococcus aureus* biofilm formation and detachment through host plasminogen activation. The Journal of Infectious Diseases, 213(1): 139-148.

Lacey, R. W., and Chopra, I. (1972). Evidence for mutation to streptomycin resistance in clinical strains of *Staphylococcus aureus*. *Journal of General Microbiology*, 73(1), 175–180.

Lakhundi, S., and Zhang, K. (2018). Methicillin-resistant *Staphylococcus aureus*: Molecular characterization, evolution, and epidemiology. *Clinical Microbiology Reviews*, 31(4), 1–103.

Lanier, L. L., Engleman, E. G., Gatenby, P., Babcock, G. F., Warner, N. L., and Herzenberg, L. A. (1983). Correlation of functional properties of human lymphoid cell subsets and surface marker phenotypes using

multiparameter analysis and flow cytometry. *Immunological Reviews*, 74(1), 143–160.

Larsen, J., Clasen, J., Hansen, J. E., Paulander, W., Petersen, A., Larsen, A. R., and Frees, D. (2016). Copresence of *tet*(K) and *tet*(M) in livestock-associated methicillin-resistant *Staphylococcus aureus* clonal complex 398 is associated with increased fitness during exposure to sublethal concentrations of tetracycline. *Antimicrobial Agents and Chemotherapy*, 60(7), 4401–4403.

Laurent, F., Chardon, H., Haenni, M., Bes, M., Reverdy, M. E., Madec, J. Y., Lagier, E., Vandenesch, F., and Tristan, A. (2012). MRSA harboring *mec*A variant gene *mec*C, France. *Emerging Infectious Diseases*, 18(9), 1465–1467.

Lavara, R., Baselga, M., Marco-Jiménez, F., and Vicente, J. S. (2015). Embryo vitrification in rabbits: Consequences for progeny growth. *Theriogenology*, 84(5), 674–680.

Lavara, R., Baselga, M., and Vicente, J. S. (2011). Does storage time in LN2 influence survival and pregnancy outcome of vitrified rabbit embryos? *Theriogenology*, 76(4), 652–657.

Leclercq, R., and Courvalin, P. (1991). Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. *Antimicrobial Agents and Chemotherapy*, 35(7), 1267–1272.

Li, M., Cha, D. J., Lai, Y., Villaruz, A. E., Sturdevant, D. E., and Otto, M. (2007). The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*. *Molecular Microbiology*, 66(5), 1136–1147.

Linhardt, F., Ziebuhr, W., Meyer, P., Witte, W., and Hacker, J. (1992). Pulsed-field gel electrophoresis of genomic restriction fragments as a tool for the epidemiological analysis of *Staphylococcus aureus* and coagulase-negative staphylococci. *FEMS Microbiology Letters*, 95(2–3), 181–185.

Liu, C., Gelius, E., Liu, G., Steiner, H., and Dziarski, R. (2000). Mammalian peptidoglycan recognition protein binds peptidoglycan with high affinity, is expressed in neutrophils, and inhibits bacterial growth. *Journal of Biological Chemistry*, 275(32), 24490–24499.

Lochmiller, R. L., and Deerenberg, C. (2000). Trade-offs in evolutionary immunology: Just what is the cost of immunity? *Oikos*, 88(1), 87–98.

Loncaric, I., Kübber-Heiss, A., Posautz, A., Stalder, G. L., Hoffmann, D., Rosengarten, R., and Walzer, C. (2013). Characterization of methicillin-resistant *Staphylococcus spp*. carrying the *mec*C gene,

isolated from wildlife. *Journal of Antimicrobial Chemotherapy*, 68(10), 2222–2225.

Loof, T. G., Mörgelin, M., Johansson, L., Oehmcke, S., Olin, A. I., Dickneite, G., Norrby-Teglund, A., Theopold, U., and Herwald, H. (2011). Coagulation, an ancestral serine protease cascade, exerts a novel function in early immune defense. *Blood*, 118(9), 2589–2598.

Lowder, B. V., Guinane, C. M., Zakour, N. L. B., Weinert, L. A., Conway-Morris, A., Cartwright, R. A., Simpson, A. J., Rambaut, A., Nübel, U., and Fitzgerald, J. R. (2009). Recent human-to-poultry host jump, adaptation, and pandemic spread of *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences of the United States of America*, 106(46), 19545–19550.

Lowy F.D. (1998). *Staphylococcus aureus* Infections. *The New England Journal of Medicine*, 339(8), 520–532.

Lozano, C., Marí, A., Aspiroz, C., Gómez-Sanz, E., Ceballos, S., Fortuño, B., Barcenilla, F., Jover-Sáenz, A., and Torres, C. (2014). Nasal carriage of coagulase positive staphylococci in patients of a Primary-Healthcare-Center: Genetic lineages and resistance and virulence genes. *Enfermedades Infecciosas y Microbiologia Clinica*, 33(6), 391–396.

Lüthje, P., and Schwarz, S. (2006). Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide – lincosamide resistance phenotypes and genotypes. *Journal of Antimicrobial Chemotherapy*, 57(5), 966–969.

Lyon, B. R., and Skurray, R. (1987). Antimicrobial resistance of *Staphylococcus aureus*: Genetic basis. *Microbiological Reviews*, 51(1), 88–134.

Ma, X., Ito, T., Tientsasitorn, C., Jamklang, M., Chongtrakool, P., Boyle-Vavra, S., Daum, R. S., and Hiramatsu, K. (2002). Novel type of staphylococcal cassette cromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. *Antimicrobial Agents and Chemotherapy*, 46(2), 1147–1152.

Madsen, S. A., Weber, P. S. D., and Burton, J. L. (2002). Altered expression of cellular genes in neutrophils of periparturient dairy cows. *Veterinary Immunology and Immunopathology*, 86(3–4), 159–175.

Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M., and Spratt, B. G. (1998). Multilocus sequence

typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences of the United States of America*, 95(6), 3140–3145.

Maiques, E., Úbeda, C., Campoy, S., Salvador, N., Lasa, Í., Novick, R. P., Barbé, J., and Penadés, J. R. (2006). β-lactam antibiotics induce the SOS response and horizontal transfer of virulence factors in *Staphylococcus aureus*. *Journal of Bacteriology*, 188(7), 2726–2729.

Malachowa, N., and DeLeo, F. R. (2010). Mobile genetic elements of *Staphylococcus aureus*. *Cellular and Molecular Life Sciences*, 67(18), 3057–3071.

Mangili, A., Bica, I., Snydman, D. R., and Hamer, D. H. (2005). Daptomycin-resistant, methicillin resistant *Staphylococcus aureus* bacteremia. *Clinical Infectious Diseases*, 40(7), 1058–1060.

Marco-Jiménez, F., Lavara, R., Jiménez-Trigos, E., and Vicente, J. S. (2013). In vivo development of vitrified rabbit embryos: Effects of vitrification device, recipient genotype, and asynchrony. *Theriogenology*, 79(7), 1124–1129.

Martin, P., Trieu-Cuot, P., and Courvalin, P. (1986). Nucleotide sequence of the *tet*M tetracycline resistance determinant of the streptococcal conjugative shuttle transposon Tnl545. *Nucleic Acids Research*, 14(17), 7047–7058.

Massey, R. C., Kantzanou, M. N., Fowler, T., Day, N. P. J., Schofield, K., Wann, E. R., Berendt, A. R., Höök, M., and Peacock, S. J. (2001). Fibronectin-binding protein A of *Staphylococcus aureus* has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells. *Cellular Microbiology*, 3(12), 839–851.

Mat Azis, N., Pung, H. P., Abdul Rachman, A. R., Amin Nordin, S., Sarchio, S. N. E., Suhaili, Z., and Mohd Desa, M. N. (2017). A persistent antimicrobial resistance pattern and limited methicillin-resistance-associated genotype in a short-term *Staphylococcus aureus* carriage isolated from a student population. *Journal of Infection and Public Health*, 10(2), 156–164.

McAdow, M., Missiakas, D. M., and Schneewind, O. (2012). *Staphylococcus aureus* secretes coagulase and von Willebrand factor binding protein to modify the coagulation cascade and establish host infections. *Journal of Innate Immunity*, 4, 141–148.

McCaig, L. F., McDonald, L. C., Mandal, S., and Jernigan, D. B. (2006). *Staphylococcus aureus*–associated skin and soft tissue infections in ambulatory care. *Emerging Infectious Diseases*, 12(11), 1715–1723.

McCarthy, A. J., Lindsay, J. A., and Loeffler, A. (2012). Are all meticillin-resistant *Staphylococcus aureus* (MRSA) equal in all hosts? Epidemiological and genetic comparison between animal and human MRSA. *Veterinary Dermatology*, 23, 267–275. https://doi.org/10.1111/j.1365-3164.2012.01072.x

McCormick, J. K., Yarwood, J. M., and Schlievert, P. M. (2001). Toxic shock syndrome and bacterial superantigens: An update. *Annual Reviews of Microbiology*, 55, 77–104.

McDevitt, D., Francois, P., Vaudaux, P., and Foster, T. J. (1994). Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. *Molecular Microbiology*, 11(2), 237–248.

McDevitt, P., Francois, D., Vaudaux, P., and Foster, T. J. (1995). Identification of the ligand-binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus*. *Molecular Microbiology*, 16(5), 895–907.

McLoughlin, R. M., Solinga, R. M., Rich, J., Zaleski, K. J., Cocchiaro, J. L., Risley, A., Tzianabos, A. O., and Lee, J. C. (2006). CD4+ T cells and CXC chemokines modulate the pathogenesis of *Staphylococcus aureus* wound infections. *Proceedings of the National Academy of Sciences of the United States of America*, 103(27), 10408–10413.

Meehl, M., Herbert, S., Götz, F., and Cheung, A. (2007). Interaction of the *Gra*RS two-component system with the *Vra*FG ABC transporter to support vancomycin-intermediate resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 51(8), 2679–2689.

Meemken, D., Blaha, T., Hotzel, H., Strommenger, B., Klein, G., Ehricht, R., Monecke, S., and Kehrenberg, C. (2013). Genotypic and phenotypic characterization of *Staphylococcus aureus* isolates from wild boars. *Applied and Environmental Microbiology*, 79(5), 1739–1742.

Merz, A., Stephan, R., and Johler, S. (2016). Genotyping and DNA microarray based characterization of *Staphylococcus aureus* isolates from rabbit carcasses. *Meat Science*, 112, 86–89.

Milheiriço, C., Oliveira, D. C., and de Lencastre, H. (2007a). Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome *mec* type IV in methicillin-resistant *Staphylococcus aureus*: "SCC*mec* IV multiplex." *Journal of Antimicrobial Chemotherapy*, 60(1), 42–48.

Milheiriço, C., Oliveira, D. C., and de Lencastre, H. (2007b). Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 51(9), 3374–3377.

Millar, M., Wilcock, A., Sanderson, Y., Kite, P., and McDonnell, M. K. (1986). Catalase negative *Staphylococcus aureus*. *Journal of Clinical Pathology*, 39, 695.

Miller, L. G., Perdreau-Remington, F., Rieg, G., Mehdi, S., Perlroth, J., Bayer, A. S., Tang, A. W., Phung, T. O., and Spellberg, B. (2005). Necrotizing fasciitis caused by community-associated methicillin-resistant: *Staphylococcus aureus* in Los Angeles. *New England Journal of Medicine*, 352(14), 1445–1453.

Misiura, A., Pigli, Y. Z., Boyle-Vavra, S., Daum, R. S., Boocock, M. R., and Rice, P. A. (2013). Roles of two large serine recombinases in mobilizing the methicillin-resistance cassette SCC*mec*. *Molecular Microbiology*, 88(6), 1218–1229.

Monecke, S., Slickers, P., Ellington, M. J., Kearns, A. M., and Ehricht, R. (2007). High diversity of Panton-Valentine leukocidin-positive, methicillin-susceptible isolates of *Staphylococcus aureus* and implications for the evolution of community-associated methicillin-resistant *S. aureus*. *Clinical Microbiology and Infection*, 13(12), 1157–1164.

Monecke, Stefan, Gavier-Widén, D., Hotzel, H., Peters, M., Guenther, S., Lazaris, A., Loncaric, I., Müller, E., Reissig, A., Ruppelt-Lorz, A., Shore, A. C., Walter, B., Coleman, D. C., and Ehricht, R. (2016). Diversity of *Staphylococcus aureus* isolates in european wildlife. *PLoS ONE*, 11(12), e0168433.

Mongkolrattanothai, K., Boyle, S., Kahana, M. D., and Daum, R. S. (2003). Severe *Staphylococcus aureus* infections caused by clonally related community-acquired methicillin-susceptible and methicillin-resistant isolates. *Clinical Infectious Diseases*, 37(8), 1050–1058.

Moore, P. C. L., and Lindsay, J. A. (2001). Genetic variation among hospital isolates of methicillin-sensitive *Staphylococcus aureus*: Evidence for horizontal transfer of virulence genes. *Journal of Clinical Microbiology*, 39(8), 2760–2767.

Moran, G. J., Krishnadasan, A., Gorwitz, R. J., Fosheim, G. E., McDougal, L. K., Carey, R. B., and Talan, D. A. (2006). Methicillin-resistant *S. aureus* infections among patients in the emergency department. *The New England Journal of Medicine*, 355(7), 666–674.

Moreno-Grúa, E., Pérez-Fuentes, S., Muñoz-Silvestre, A., Viana, D., Fernández-Ros, A. B., Sanz-Tejero, C., Corpa, J. M., and Selva, L. (2018). Characterization of livestock-associated methicillin-resistant *Staphylococcus aureus* isolates obtained from commercial rabbitries located in the Iberian Peninsula. *Frontiers in Microbiology*, 9, 1812.

Moreno-Grúa, Elena, Pérez-Fuentes, S., Viana, D., Cardells, J., Lizana, V., Aguiló, J., Selva, L., and Corpa, J. M. (2020). Marked presence of methicillin-resistant *Staphylococcus aureus* in wild lagomorphs in Valencia, Spain. *Animals*, 10(7), 1109.

Murugesan, S., Perumal, N., Mahalingam, S. P., Dilliappan, S. K., and Krishnan, P. (2015). Analysis of antibiotic resistance genes and its associated SCC*mec* types among nasal carriage of methicillin resistant coagulase negative Staphylococci from community settings, Chennai, Southern India. *Journal of Clinical and Diagnostic Research*, 9(8), DC01–DC5.

Muñoz-Silvestre, A., Penadés, M., Selva, L., Pérez-Fuentes, S., Moreno-Grua, E., García-Quirós, A., Pascual, J. J., Arnau-Bonachera, A., Barragán, A., Corpa, J. M., and Viana, D. (2020). Pathogenesis of intradermal Staphylococcal infections: Rabbit experimental approach to natural *Staphylococcus aureus* skin infections. *American Journal of Pathology*, 190(6), 1188–1210.

Mwangi, M. M., Shang, W. W., Zhou, Y., Sieradzki, K., De Lencastre, H., Richardson, P., Bruce, D., Rubin, E., Myers, E., Siggia, E. D., and Tomasz, A. (2007). Tracking the in vivo evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. *Proceedings of the National Academy of Sciences of the United States of America*, 104(22), 9451–9456.

Naimi, T. S., LeDell, K. H., Como-Sabetti, K., Borchardt, S. M., Boxrud, D. J., Etienne, J., Johnson, S. K., Vandenesch, F., Fridkin, S., O'Boyle, C., Danila, R. N., and Lynfield, R. (2003). Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. *Journal of the American Medical Association*, 290(22), 2976–2984.

Nilsson, I. M., Lee, J. C., Bremell, T., Rydén, C., and Tarkowski, A. (1997). The role of staphylococcal polysaccharide microcapsule expression

in septicemia and septic arthritis. *Infection and Immunity*, 65(10), 4216–4221.

Nilsson, I., Patti, J. M., Bremell, T., Höök, M., and Tarkowski, A. (1998). Vaccination with a recombinant fragment of collagen adhesin provides protection against *Staphylococcus aureus* – mediated septic death. *Journal of Clinical Investigation*, 101, 2640–2649.

Novick, R. P., and Subedi, A. (2007). The SaPIs: mobile pathogenicity islands of Staphylococcus. *Chemical Immunology and Allergy*, 93, 42–57.

Novick, R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology*, 48(6), 1429–1449.

Obaidat, M. M., Bani Salman, A. E., and Roess, A. A. (2018). High prevalence and antimicrobial resistance of *mec*A *Staphylococcus aureus* in dairy cattle, sheep, and goat bulk tank milk in Jordan. *Tropical Animal Health and Production*, 50(2), 405–412.

Okuma, K., Iwakawa, K., Turnidge, J. D., Grubb, W. B., Bell, J. M., O'Brien, F. G., Coombs, G. W., Pearman, J. W., Tenover, F. C., Kapi, M., Tiensasitorn, C., Ito, T., and Hiramatsu, K. (2002). Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. *Journal of Clinical Microbiology*, 40(11), 4289–4294.

Oliveira, D. C., and De Lencastre, H. (2002). Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 46(7), 2155–2161.

Oliveira, D. C., MilheiriÇo, C., Vinga, S., and de Lencastre, H. (2006). Assessment of allelic variation in the *ccr*AB locus in methicillin-resistant *Staphylococcus aureus* clones. *Journal of Antimicrobial Chemotherapy*, 58(1), 23–30.

Oliveira, D. C., Tomasz, A., and De Lencastre, H. (2001). The evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*: Identification of two ancestral genetic backgrounds and the associated *mec* elements. *Microbial Drug Resistance*, 7(4), 349-361.

Olsen, J. E., Christensen, H., and Aarestrup, F. M. (2006). Diversity and evolution of *bla*Z from *Staphylococcus aureus* and coagulase-negative staphylococci. *Journal of Antimicrobial Chemotherapy*, 57(3), 450–460.

Pantosti, A, Sanchini, A., and Monaco, M. (2007). Microbiology of antibiotic resistance in *Staphylococcus aureus*. *Future Microbiology*, 2(3), 323–334.

Pantosti, Annalisa. (2012). Methicillin-resistant *Staphylococcus aureus* associated with animals and its relevance to human health. *Frontiers in Microbiology*, 3, 127.

Pantůček, R., Götz, F., Doškař, J., and Rosypal, S. (1996). Genomic variability of *Staphylococcus aureus* and the other coagulase-positive *Staphylococcus* species estimated by macrorestriction analysis using pulsed-field gel electrophoresis. *International Journal of Systematic Bacteriology*, 46(1), 216–222.

Parra, D., Korytář, T., Takizawa, F., and Sunyer, J. O. (2016). B cells and their role in the teleost gut. *Developmental and Comparative Immunology*, 64, 150–166.

Pascual, J. J. (2010). The role of body condition on new feeding and breeding programmes for reproductive rabbit does. *Proceedings of 22nd Hungarian Conference on Rabbit Production, Kaposvar, Hungary*, 1, 11–32.

Pascual, J. J., Savietto, D., Cervera, C., and Baselga, M. (2013). Resources allocation in reproductive rabbit does: A review of feeding and genetic strategies for suitable performance. *World Rabbit Science*, 21(3), 123–144.

Paterson, G. K., Harrison, E. M., and Holmes, M. A. (2014a). The emergence of *mec*C methicillin-resistant *Staphylococcus aureus*. *Trends in Microbiology*, 22(1), 42–47.

Paterson, G. K., Larsen, A. R., Robb, A., Edwards, G. E., Pennycott, T. W., Foster, G., Mot, D., Hermans, K., Baert, K., Peacock, S. J., Parkhill, J., Zadoks, R. N., and Holmes, M. A. (2012). The newly described *mec*A homologue, *mec*ALGA251, is present in methicillin-resistant *Staphylococcus aureus* isolates from a diverse range of host species. *Journal of Antimicrobial Chemotherapy*, 67(12), 2809–2813.

Paterson, G. K., Morgan, F. J. E., Harrison, E. M., Cartwright, E. J. P., Török, M. E., Zadoks, R. N., Parkhill, J., Peacock, S. J., and Holmes, M. A. (2014b). Prevalence and characterization of human *mec*C methicillin-resistant *Staphylococcus aureus* isolates in England. *Journal of Antimicrobial Chemotherapy*, 69(4), 907–910.

Peacock, S. J., Moore, C. E., Justice, A., Kantzanou, M., Story, L., Mackie, K., O'Neill, G., and Day, N. P. J. (2002). Virulent combinations of

adhesin and toxin genes in natural populations of *Staphylococcus aureus*. *Infection and Immunity*, 70(9), 4987–4996.

Penadés, M., Arnau-Bonachera, A., García-Quirós, A., Viana, D., Selva, L., Corpa, J. M., and Pascual, J. J. (2017). Long-term implications of feed energy source in different genetic types of reproductive rabbit females. II. Immunologic status. *Animal*, 12(9), 1877–1885.

Penadés, M., Viana, D., García-Quirós, A., Muñoz-Silvestre, A., Moreno-Grua, E., Pérez-Fuentes, S., Pascual, J. J., Corpa, J. M., and Selva, L. (2020). Differences in virulence between the two more prevalent *Staphylococcus aureus* clonal complexes in rabbitries (CC121 and CC96) using an experimental model of mammary gland infection. *Veterinary Research*, 51(1), 11.

Pérez-Fuentes, S. (2019). Study about two challenges of rabbit production: characterization of recent *Staphylococcus aureus* outbreaks and assessment of the health of rabbit does (*Oryctolagus cuniculus*) in five different housing systems (Tesis doctoral). Universidad CEU Cardenal Herrera.

Perfetto, S. P., Chattopadhyay, P. K., and Roederer, M. (2004). Seventeen-colour flow cytometry: Unravelling the immune system. *Nature Reviews Immunology*, 4(8), 648–655.

Perry, J. D., Davies, A., Butterworth, L. A., Hopley, A. L. J., Nicholson, A., and Gould, F. K. (2004). Development and evaluation of a chromogenic agar medium for methicillin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology*, 42(10), 4519–4523.

Pesavento, G., Ducci, B., Comodo, N., and Lo Nostro, A. (2007). Antimicrobial resistance profile of *Staphylococcus aureus* isolated from raw meat: A research for methicillin resistant *Staphylococcus aureus* (MRSA). *Food Control*, 18(3), 196–200.

Petinaki, E., and Spiliopoulou, I. (2012). Methicillin-resistant *Staphylococcus aureus* among companion and food-chain animals: impact of human contacts. *Clinical Microbiology and Infection*, 18(7), 626–634.

Piles, M., and Blasco, A. (2003). Response to selection for growth rate in rabbits estimated by using a control cryopreserved population. *World Rabbit Science*, 11, 53–62.

Pinho, M. G., de Lencastre, H., and Tomasz, A. (2001). An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. *Proceedings of the National Academy of Sciences of the United States of America*, 98(19), 10886–10891.

Poddar, S. K., and McClelland, M. (1991). Restriction fragment fingerprint and genome sizes of *Staphylococcus* species using pulsed-field gel electrophoresis and infrequent cleaving enzymes. *DNA and Cell Biology*, 10(9), 663–669.

Porrero, M. C. (2014a). Detección y caracterización de *Staphylococcus aureus* procedentes de animales y aguas (Tesis doctoral). Universidad Complutense de Madrid.

Porrero, M. C., Harrison, E., Fernández-Garayzábal, J. F., Paterson, G. K., Díez-Guerrier, A., Holmes, M. A., and Domínguez, L. (2014b). Detection of *mec*C-Methicillin-resistant *Staphylococcus aureus* isolates in river water: A potential role for water in the environmental dissemination. *Environmental Microbiology Reports*, 6(6), 705–708.

Porrero, M. C., Mentaberre, G., Sánchez, S., Fernández-Llario, P., Casas-Díaz, E., Mateos, A., Vidal, D., Lavín, S., Fernández-Garayzábal, J. F., and Domínguez, L. (2014c). Carriage of *Staphylococcus aureus* by free-living wild animals in Spain. *Applied and Environmental Microbiology*, 80(16), 4865–4870.

Porrero, M. C., Valverde, A., Fernández-Llario, P., Díez-Guerrier, A., Mateos, A., Lavín, S., Cantón, R., Fernández-Garayzabal, J. F., and Domínguez, L. (2014d). *Staphylococcus aureus* carrying *mec*C gene in animals and urban wastewater, Spain. *Emerging Infectious Diseases*, 20(5), 899–901.

Prevost, G., Couppie, P., Prevost, P., Gayett, S., Petiaut, P., Cribier, B., Monteil, H., and Piemont, Y. (1995). Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. *Journal of Medical Microbiology*, 42, 237–245.

Price, L. B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, P. S., Pearson, T., Walters, A. E., Foster, J. T., Schupp, J., Gillece, J., Driebe, E., Liu, C. M., Springer, B., Zdovc, I., Battisti, A., Franco, A., Zmudzki, J., Schwarz, S., Butaye, P., Jouy, E., Pomba, C., Porrero, M. C., Ruimy, R., Smith, T. C., Robinson, D. A., Weese, J. S., Arriola, C. S., Yu, F., Laurent, F., Keim, P., Skov, R., Aarestrup, F. M. (2012). *Staphylococcus aureus* CC398: Host adaptation and emergence of methicillin resistance in livestock. *MBio*, 3(1), e00305-11.

Puon Peláez, X.-H., McEwan, N. R., and Olvera-Ramírez, A. M. (2018). Epizootic rabbit enteropathy (ERE): A review of current knowledge. *European Scientific Journal*, 14(36), 137–149.

Quinn, M. T., Ammons, M. C. B., and DeLeo, F. R. (2006). The expanding role of NADPH oxidases in health and disease: No longer just agents of death and destruction. *Clinical Science*, 111(1), 1–20.

Rahi, A., Kazemeini, H., Jafariaskari, S., Seif, A., Hosseini, S., and Dehkordi, F. S. (2020). Genotypic and phenotypic-based assessment of antibiotic resistance and profile of Staphylococcal cassette chromosome *mec* in the methicillin-resistant *Staphylococcus aureus* recovered from raw milk. *Infection and Drug Resistance*, 13, 273–283.

Rall, W. F., Schmidt, P. M., Lin, X., Brown, S. S., Ward, A. C., and Hansen, C. T. (2000). Factors affecting the efficiency of embryo cryopreservation and rederivation of rat and mouse models. *ILAR Journal*, 41(4), 221–227.

Rao, Q., Shang, W., Hu, X., and Rao, X. (2015). *Staphylococcus aureus* ST121: A globally disseminated hypervirulent clone. *Journal of Medical Microbiology*, 64(12), 1462–1473.

Rashwan, A. A., and Marai, I. F. M. (2000). Mortality in young rabbits: a review. *World Rabbit Science*, 8(3), 111–124.

Rauw, W M, Kanis, E., Noordhuizen-Stassen, E. N., and Grommers, F. J. (1998). Undesirable side effects of selection of high production efficiency in farm animals: a review. *Livestock Production Science*, 56(1), 15–33.

Rauw, Wendy M. (2012). Immune response from a resource allocation perspective. *Frontiers in Genetics*, 3, 1–14.

Reinoso, E., Bettera, S., Frigerio, C., DiRenzo, M., Calzolari, A., and Bogni, C. (2004). RAPD-PCR analysis of *Staphylococcus aureus* strains isolated from bovine and human hosts. *Microbiological Research*, 159(3), 245–255.

Rennermalm, A., Li, Y., Bohaufs, L., Jarstrand, C., Brauner, A., Brennan, F. R., and Flock, J.-I. (2001). Antibodies against a truncated *Staphylococcus aureus* fibronectin-binding protein protect against dissemination of infection in the rat. *Vaccine*, 19, 3376–3383.

Reynolds, P. E., and Brown, D. F. J. (1985). Penicillin-binding proteins of β-lactam-resistant strains of *Staphylococcus aureus*. Effect of growth conditions. *FEBS Letters*, 192(1), 28–32.

Rigby, K. M., and DeLeo, F. R. (2012). Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Seminars in Immunopathology*, 34(2), 237–259.

Robinson, D. A., and Enright, M. C. (2004). Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. *Clinical Microbiology and Infection*, 10(2), 92–97.

Robinson, D. Ashley, and Enright, M. C. (2003). Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 47(12), 3926–3934.

Rooijakkers, S. H. M., van Wamel, W. J. B., Ruyken, M., van Kessel, K. P. M., and van Strijp, J. A. G. (2005). Anti-opsonic properties of staphylokinase. *Microbes and Infection*, 7, 476–484.

Rosell, J. M. (1996). Situación actual y perspectivas de la Cunicultura. In C.Buxadé Zootecnia. Bases de la Produccidn Animal. Ediciones Mundi-Prensa, Madrid, 13-28.

Rosell, J. M., and de la Fuente, L. F. (2009). Culling and mortality in breeding rabbits. *Preventive Veterinary Medicine*, 88(2), 120–127.

Rozalska, B., and Wadström, T. (1993). Protective opsonic activity of antibodies against fibronectin-binding proteins (FnBPs) of *Staphylococcus aureus*. *Scandinavian Journal of Immunology*, 37, 575–580.

Ruiz-Ripa, L., Alcalá, L., Simón, C., Gómez, P., Mama, O. M., Rezusta, A., Zarazaga, M., and Torres, C. (2019a). Diversity of *Staphylococcus aureus* clones in wild mammals in Aragon, Spain, with detection of MRSA ST130-*mec*C in wild rabbits. *Journal of Applied Microbiology*, 127(1), 284–291.

Ruiz-Ripa, L., Gómez, P., Andrea Alonso, C., Camacho, M. C., de la Puente, J., Fernández-Fernández, R., Ramiro, Y., Quevedo, M. A., Blanco, J. M., Zarazaga, M., Höfle, U., Torres, C. (2019b). Detection of MRSA of lineages CC130-*mec*C and CC398-*mec*A and *Staphylococcus delphini*-*lnu*(A) in magpies and cinereous vultures in Spain. *Microbial Ecology*, 78(2), 409-415.

Sabat, A., Melles, D. C., Martirosian, G., Grundmann, H., van Belkum, A., and Hryniewicz, W. (2006). Distribution of the serine-aspartate repeat protein-encoding *sdr* genes among nasal-carriage and invasive *Staphylococcus aureus* strains. *Journal of Clinical Microbiology*, 44(3), 1135–1138.

Saïd-Salim, B., Mathema, B., and Kreiswirth, B. N. (2003). Community-acquired methicillin-resistant *Staphylococcus aureus*: An emerging pathogen in orthopaedics. *Infection Control and Hospital Epidemiology*, 24(6), 451–455.

Sanford, M. D., Widmer, A. F., Bale, M. J., Jones, R. N., and Wenzel, R. P. (1994). Efficient detection and long-term persistence of the carriage of methicillin-resistant *Staphylococcus aureus*. *Clinical Infectious Diseases*, 19(6), 1123–1128.

Schmitz, F.-J., Krey, A., Sardurski, R., Verhoef, J., Milatovid, D., and Fluit, A. C. (2001). Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. *Journal of Antimicrobial Chemotherapy*, 47, 239–240.

Schnellmann, C., Gerber, V., Rossano, A., Jaquier, V., Panchaud, Y., Doherr, M. G., Thomann, A., Straub, R., and Perreten, V. (2006). Presence of new *mec*A and *mph* (C) variants conferring antibiotic resistance in *Staphylococcus spp.* isolated from the skin of horses before and after clinic admission. *Journal of Clinical Microbiology*, 44(12), 4444–4454.

Schwartz, D. C., and Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*, 37(1), 67–75.

Schwarz, S., Kehrenberg, C., Doublet, B., and Cloeckaert, A. (2004). Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiology Reviews*, 28(5), 519–542.

Segura, P., Martinez, J., Peris, B., Selva, L., Viana, D., Penades, J. R., and Corpa, J. M. (2007). Staphylococcal infections in rabbit does on two industrial farms. *Veterinary Record*, 160(25), 869–873.

Selva, L., Viana, D., and Corpa, J. M. (2015). *Staphylococcus aureus* nasal carriage could be a risk for development of clinical infections in rabbits. *World Rabbit Science*, 23(3), 181–184.

Severin, A., Wu, S. W., Tabei, K., and Tomasz, A. (2005). High-level beta-lactam resistance and cell wall synthesis catalyzed by the *mec*A homologue of *Staphylococcus sciuri* introduced into *Staphylococcus aureus*. *Journal of Bacteriology*, 187(19), 6651–6658.

Shannon, K., and Phillips, I. (1982). Mechanisms of resistance to aminoglycosides in clinical isolates. *Journal of Antimicrobial Chemotherapy*, 9(2), 91–102.

Shaw, K. J., Rather, P. N., Hare, R. S., and Miller, G. H. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiological Reviews*, 57(1), 138–163.

Shopsin, B., Gomez, M., Montgomery, S. O., Smith, D. H., Waddington, M., Dodge, D. E., Bost, D. A., Riehman, M., Naidich, S., and

Kreiswirth, B. N. (1999). Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *Journal of Clinical Microbiology*, 37(11), 3556–3563.

Shore, A. C., Deasy, E. C., Slickers, P., Brennan, G., O'Connell, B., Monecke, S., Ehricht, R., and Coleman, D. C. (2011a). Detection of staphylococcal cassette chromosome *mec* type XI carrying highly divergent *mec*A, *mec*I, *mec*R1, *bla*Z, and *ccr* genes in human clinical isolates of clonal complex 130 methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 55(8), 3765–3773.

Shore, A. C., Rossney, A. S., Brennan, O. M., Kinnevey, P. M., Humphreys, H., Sullivan, D. J., Goering, R. V, Ehricht, R., Monecke, S., and Coleman, D. C. (2011b). Characterization of a novel arginine catabolic mobile element (ACME) and staphylococcal chromosomal cassette *mec* composite island with significant homology to *Staphylococcus epidermidis* ACME type II in methicillin-resistant *Staphylococcus aureus* genotype. *Antimicrobial Agents and Chemotherapy*, 55(5), 1896–1905.

Shurland, S. M., Stine, O. C., Venezia, R. A., Johnson, J. K., Zhan, M., Furuno, J. P., Miller, R. R., Johnson, T., and Roghmann, M.-C. (2009). Colonization sites of USA300 methicillin-resistant *Staphylococcus aureus* in residents of extended care facilities. *Infection Control and Hospital Epidemiology*, 30(4), 313–318.

Siboo, I. R., Cheung, A. L., Bayer, A. S., and Sullam, P. M. (2001). Clumping factor A mediates binding of *Staphylococcus aureus* to human platelets. *Infection and Immunity*, 69(5), 3120–3127.

Siegel, P. B., and Honaker, C. F. (2009). Impact of genetic selection for growth and immunity on resource allocations. *Journal of Applied Poultry Research*, 18(1), 125–130.

Siemsen, D. W., Malachowa, N., Schepetkin, I. A., Whitney, A. R., Kirpotina, L. N., Lei, B., DeLeo, F. R., and Quinn, M. T. (2014). Neutrophil isolation from nonhuman species. *Methods in Molecular Biology*, 1124, 19–37.

Sieweke, M. H., and Allen, J. E. (2013). Beyond stem cells: Self-renewal of differentiated macrophages. *Science*, 342, 1242974.

Simpson, K. H., Bowden, G., Höök, M., and Anvari, B. (2003). Measurement of adhesive forces between individual *Staphylococcus aureus* MSCRAMMs and protein-coated surfaces by use of optical tweezers. *Journal of Bacteriology*, 185(6), 2031–2035.

Simpson, V. R., Davison, N. J., Kearns, A. M., Pichon, B., Hudson, L. O., Koylass, M., Blackett, T., Butler, H., Rasigade, J. P., and Whatmore, A. M. (2013). Association of a *luk* M-positive clone of *Staphylococcus aureus* with fatal exudative dermatitis in red squirrels (*Sciurus vulgaris*). *Veterinary Microbiology*, 162(2–4), 987–991.

Smith, T. C. (2015). Livestock-associated *Staphylococcus aureus*: The United States experience. *PLoS Pathogens*, 11(2), e1004564.

Somerville, G. A., and Proctor, R. A. (2009). The Biology of Staphylococci. In The staphylococci in human disease (pp. 1–18). Blackwell Publishing Ltd.

Sordillo, L. M., Contreras, G. A., and Aitken, S. L. (2009). Metabolic factors affecting the inflammatory response of periparturient dairy cows. *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases*, 10(1), 53–63.

Spoor, L. E., McAdam, P. R., Weinert, L. A., Rambaut, A., Hasman, H., Aarestrup, F. M., Kearns, A. M., Larsen, A. R., Skov, R. L., and Fitzgerald, J. R. (2013). Livestock origin for a human pandemic clone of community- associated methicillin-resistant *Staphylococcus aureus*. *MBio*, 4, e00356-13.

Sreedharan, S., Oram, M., Jensen, B., Peterson, L. R., and Fisher, L. M. (1990). DNA gyrase *gyr*A mutations in ciprofloxacin-resistant strains of *Staphylococcus aureus*: Close similarity with quinolone resistance mutations in *Escherichia coli*. *Journal of Bacteriology*, 172(12), 7260–7262.

Stegger, M., Liu, C. M., Larsen, J., Soldanova, K., Aziz, M., Contente-Cuomo, T., Petersen, A., Vandendriessche, S., Jiménez, J. N., Mammina, C., Van Belkum, A., Salmenlinna, S., Laurent, F., Skov, R. L., Larsen, A. R., Andersen, P. S., and Price, L. B. (2013). Rapid differentiation between livestock-associated and livestock-independent *Staphylococcus aureus* CC398 clades. *PLoS ONE*, 8(11), e79645.

Stephens, A. J., Huygens, F., and Giffard, P. M. (2007). Systematic derivation of marker sets for staphylococcal cassette chromosome *mec* typing. *Antimicrobial Agents and Chemotherapy*, 51(8), 2954–2964.

Stone, K. J., and Strominger, J. L. (1971). Mechanism of action of bacitracin: Complexation with metal ion and C55-Isoprenyl pyrophosphate. *Proceedings of the National Academy of Sciences of the United States of America*, 68(12), 3223–3227.

Swenson, J. M., Tenover, F. C., and Group Cefoxitin Disk Study. (2005). Results of disk diffusion testing with cefoxitin correlate with presence of *mec*A in *Staphylococcus spp*. *Journal of Clinical Microbiology*, 43(8), 3818–3823.

Taherirad, A., Jahanbakhsh, R., Shakeri, F., Anvary, S., and Ghaemi, E. A. (2016). Staphylococcal cassette chromosome *mec* types among methicillin-resistant *Staphylococcus aureus* in northern Iran. *Jundishapur Journal of Microbiology*, 9, e33933.

Takeuchi, O., and Akira, S. (2010). Pattern Recognition Receptors and Inflammation. *Cell*, 140(6), 805–820.

Tallent, S. M., Langston, T. B., Moran, R. G., and Christie, G. E. (2007). Transducing particles of *Staphylococcus aureus* pathogenicity island SaPI1 are comprised of helper phage-encoded proteins. *Journal of Bacteriology*, 189(20), 7520–7524.

Tegmark, K., Morfeldt, E., and Arvidson, S. (1998). Regulation of *agr*-dependent virulence genes in *Staphylococcus aureus* by RNAIII from coagulase-negative staphylococci. *Journal of Bacteriology*, 180(12), 3181–3186.

Thomas, C. M., and Nielsen, K. M. (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews*, 3(9), 711–721.

Thomas, D., Chou, S., Dauwalder, O., and Lina, G. (2007). Diversity in *Staphylococcus aureus* enterotoxins. *Chemical Immunology and Allergy*, 93(1), 24–41.

Tormo, M. Á., Ferrer, M. D., Maiques, E., Úbeda, C., Selva, L., Lasa, Í., Calvete, J. J., Novick, R. P., and Penadés, J. R. (2008). *Staphylococcus aureus* pathogenicity island DNA is packaged in particles composed of phage proteins. *Journal of Bacteriology*, 190(7), 2434–2440.

Torres, C., Esteve, C., Nieto, M. P., Burgui, J. M. (2019). Estadísticas cinegéticas de la Comunitat Valenciana. Memoria 2018. Temporada 2017/2018 [Hunting statistics of the Valencian Community. Annual Report 2018. 2017/2018 Season]. Hunting and Fishing Service; Valencia Region Government: Valencia, Spain, 2019.

Traber, K. E., Lee, E., Benson, S., Corrigan, R., Cantera, M., Shopsin, B., and Novick, R. P. (2016). *agr* function in clinical *Staphylococcus aureus* isolates. *Microbiology* (Reading), 154(8), 2265–2274.

Trad, S., Allignet, J., Frangeul, L., Davi, M., Vergassola, M., Couve, E., Morvan, A., Kechrid, A., Buchrieser, C., Glaser, P., and Solh, N. El. (2004). DNA macroarray for identification and typing of *Staphylococcus aureus* isolates. *Journal of Clinical Microbiology*, 42(5), 2054–2064.

Traversa, A., Gariano, G. R., Gallina, S., Bianchi, D. M., Orusa, R., Domenis, L., Cavallerio, P., Fossati, L., Serra, R., and Decastelli, L. (2015). Methicillin resistance in *Staphylococcus aureus* strains isolated from food and wild animal carcasses in Italy. *Food Microbiology*, 52, 154–158.

Trzcinski, K., Cooper, B. S., Hryniewicz, W., and Dowson, C. G. (2000). Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, 45, 763–770.

Tsiodras, S., Gold, H. S., Sakoulas, G., Eliopoulos, G. M., Wennersten, C., Venkataraman, L., Moellering, R. C., and Ferraro, M. J. (2001). Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. *The Lancet*, 358(9277), 207–208.

Tsubakishita, S., Kuwahara-Arai, K., Baba, T., and Hiramatsu, K. (2010). Staphylococcal cassette chromosome *mec*-like element in *Macrococcus caseolyticus*. *Antimicrobial Agents and Chemotherapy*, 54(4), 1469–1475.

Tzianabos, A. O., Wang, J. Y., and Lee, J. C. (2001). Structural rationale for the modulation of abscess formation by *Staphylococcus aureus* capsular polysaccharides. *Proceedings of the National Academy of Sciences of the United States of America*, 98(16), 9365–9370.

Úbeda, C., Maiques, E., Knecht, E., Lasa, Í., Novick, R. P., and Penadés, J. R. (2005). Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. *Molecular Microbiology*, 56(3), 836–844.

Urushibara, N., Aung, M. S., Kawaguchiya, M., and Kobayashi, N. (2020). Novel staphylococcal cassette chromosome *mec* (SCC *mec*) type XIV (5A) and a truncated SCC *mec* element in SCC composite islands carrying *spe*G in ST5 MRSA in Japan. *Journal of Antimicrobial Chemotherapy*, 75, 46–50.

van Wamel, W. J. B., Rooijakkers, S. H. M., Ruyken, M., van Kessel, K. P. M., and van Strijp, J. A. G. (2006). The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-

converting bacteriophages. *Journal of Bacteriology*, 188(4), 1310–1315.

Vancraeynest, D, Haesebrouck, F., Deplano, A., Denis, O., Godard, C., Wildemauwe, C., and Hermans, K. (2006). International dissemination of a high virulence rabbit *Staphylococcus aureus* clone. *Journal of Veterinary Medicine*, 53, 418–422.

Vancraeynest, Dieter, Hermans, K., and Haesebrouck, F. (2004). Genotypic and phenotypic screening of high and low virulence *Staphylococcus aureus* isolates from rabbits for biofilm formation and MSCRAMMs. *Veterinary Microbiology*, 103(3–4), 241–247.

Vandendriessche, S., Vanderhaeghen, W., Soares, F. V., Hallin, M., Catry, B., Hermans, K., Butaye, P., Haesebrouck, F., Struelens, M. J., and Denis, O. (2013). Prevalence, risk factors and genetic diversity of methicillin-resistant *Staphylococcus aureus* carried by humans and animals across livestock production sectors*. Journal of Antimicrobial Chemotherapy*, 68(7), 1510–1516.

Vandenesch, F., Lina, G., and Thomas, H. (2012). *Staphylococcus aureus* hemolysins, bi-component leukocidins, and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors? *Frontiers in Cellular and Infection Microbiology*, 2(12), 1–15.

Vanderhaeghen, W., Hermans, K., Haesebrouck, F., and Butaye, P. (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) in food production animals. *Epidemiology and Infection*, 138, 606–625.

Vautor, E., Abadie, G., Guibert, J. M., Chevalier, N., and Pépin, M. (2005). Nasal carriage of *Staphylococcus aureus* in dairy sheep. *Veterinary Microbiology*, 106(3–4), 235–239.

Veerkamp, R. F., Windig, J. J., Calus, M. P. L., Ouweltjes, W., De Haas, Y., and Beerda, B. (2009). Selection for high production in dairy cattle. In Rauw, W. (Ed.) Resource allocation theory applied to farm animal production.CABI. Wallingford, Oxfordshire. UK.

Verkade, E., and Kluytmans, J. (2014). Infection, genetics and evolution livestock-associated *Staphylococcus aureus* CC398: Animal reservoirs and human infections. *Infection, Genetics and Evolution*, 21, 523–530.

Vestergaard, M., Leng, B., Haaber, J., Bojer, M. S., Vegge, C. S., and Ingmer, H. (2016). Genome-wide identification of antimicrobial intrinsic resistance determinants in *Staphylococcus aureus*. *Frontiers in Microbiology*, 7, 1–10.

Viana, D., Comos, M., McAdam, P. R., Ward, M. J., Selva, L., Guinane, C. M., González-Muñoz, B. M., Tristan, A., Foster, S. J., Fitzgerald, J. R., and Penadés, J. R. (2015a). A single natural nucleotide mutation alters bacterial pathogen host tropism. *Nature Genetics*, 47, 361–366.

Viana, D., Selva, L., Callanan, J. J., Guerrero, I., Ferrian, S., and Corpa, J. M. (2011). Strains of *Staphylococcus aureus* and pathology associated with chronic suppurative mastitis in rabbits. *The Veterinary Journal*, 190, 403–407.

Viana, D., Selva, L., Penadés, M., and Corpa, J. M. (2015b). Screening of virulence genes in *Staphylococcus aureus* isolates from rabbits. *World Rabbit Science*, 23(3), 185–195.

Viana, D., Selva, L., Segura, P., Penadés, J. R., and Corpa, J. M. (2007). Genotypic characterization of *Staphylococcus aureus* strains isolated from rabbit lesions. *Veterinary Microbiology*, 121(3–4), 288–298.

Vicente, J. S., Llobat, L., Viudes-de-Castro, M. P., Lavara, R., Baselga, M., and Marco-Jiménez, F. (2012). Gestational losses in a rabbit line selected for growth rate. *Theriogenology*, 77(1), 81–88.

Vicente, José Salvador, Viudes-de-Castro, M. P., García, M. de la L., and Baselga, M. (2003). Effect of rabbit line on a program of cryopreserved embryos by vitrification. *Reproduction Nutrition Development*, 43, 137–143.

von Eiff, C., Becker, K., Machka, K., Stammer, H., and Peters, G. (2001). Nasal carriage as a source of *Staphylococcus aureus* bacteremia. *The New England Journal of Medicine*, 344(1), 11–16.

Voyich, J. M., Braughton, K. R., Sturdevant, D. E., Whitney, A. R., Saïd-Salim, B., Porcella, S. F., Long, R. D., Dorward, D. W., Gardner, D. J., Kreiswirth, B. N., Musser, J. M., and DeLeo, F. R. (2005). Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *The Journal of Immunology*, 175, 3907–3919.

Vuong, C., Kocianova, S., Voyich, J. M., Yao, Y., Fischer, E. R., DeLeo, F. R., and Otto, M. (2004a). A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *Journal of Biological Chemistry*, 279(52), 54881–54886.

Vuong, C., Voyich, J. M., Fischer, E. R., Braughton, K. R., Whitney, A. R., DeLeo, F. R., and Otto, M. (2004b). Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major

components of the human innate immune system. *Cellular Microbiology*, 6(3), 269–275.

Walev, I., Weller, U., Strauch, S., Foster, T., and Bhakdi, S. (1996). Selective killing of human monocytes and cytokine release provoked by sphingomyelinase (beta-toxin) of *Staphylococcus aureus*. *Infection and Immunity*, 64(8), 2974–2979.

Wang, L., and Archer, G. L. (2010). Roles of *Ccr*A and *Ccr*B in excision and integration of staphylococcal cassette chromosome *mec*, a *Staphylococcus aureus* genomic island. *Journal of Bacteriology*, 192(12), 3204–3212.

Weese, J. S., Dick, H., Willey, B. M., McGeer, A., Kreiswirth, B. N., Innis, B., and Low, D. E. (2006). Suspected transmission of methicillin-resistant *Staphylococcus aureus* between domestic pets and humans in veterinary clinics and in the household. *Veterinary Microbiology*, 115(1–3), 148–155.

Weese, J. Scott, and Lefebvre, S. L. (2007). Risk factors methicillin-resistant *Staphylococcus aureus* colonization in horses admitted to a veterinary teaching hospital. *Canadian Veterinary Journal*, 48(9), 921–926.

Weese, J Scott. (2010). Methicillin-resistant *Staphylococcus aureus* in animals. *ILAR Journal*, 51(3), 233–244.

Weisblum, B. (1995). Erythromycin resistance by ribosome modification. *Antimicrobial Agents and Chemotherapy*, 39(3), 577–585.

Weissman, I. L., Anderson, D. J., and Gage, F. (2001). Stem and progenitor cells: Origins, and transdifferentiations. *Cell and Develeopmental Biology*, 17, 387–403.

Wells, M. Y., Decobecq, C. P. M., Decouvelaere, D. M., Justice, C., and Guittin, P. (1999). Changes in clinical pathology parameters during gestation in the new zealand white rabbit. *Toxicologic Pathology*, 27(3), 370–379.

Wertheim, H. F. L., Melles, D. C., Vos, M. C., van Leeuwen, W., van Belkum, A., Verbrugh, H. A., and Nouwen, J. L. (2005). The role of nasal carriage in *Staphylococcus aureus* infections. *The Lancet*, 5, 751–762.

Wertheim, H. F. L., Vos, M. C., Ott, A., van Belkum, A., Voss, A., Kluytmans, J. A. J. W., van Keulen, P. H. J., Vandenbroucke-Grauls, C. M. J. E., Meester, M. H. M., and Verbrugh, H. A. (2004). Risk and outcome of nosocomial *Staphylococcus aureus*

bacteraemia in nasal carriers versus non-carriers. *The Lancet*, 364, 703–705.

Wideman, R. F., Rhoads, D. D., Erf, G. F., and Anthony, N. B. (2013). Pulmonary arterial hypertension (ascites syndrome) in broilers: A review. *Poultry Science*, 92(1), 64–83.

Wiesenthal, A. M., Ressman, M., Caston, S. A., and Todd, J. K. (1985). Toxic shock syndrome. *Americal Journal of Epidemiology*, 122(5), 847–856.

Williams, R. E. O. (1963). Healthy carriage of *Staphylococcus aureus*: its prevalence and importance. *Bacteriological Reviews*, 27, 56–71.

Witte, W., Strommenger, B., and Werner, W. (2014). Diagnostics, typing, and taxonomy. In Gram-positive pathogens (pp. 371–380).

Witte, Wolfgang, Strommenger, B., Stanek, C., and Cuny, C. (2007). Methicillin-resistant *Staphylococcus aureus* ST398 in humans and animals, central Europe. *Emerging Infectious Diseases*, 13(2), 255–258.

Wolz, C., McDevitt, D., Foster, T. J., and Cheung, A. L. (1996). Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* newman. *Infection and Immunity*, 64(8), 3142–3147.

Wu, S., Piscitelli, C., de Lencastre, H., and Tomasz, A. (1996). Tracking the evolutionary origin of the methicillin resistance gene: Cloning and sequencing of a homologue of *mec*A from a methicillin susceptible strain of *Staphylococcus sciuri*. *Microbial Drug Resistance*, 2(4), 435–441.

Wu, Z., Li, F., Liu, D., Xue, H., and Zhao, X. (2015). Novel type XII staphylococcal cassette chromosome *mec* harboring a new cassette chromosome recombinase, *Ccr*C2. *Antimicrobial Agents and Chemotherapy*, 59(12), 7597–7601.

Xing, J., Luo, K., Xiao, Y., Tang, X., and Zhan, W. (2018). Influence of CD4-1+, CD4-2+ and CD8+ T lymphocytes subpopulations on the immune response of B lymphocytes in flounder (*Paralichthys olivaceus*) immunized with thymus-dependent or thymus-independent antigen. *Fish and Shellfish Immunology*, 84, 979–986.

Xing, J., Xiao, Y., Tang, X., Sheng, X., and Zhan, W. (2017). Inhibition of Cyclosporine A or rapamycin on T lymphocyte counts and the influence on the immune responses of B lymphocytes in flounder (*Paralichthys olivaceus*). *Fish and Shellfish Immunology*, 66, 78–85.

Xiong, Y., and Bosselut, R. (2012). CD4-CD8 differentiation in the thymus: connecting circuits and building memories. *Current Opinion in Immunology*, 24(2), 139–145.

Xue, H., Lu, H., and Zhao, X. (2011). Sequence diversities of serine-aspartate repeat genes among *Staphylococcus aureus* isolates from different hosts presumably by horizontal gene transfer. *PLoS ONE*, 6(5), e20332.

Xue, H., Wu, Z., Li, L., Li, F., Wang, Y., and Zhao, X. (2015). Coexistence of heavy metal and antibiotic resistance within a novel composite staphylococcal cassette chromosome in a *Staphylococcus haemolyticus* isolate from bovine mastitis milk. *Antimicrobial Agents and Chemotherapy*, 59(9), 5788–5792.

Yamane, K., and Leung, K. P. (2016). Rabbit M1 and M2 macrophages can be induced by human recombinant GM-CSF and M-CSF. *FEBS Open Bio*, 6(9), 945–953.

Yarwood, J. M., McCormick, J. K., Paustian, M. L., Orwin, P. M., Kapur, V., and Schlievert, P. M. (2002). Characterization and expression analysis of *Staphylococcus aureus* pathogenicity island 3. *Journal of Biological Chemistry*, 277(15), 13138–13147.

Yoshida, H., Bogaki, M., Nakamura, S., Ubukata, K., and Konno, M. (1990). Nucleotide sequence and characterization of the *Staphylococcus aureus nor*A gene, which confers resistance to quinolones. *Journal of Bacteriology*, 172(12), 6942–6949.

Yoshida, Y., Matsuo, M., Oogai, Y., Kato, F., Nakamura, N., Sugai, M., and Komatsuzawa, H. (2011). Bacitracin sensing and resistance in *Staphylococcus aureus*. *FEMS Microbiology Letters*, 320(1), 33–39.

Zetola, N., Francis, J. S., Nuermberger, E. L., and Bishai, W. R. (2005). Community-acquired meticillin-resistant *Staphylococcus aureus*: An emerging threat. *The Lancet Infectious Diseases*, 5, 275–286.

Zhang, K., McClure, J. A., Elsayed, S., Louie, T., and Conly, J. M. (2005). Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology*, 43(10), 5026–5033.

Zinner, S. H., and Mayer, K. H. (2000). Sulfonamides and trimethoprim. In Mandell GL, Bennett JE, Dolin R, eds. Principles and practice of infectious diseases (pp. 394–404). New York: Churchill Livingstone.