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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

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“Yo soy optimista por naturaleza,
Creo que la vida vale la pena,
Que las cosas saldrán adelante.
[…]
Pero me interesa mucho también el concepto de "fracaso",
Porque tiene que ver con intentar algo,
Con haber probado, con aproximarse a algo.
La ciencia no progresaría sin la noción de fracaso.
Sin dudas, vuelvo a repetir:
Entre hacer o no hacer, haz siempre”.

Falsalarma. “Oro y arena”. 2019.

ABSTRACT

Rabbit production faces a wide variety of challenges today, being staphylococcal infections and welfare of rabbit does two of the most important ones. In rabbits, *Staphylococcus aureus* is a bacterium that mainly produces mastitis, pododermatitis, abscesses and pyoderma, generating numerous economic losses in the affected farms. But the population of *S. aureus* is very heterogeneous, and it is necessary to perform a typing to characterize the strains present in each farm and to help its prevention and treatment. The most frequent clones in rabbitries were ST121 (high virulent strains) and ST96 (low virulent strains). However, in recent years, veterinarians and farmers have expressed their concern about the emergence of more virulent and persistent outbreaks. Considering that *S. aureus* has a great adaptation capacity, it was suggested that the clones could have changed or acquired new virulence factors or even new clones could have appeared, causing this increase in the number and virulence of the outbreaks in rabbit farms. Therefore, the objectives of the first part of this thesis were to characterize genetically the isolated strains from rabbit farms, to study their geographical distribution and to test *in vivo* some of these strains to verify whether the observed genetic changes correlated with a greater infective capacity. A new clone was detected, the ST3764, reaching the 19% of all isolates. This clone is a variant of the ST121, which was detected for the first time in 2014 in the northwest of the Iberian Peninsula and later it spread to the east. Similarly, ST2855 strains, a variant of ST96 clone, were isolated from staphylococcal infections throughout the whole Iberian Peninsula. Therefore, the percentage of strains belonging to clone ST121 suffered a decrease as a result of a high number of ST3764 and ST2855 strains. In addition, new clones not described previously in rabbits in Spain have also isolated, such as ST1, ST146 and ST398. The complete sequencing of these new clones has evidenced the acquisition of mobile genetic elements, with the exception of ST2855 strains. Most of them had lost the phage with type 3 integrase, which not also carried the human IEC with the genes *sak*, *chp* and *scn*, but also truncated the sequence of β-

haemolysin, causing these strains to express it. Finally, mutations in the *dltb* gene were found between strains ST96 and ST2855. All these changes could suggest an increase in virulence of ST2855 strains compared to ST96, but ST2855 strains were not able to infect either. In contrast, ST3764 strains infected a greater number of animals than their predecessor ST121, although it was not a significant increase. Therefore, the dissemination of ST2855 strains could be due to genetic changes not detected here or due to other factors extrinsic to the strains.

The presence of *S. aureus* in the farms is closely related to the second challenge studied in this thesis, since the lesions produced by this bacterium greatly reduce animal welfare. In recent decades, concern about rabbit welfare and sustainability has increased. The housing system is a very important factor for animal welfare. However, information about how different available housing types for female rabbits affect their health status is scarce, but this is an important factor for their welfare. Hence the objective of this study was to evaluate the health status of female rabbits in five common housing systems: three different single-housing systems with distinct available surfaces and heights, a single-housing system with a platform and a collective system. The female rabbits in the collective and platform cages had higher cortisol concentrations in hair than those in the single-housing systems with no platform. The haptoglobin concentrations and kit mortality rates during lactation were higher for the collective-cage female rabbits. The collective group had more culled females and more lesions than the females in the other groups. The main reasons for culling in all the groups were reproductive problems and presence of abscesses, and the collective group of females was the most affected. In conclusion, keeping females in these collective systems negatively affected their health status and, therefore, their welfare. In contrast, individual housings caused lower concentrations of haptoglobin and hair cortisol (except for the cage with platform), less kit mortality rates and fewer culled females. This means a better sanitary status and greater welfare in individual housings, and therefore, they would be more indicated for female rabbits.

RESUMEN

La cunicultura afronta diferentes retos hoy en día, siendo las estafilococias y la mejora del bienestar de las conejas dos de los más importantes. *Staphylococcus aureus* es una bacteria que en conejos produce principalmente mastitis, pododermatitis, abscesos y piodermas, generando numerosas pérdidas económicas en las granjas afectadas. Pero la población de *S. aureus* es muy heterogénea y es necesario realizar un tipado para caracterizar las cepas presentes en cada granja y ayudar a su prevención y tratamiento. Los clones más frecuentes en cunicultura eran el ST121 y el ST96, el primero considerado de alta virulencia y el segundo, de baja virulencia. Sin embargo, en los últimos años, veterinarios y ganaderos han mostrado su preocupación por la aparición de brotes más virulentos y persistentes. Considerando que *S. aureus* tiene una gran capacidad de adaptación, se planteó la posibilidad de que los clones hubieran cambiado o adquirido nuevos factores de virulencia o incluso hubieran aparecido clones nuevos que estuvieran provocando este aumento del número y virulencia de los brotes en granjas cunícolas. Por tanto, los objetivos de la primera parte de esta tesis fueron la caracterización genética de las cepas aisladas de granjas de conejos y su distribución geográfica y testar *in vivo* algunas de estas cepas para comprobar si los cambios genéticos observados se correlacionaban con una mayor capacidad infectiva. Se detectó un nuevo clon, el ST3764, llegando a suponer el 19% de todos los aislados. Este clon es una variante del ST121, que se detectó por primera vez en el 2014 en el noroeste de la Península Ibérica y que posteriormente se diseminó hacia el este. Del mismo modo, también se detectaron casos de estafilococia por cepas ST2855, una variante del clon ST96, en toda la Península. Por tanto, el porcentaje de cepas pertenecientes al clon ST121 ha sufrido una disminución como consecuencia de un elevado número de cepas ST3764 y ST2855. Además, también se han detectado nuevos clones no descritos hasta el momento en conejos en España, como el ST1, ST146 y ST398. La secuenciación completa de estos nuevos clones ha evidenciado la

adquisición de elementos genéticos móviles, a excepción de las cepas ST2855. La mayoría habían perdido el profago con integrasa tipo 3, que además de portar el IEC humano con los genes *sak*, *chp* y *scn*, trunca la secuencia de la β-hemolisina, haciendo que estas cepas la expresen. Finalmente, se encontraron mutaciones en el gen *dltb* entre las cepas ST96 y ST2855. Todos estos cambios podrían sugerir un aumento de la virulencia de las cepas ST2855 respecto a las ST96, pero las cepas ST2855 no tampoco fueron capaces de infectar. Por el contrario, las cepas ST3764 infectaron a un mayor número de animales que su antecesora ST121, aunque no fue un aumento significativo. Por tanto, la diseminación de las cepas ST2855 podría deberse a cambios genéticos no detectados aquí o a otros factores extrínsecos a las cepas.

La presencia de *S. aureus* en las granjas está muy relacionada con el segundo reto estudiado en esta tesis, ya que las lesiones producidas por esta bacteria merman enormemente el bienestar de los animales. En las últimas décadas, la preocupación por el bienestar de las conejas ha aumentado y el sistema de alojamiento es un factor directamente relacionado con el bienestar. Sin embargo, la información científica sobre cómo los diferentes tipos de alojamiento afectan al bienestar desde el punto de vista de la salud es escasa. Por tanto, el objetivo de este estudio fue evaluar el estado de salud de las conejas en cinco sistemas de alojamiento: tres sistemas diferentes de alojamiento individual con distintas superficies y alturas disponibles; un sistema de vivienda individual con plataforma y un sistema de alojamiento para 6 conejas. Se observó que las conejas alojadas en grupo y en la jaula con plataforma tenían concentraciones de cortisol en el pelo más altas que las de los otros 3 sistemas de alojamiento individual. Las concentraciones de haptoglobina y las tasas de mortalidad de los gazapos antes del destete fueron más altas en el alojamiento colectivo. Además, este alojamiento ocasionó la eliminación de más conejas que el resto. Las principales causas de eliminación fueron los problemas reproductivos y la presencia de abscesos, siendo el alojamiento colectivo el que presentó un mayor número. En conclusión, mantener a las

hembras en estos sistemas colectivos afecta negativamente su estado de salud y, por tanto, a su bienestar. Por el contrario, los alojamientos individuales provocaron concentraciones más bajas de haptoglobina y cortisol (a excepción de la jaula con plataforma), menos mortalidad en gazapos y menor número de conejas eliminadas. Esto supone un mejor estado sanitario y mayor bienestar, con lo que estos alojamientos serían más indicados para las conejas reproductoras.

ABBREVIATIONS

1AI	first artificial insemination
5P	fifth parturition
aa	amino acid
APP	acute phase protein
attB	attachment site of bacteriophage
CC	clonal complex
CFH	complement factor H
CFU	colony-forming unit
cm	centimetre
COL	collective
CRISPR	clustered regularly interspaced short palindromic repeats
CV	coefficient of variability
DAPI	4 ',6-diamidino-2-fenilindol
DLV	double locus variant
DM	dry matter
DNA	deoxyribonucleic acid
DPBS	dulbecco's phosphate-buffered saline solution
dpp	days post-partum
EDTA	ethylenediaminetetraacetic acid
egc	enterotoxin gene cluster
FITC	fluorescein isothiocyanate
g	gram
h	hour
H/L	heterophil/lymphocyte ratio
HD	higher and deeper
HPA	hypothalamic–pituitary–adrenal
HV	high virulence strains
IEC	immune evasion cluster
kb	kilo base
L	litre
LV	low virulence strains
MGE	Mobile genetic element
min	minute
mL	millilitre
MLST	multilocus sequence typing
mM	millimolar
MRSA	Methicillin-resistant *Staphylococcus aureus*

MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MSSA	Methicillin-sensible *Staphylococcus aureus*
NETs	neutrophil extracellular traps
ng	nanogram
nM	nanomolar
o/n	over night
ºC	degrees Celsius
$OD_{(nm)}$	optical density at a given wavelength (nm)
ORF	open reading frame
P	*P-value*
pb	pairs of bases
PCR	polymerase chain reaction
PF	platform
PFGE	Pulsed Field Gel Electrophoresis
PMN	polymorphonuclear leukocytes
PSM	Phenol Soluble Modulin
PV	polyvalent
PVL	Panton-Valentine Leucocidin
RAPD	Randomly Amplified Polymorphic DNA
RFLP	restriction fragment length polymorphism
RNB	rabbit neutrophil buffer
rpm	revolutions per minute
s	second
Saint	*Staphylococcus aureus* bacteriophage
SaPIs	*Staphylococcus aureus* Pathogenicity Islands
SCC*mec*	chromosomal cassette *mec*
SLV	single locus variant
ST	sequence type
TNFR	tumour necrosis factor receptor 1
TR	traditional
TSA	tryptic soy agar
TSB	tryptic soy broth
U	international units
UV	ultra violet
µg	microgram
µL	microliter
µm	micrometre
µM	micromolar

CONTENTS

I- INTRODUCTION

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1.1. *Staphylococcus aureus*

1.1.1. General information

Staphylococcus aureus is a bacterium belonging to the genus *Staphylococcus* and phylum Firmicutes. *S. aureus* is characterized by being small (diameter of 0.5-1.5 µm), rounded and grouped as a grape cluster. It does not form spores and lack flagellum. It is a positive catalase and facultative anaerobic bacteria, and it uses carbohydrates both by oxidation and by fermentation. Despite its inability to produce spores, its cell wall contains peptidoglycans and teichoic acid. Therefore, they are able to withstand adverse environmental conditions such as drying, temperatures of 60°C for 30 minutes, pH values between 4 and 9.5 and salt concentrations of 7.5% (Quinn *et al.*, 2011).

It is widely distributed in nature and is considered as habitual host of the skin and mucous membranes, mainly the nasal cavity (Quinn *et al.*, 2011). About the 30% of the human population carry this bacteria in the nostrils, armpits, mucosae or inguinal region (Kluytmans *et al.*, 1997; Peacock *et al.*, 2001). However, *S. aureus* is an opportunistic pathogen, which means that it needs predisposing factors to produce lesions, like immunosuppression, a wound or another concomitant disease.

But the most important characteristic of *S. aureus* is the ability to produce the protein coagulase, which binds to fibrinogen and induces the coagulation of plasma. There are other *Staphylococcus* that also produce coagulase, as *Staphylococcus intermedius* and some strains of *Staphylococcus hyicus*. Although the most pathogenic species is *S. aureus,* within the genus *Staphylococcus* there are also some coagulase negative species, as *S. epidermidis*, *S. saprophyticus* and *S. lugdunensis* which cause several diseases in people and animals (Quinn *et al.*, 2011). For this

reason, coagulase is widely used for diagnostic tests, since it is secreted by almost all strains of *S. aureus* and it is an easy test to perform.

1.1.2. Clinical and livestock relevance

S. aureus is a great threat for human and animal health (Gijón *et al.*, 2016; Vogel *et al.*, 2016; Aires-de-Sousa, 2017). In the last decades strains with resistance to different antibiotics have emerged, making the treatment more difficult (Hassoun *et al.*, 2017). The most important strains causing these infections are those called Methicillin-resistant *Staphylococcus aureus* (MRSA). This resistance is given by the *mecA* or *mecC* genes within a chromosomal cassette called SCC*mec* (Boyce *et al.*, 2005). In addition to the resistance to methicillin, the different SCC*mec* carry other genes involved in the resistance to other antibiotics (Partridge *et al.*, 2018). However, there is a decreasing trend in the MRSA bacteraemia cases, while MSSA cases have increased since 2005 (Jokinen *et al.*, 2018).

In animal production, *S. aureus* affects practically all the production species (Aires-de-Sousa, 2017). It is an important cause of economic losses due to infections of the mammary gland of lactating females. In dairy cattle it is one of the main agents of mastitis and the mammary infection usually progresses to chronic or subclinical forms (Cucarella *et al.*, 2004; Luini *et al.*, 2015). In sheep and goats *S. aureus* is the most common agent producing acute gangrenous mastitis, as well as skin disorders such as dermatitis and folliculitis (Menzies and Ramanoon, 2001; Islam *et al.*, 2012). The pig production is also affected by *S. aureus* which is found in the 51% of the sampled farms and isolated from the 30% of the animals. But pigs seem to be carriers and they do not usually develop lesions (Meemken *et al.*, 2010; Osadebe *et al.*, 2013). Finally, in poultry *S. aureus* produces several diseases, such as chondronecrosis and septicaemia (Lowder *et al.*, 2009; Aires-de-Sousa, 2017).

S. aureus also affects commercial rabbits, being isolated from both farms with severe staphylococcal problems and clinically healthy farms (Hermans

et al., 1999). The problem started about the 1980s, when severe outbreaks were reported in different European countries, probably due to the intensification in animal production (Okerman *et al.*, 1984; Hermans *et al.*, 2000b). The most common lesions produced by *S. aureus* are mastitis in females, pododermatitis in adult animals, abscesses in young and adult rabbits and pyodermas in kits (Segura *et al.*, 2007).

Carrier animals are important in the epidemiology of the infections. Previous studies indicated elevated rates of nasal carriers in rabbits, up to a prevalence of 56% in the studied farms. This high number of carriers could explain the difficulties in the elimination of *S. aureus* from the farms. And it has also been demonstrated that the isolated strains of nasal cavity and lesion were genetically related in 91.7% of the animals, which suggested the possible nasal origin of the disease. Therefore, the presence of carrier animals in a farm could be a risk for lesion development (Selva *et al.*, 2015).

In recent years, both veterinarians and farmers have expressed increasing concern about more virulent outbreaks of this bacteria. Moreover, they emphasize that the outbreaks are recurrent and some of them do not respond to antibiotic treatment. Because it has been shown that single mutations can change the behaviour of a strain (Viana *et al.*, 2015a), these new outbreaks may be due to mutations or acquisition of new virulence factors or resistance to antibiotics.

1.1.3. Characterization of *S. aureus* strains

The *S. aureus* population is highly heterogenic, which means that there are many different strains, each one with its own characteristics, virulence factors and resistance genes. For this reason, in the last decades, the characterization of *S. aureus* isolates has become more important. Before the routinely use of molecular typing, phage typing (Blair, 1961) and biotyping (Devriese, 1984) were commonly used. In addition, antibiograms and immunoblotting were also used, but all these techniques provided more or less the same discrimination level between isolates and it was

recommended the use of two assays at the same time to characterize properly the isolates (Tenover *et al.*, 1994). Pulsed Field Gel Electrophoresis (PFGE), developed by Schwartz and Cantor (1984), became one of the most used techniques to characterize *S. aureus* strains. However, comparing results between laboratories was not possible and a harmonization of the technique was necessary. It was standardized in 2003, creating a database and allowing the comparison between different laboratories (Murchan *et al.*, 2003). In 1990, Williams *et al.* (1990) developed a technique suited for rapid detection of genomic polymorphisms, called Randomly Amplified Polymorphic DNA (RAPD)-typing. It was based on a polymerase chain reaction with a single short oligonucleotide primer of arbitrary sequence. Rabbit isolates were also classified into high and low virulence strains according to the number of animals affected, the severity of the lesions, the biotype and the RAPD type (Hermans *et al.*, 2000b).

Currently, the most frequent typing technique is called Multi Locus Sequence Typing (MLST), developed by Enright *et al.* (2000). It is based in the detection of variations in seven highly conserved genes (*arc*, *aro*, *glp*, *gmk*, *pta*, *tpi* and *yqi*) and thus it allows to know the phylogenetic relationships between different strains and to discern between different clonal lineages. Although it is expensive, it has a wide database, which provides a rigorous and standardized technique.

Viana *et al.* (2007) described a molecular typing based on the Restriction Fragment Length Polymorphism (RFLP) of the genes *coa* and *spa* of *S. aureus*. They also included the analysis by PCR of the polymorphic gene *clfB*. With this technique, greater discrimination is obtained than with the previous techniques, since it discriminates between different isolates with the same MLST. This technique allows a quickly and economically typing of rabbit strains and also strains from other species.

Based on the genotyping described by Viana *et al.* (2007), the most widespread *S. aureus* clone in rabbit farms was the A1 II1 δ (70% of the

isolates). This genotype belonged to ST121 clone and it was classified as high virulent strain because it was widely disseminated in rabbit farms where it produced chronic problems of staphylococcosis (Vancraeynest *et al.*, 2006). There were other less common genotypes affecting the rabbits, such the B1 I1 α, B1 IV1 α that belonged to the ST96 (Viana *et al.*, 2011). However, Viana *et al.* (2007) reported that a low virulence strain previously isolated and described by Hermans *et al.* (2000b) infected a high number of animals, producing huge economic losses. Therefore, the classification according to clinical observations should be complemented with molecular techniques.

1.1.4. Pathogenesis of *S. aureus*

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 favourable conditions, like wounds, immunosuppression or other concomitant diseases, to multiply and to produce an infection (Figure I1-1) (Kobayashi *et al.*, 2015).

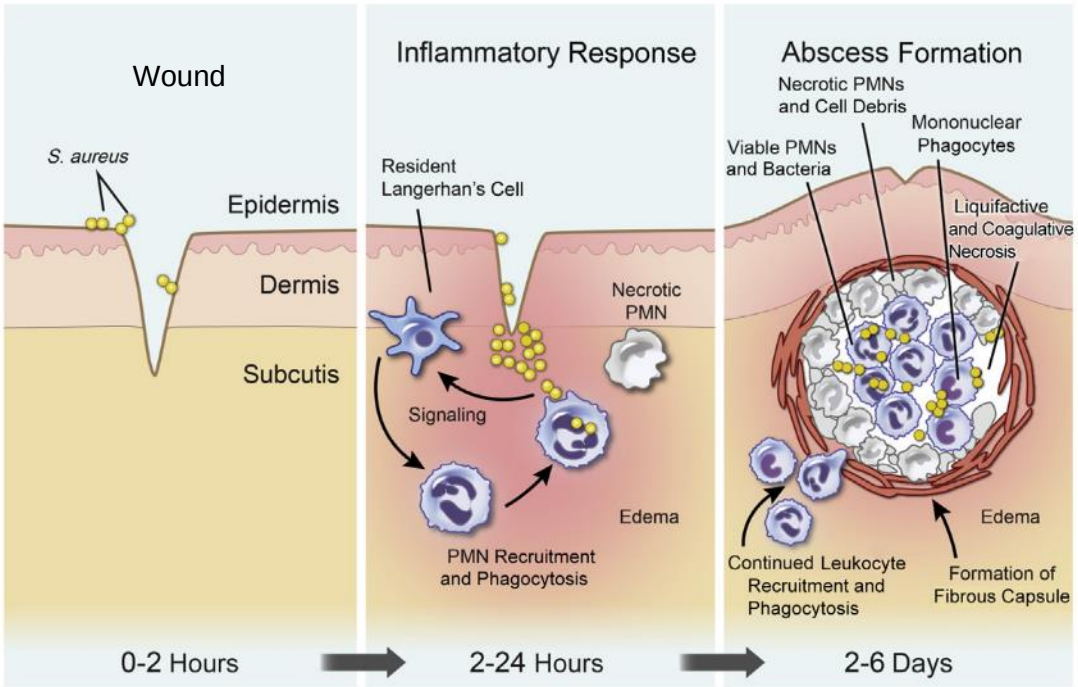

Figure I1-1. Beginning and evolution of the infection by *S. aureus*. PMN: polymorphonuclear leukocytes (Adapted from Kobayashi *et al.*, 2015).

S. aureus produces mainly suppurative lesions, especially abscesses. It can penetrate through the skin, mucous membranes and gastrointestinal tract. When the bacterium enter the host, a complex cellular response begins (Kobayashi *et al.*, 2015). Neutrophils are the first to reach the site of infection, starting to phagocytize bacteria and producing different toxins and cytokines to eliminate them. Finally, degranulation and lysis of neutrophils occur with the releasing of neutrophil extracellular traps (NETs) (Figure I1-2) (Amulic *et al.*, 2012).

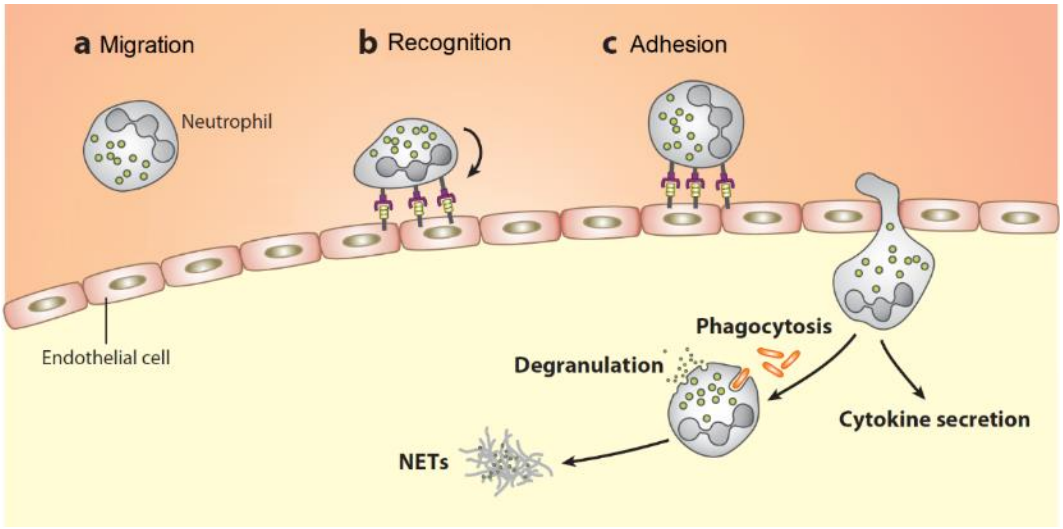

Figure I1-2. Neutrophil recruitment and function stages (Adapted from Amulic, 2012).

On the other side, *S. aureus* has numerous mechanisms to evade the immune response. If *S. aureus* succeeds, a layer of fibrin forms around the bacterial community within 4 days, forming the pseudocapsule of the abscess. Meanwhile, the recruitment of neutrophils and macrophages continues and *S. aureus* causes the lysis of all these leukocytes and the necrosis of the surrounding tissue, turning all this into a purulent content (Thammavongsa *et al.*, 2013). Later, the pseudocapsule matures, becoming fibrous. Once formed, the abscess can open, with the consequent release of bacteria into the bloodstream and invasion of other organs and tissues (Cheng *et al.*, 2011). In the absence of antibiotic treatment, sepsis and death of the individual may occurs.

1.1.5. Virulence factors

In order to multiply, produce lesions and invade the host, *S. aureus* has developed numerous virulence factors, which are necessary for its pathogenic mechanism. But not all of them are expressed at the same time. It has a wide range of regulatory genes, and its expression depends, among other factors, on the environmental conditions, the infection phase in which it is found and the immune response of the host (Figure I1-3). One of the most important regulatory genes is the *agr* gene. It participates in the regulation of the expression of virulence genes, such the coagulases, protein A and haemolysins (Wolz *et al.*, 1996; Traber *et al.*, 2008).

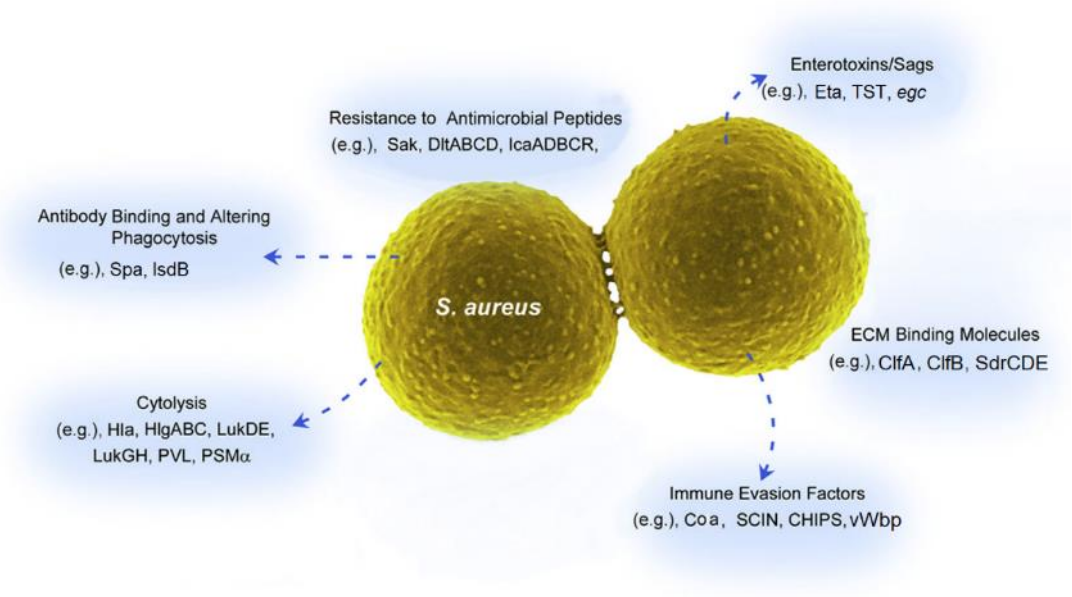

Figure I1-3. Virulence factors produced by *S. aureus* (Adapted from Kobayashi *et al.*, 2015).

To date, two coagulases have been described: coagulase (Coa) and von Willebrand Factor Binding Protein (Vwbp). Coagulase is a protein that has been known for more than 100 years and it has multiple allelic variants (Watanabe *et al.*, 2005). It is encoded by the *coa* gene and is expressed during infection. As explained above, coagulase binds to prothrombin and activates it, triggering the transformation of fibrinogen into fibrin and forming the clot. However, coagulase shows different activity against plasma from different hosts, indicating a host specific clotting (Bjerketorp *et al.*, 2006).

On the other hand, coagulase has an important antigenic effect, developing an effective immune response that causes a decrease in the effects and injuries, which can prompt its use in a possible vaccine (Cheng *et al.*, 2010).

The Vwbp was discovered at the beginning of this century (Bjerketorp *et al.*, 2002). It binds to the von Willebrand factor and also to the prothrombin, triggering the coagulation as well. It has been found that Vwbp is very host specific in part because the *vwb* gene has different alleles. In addition, is carried by highly mobile pathogenicity islands, which facilitate the strains to adapt to different hosts (Viana *et al.*, 2010). On the other hand, Vwbp is also a potent antigen and it is very important for the host to develop immunity (Cheng *et al.*, 2010). 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).

Another important group of virulence factors are the MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules). These molecules facilitate *S. aureus* adhesion to different cells and components of the host. Within MSCRAMM group are the Sdr's, the protein A and the clumping factor A and B (Sivaraman *et al.*, 2009; Foster *et al.*, 2014).

There are 3 different Sdr genes: *sdrC*, *sdrD* and *sdrE*. All strains of *S. aureus* have at least one of these genes (Josefsson *et al.*, 1998; Viana *et al.*, 2015b). In addition to these tree genes, *sdrE* has an allelic variant called *bbp*, a bone sialoprotein-binding protein, which could be host-specific (Peacock *et al.*, 2002; Vazquez *et al.*, 2011). It has recently been discovered that SdrE acts as a clamp to capture the C-terminal tail of complement factor H (CFH), inhibiting the alternative path of the complement system and allowing evasion of the immune system (Zhang *et al.*, 2017). It has been observed that the levels of antibodies against SdrE are higher in the carriers. These levels are also higher in non-carriers who subsequently

acquire the bacteria than in those who do not acquire it (Ghasemzadeh-Moghaddam *et al.*, 2017).

The staphylococcal protein A (SpA), encoded by the *spa* gene, is a sortase-anchored product that is released from the bacterial envelope in large amounts and has several functions. First, SpA impedes phagocytosis by binding the Fcg component of immunoglobulin; second, it activates platelet aggregation via its binding to von Willebrand factor and third, it functions as a B-cell superantigen (Foster *et al.*, 2014). In addition, it binds to tumour necrosis factor receptor 1 (TNFR1) on lung epithelial cells, which leads to the activation of intracellular signalling, the expression of cytokines and the recruitment of neutrophils (Gómez *et al.*, 2004).

The clumping factor A (ClfA) is an important virulence factor of *S. aureus* involved in the pathogenesis of the infection. It binds to the C-terminal region of the fibrinogen γ-chain and produces the platelet aggregation or clumping of bacteria in plasma (Mcdevitt *et al.*, 1997). ClfA protects bacteria from neutrophil phagocytosis through the recruitment of fibrinogen to the bacterial cell surface (Higgins *et al.*, 2006). *S. aureus* has another fibrinogen-binding protein, called ClfB (Ní Eidhin *et al.*, 1998). This protein facilitates the attachment to the anterior nares during colonisation due to the high affinity for the keratinized epithelium, specifically cytokeratin 10, which is the dominant component of the interior of squamous cells, and loricrin, which is the most abundant protein of the keratinized epithelial cells (Walsh *et al.*, 2004).

Regarding to the toxins, haemolysins also play an important role in the development of the infection, as they cause the lysis of blood cells. Haemolysins, as well as other virulence factors such as coagulase and protein A, are regulated by *agr* (accessory gene regulator). *S. aureus* can produce three different haemolysins: α-haemolysin, β-haemolysin and δ-haemolysin. While α-haemolysin is particularly active against rabbit erythrocytes (Bhakdi *et al.*, 1984), β-haemolysin is highly haemolytic for sheep but not rabbit erythrocytes (Glenny and Stevens, 1935). In sheep

blood agar plates, the β-haemolysin increases the haemolysis produced by the δ-haemolysin, while it inhibits the haemolysis of the α-haemolysin. Because of this, it is possible to know which haemolysins are expressing the strains with a simple haemolysis test (Figure I1-4) (Traber *et al.*, 2008).

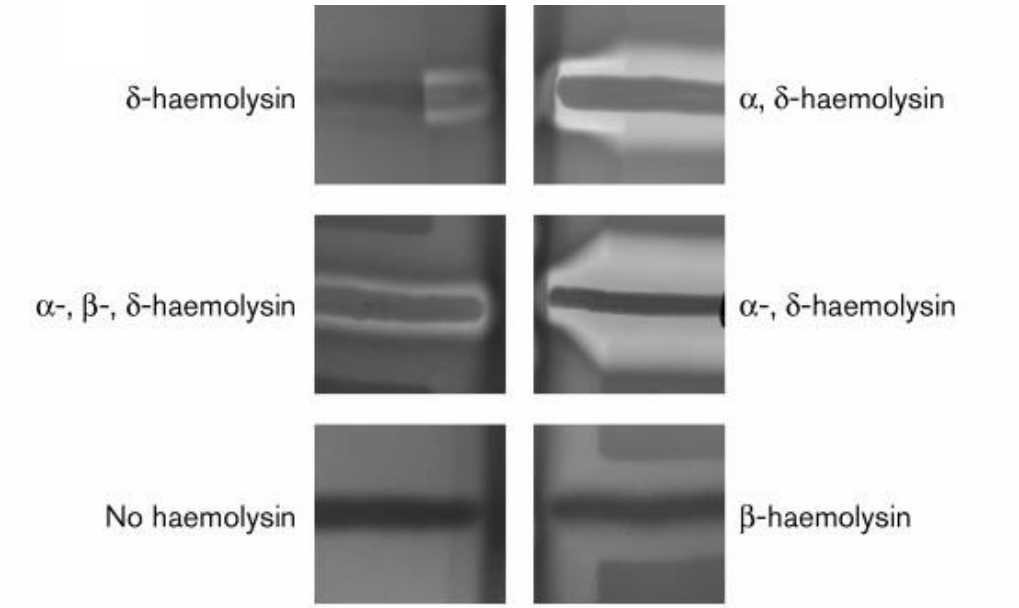

Figure I1-4. Different haemolysis patterns produced by *S. aureus* in blood agar (Adapted from Traber *et al.*, 2008).

Other toxins are the Phenol-Soluble Modulins (PSMs), small peptides capable to recruit, activate and lyse neutrophils and T-cells (Collins, J. *et al.*, 2008; Peschel and Otto, 2013). Another important group of toxins are the leucocidins. The leucocidins are two component pore-forming toxins, known to produce a rapid cytotoxic activity against neutrophils (Miles *et al.*, 2002; Löffler *et al.*, 2010). All *S. aureus* strains produce LukAB, whereas other strains may also secrete Panton-Valentine Leucocidin (PVL) and LukED (Alonzo and Torres, 2014). Gamma-toxin or gamma haemolysin is another bicomponent leukocidin, widely distributed in *S. aureus* strains (Peacock *et al.*, 2002; Viana *et al.*, 2015b). It is encoded by the locus *hlgABC*, with two possible combinations, the HlgAB and the HlgCB. Only

HlgAB shows high haemolytic activity toward both rabbit and human erythrocytes and also acts like a leukotoxin (Thammavongsa *et al.*, 2015).

1.1.6. *Staphylococcus aureus* bacteriophages

Mobile genetic elements (MGEs) are genetic material with the ability of being transferred from one bacterium to another or even between different species. This is called horizontal transfer. The most important MGEs in *S. aureus* are plasmids, transposons, bacteriophages and pathogenicity islands (SaPIs) (Partridge *et al.*, 2018). These elements play an essential role in the transmission of several genes like antibiotic resistance genes and virulence factors and they are also involved in the host adaptation (Viana *et al.*, 2010).

The most widely studied MGEs are the bacteriophages (or simply phages), since a large number of them have been identified and sequenced (Kwan *et al.*, 2005; Goerke *et al.*, 2009). Bacteriophages have been found in all the sequenced strains of *S. aureus* and their information contributes up to 4 or 5% of the total genome of the bacterium (Kuroda *et al.*, 2001). This may demonstrate their importance in the pathogenic process. In fact, Mu50 and N315, two of the sequenced strains of *S. aureus*, have a very similar phage encoding for known virulence factors: the *sep* gene, encoding the enterotoxin P, which is a superantigen involved in food poisoning, and a gene encoding a staphylokinase (*sak*) (Kuroda *et al.*, 2001). Within the strain 8325, another sequenced strain, three phages have been described and one of them also carries the *sak* gene (Iandolo *et al.*, 2002).

In *Staphylococcus* genus, most of the bacteriophages belong to the order Caudovirals, mainly to the family Syphoviridae. They are composed of an icosahedral capsid and they have a long non-contractile tail (Ackermann, 1998; Deghorain and Van Melderen, 2012). Its genetic information is transported inside the capsids as double-stranded DNA and they have the genetic information structured in conserved modules. *S. aureus* phages are mostly lysogenic, which means that the replication of their DNA occurs

passively when the chromosome of the bacterium replicates. To lysogenize a bacterium, the phage has to integrate into the bacterial chromosome at a specific point in the genome called *att*. Twelve integration sites have been identified for bacteriophages of *S. aureus* and each one of them is identified by a different integrase, which recognizes the sequence and performs the recombination (Goerke *et al.*, 2009).

Under stress situations, such as UV light, agents that damage DNA and some antibiotics, phages are induced and go through a lytic cycle (Ubeda *et al.*, 2005). During induction, the phages are excised from the bacterial chromosome and begin to replicate, transferring horizontally to other strains of *S. aureus*. Recently, it has been demonstrated that during this lytic cycle a large quantity of bacterial genomic information can also be transferred. This is related with an alteration of the normal steps of the lytic cycle (excision-replication-packaging), causing that DNA packaging initiates before the excision, directly from integrated prophages. This process was called genome hypermobility by lateral transduction and it may explain a broad amount of previously unknown mechanisms, being a really important driving force for both bacterial and phage evolution (Chen *et al.*, 2018).

Virulence factors within the phages are usually encoded in the farthest region from the integrase, after the lysis module, although in some cases they occur in other locations (Sumby and Waldor, 2003). These virulence factors can inhibit the host's immune system, such as Sak, that modulates the immune response responsible for the tissue destruction of the host (Jin *et al.*, 2004); Chp, an inhibitory protein of chemotaxis (de Haas et al., 2004) or SCIN, which inhibits the complement system (Rooijakkers *et al.*, 2005). Other virulence factors are superantigens (Sea, Seq, Sek, Sek2, Sep, Seq), which are enterotoxins that cause food poisoning and necrotizing fasciitis (Iandolo *et al.*, 2002). In addition, phages can also encode the PVL, which is a cytotoxin composed of lukF-PV and lukS-PV and the related leukocidins lukM and lukF that form pores in leukocytes and cause necrotic infections (Diep *et al.*, 2010; Löffler *et al.*, 2010). Another toxin found in the pages is

the exfoliative toxin A, which is involved in severe skin infections (Bukowski *et al.*, 2010).

Therefore, when a phage is temperated in a strain, it provides to the bacteria a new arsenal. But, on the other hand, bacteriophages can also truncate the sequence of genes encoding virulence-related proteins, such as the *hlb* gene, which eliminates the ability of the strain to be β-haemolytic. Specifically, the bacteriophages that are integrated in this gene codify for the type III integrase and sometimes for the virulence factors Sak, SCIN, Sea and CHIPS. In these bacteria there is a compromise between the functionality of *hlb* and the presence of other virulence factors, then they are selected based on the presence or absence of the phage, according to the needs imposed by the host in which they are found (van Wamel *et al.*, 2006).

1.1.7. *Staphylolcoccus aureus* Pathogenicity Islands

Other very important MGE in *S. aureus* are the *S. aureus* Pathogenicity Islands (SaPIs). This pathogenicity islands are accessory genetic elements with a size between 14 and 17 kb with a highly conserved genetic organization. This information is divided into about 10 to 12 ORF and SaPIs can integrate in six different chromosomal sites. Their integrases have a similar activity to the phage integrases, recognizing specific integration sites different from those in phages, and they also contain different virulence factors (Novick and Subedi, 2007). They may play an important role in the evolution of microorganisms by allowing the acquisition of large fragments genomes by horizontal transfer (Hacker and Kaper, 2000). Like phages, all strains of *S. aureus* sequenced presented at least one island, which demonstrates the importance of the SaPIs in the biology of the bacteria (Ram *et al.*, 2014).

To summarize the islands mechanism, they are able to identify the moment of bacteriophage replication since they use a protein encoded by the phage as inductors of its cycle (Tormo-Más *et al.*, 2010). Once induced, the islands are transferred generating infective particles from the proteins encoded by the phage (Tormo *et al.*, 2008). After the lysis of the bacteria by the enzymes

encoded by the phage, the islands infect a new host cell, where they are integrated into the *attB* of the bacterial chromosome by integrase activity encoded on the island (Ubeda *et al.*, 2003). Therefore, a strain could arrive to carry simultaneously five islands and twelve prophages, each one of them integrated in its specific *attB*.

Although the integrases of the islands and phages have similar mechanisms, their sequences and the *attB* they recognize are different. In this way, the islands and the phage have evolved differentiate themselves so as not to have to compete for their integration sites, but both have mechanisms to interfere in the function of the other. For example, when the bacterial SOS response is activated, the horizontal transfer of SaPIs and the virulence factors encoded in them is produced. In the opposite way, the island can promote the lytic cycle of the phage and facilitate the packaging of smaller capsids to avoid the phage genome to fit inside these capsids, which are used by the island (Poliakov *et al.*, 2008). In addition, SaPIs can protect the bacteria from the infection of new lytic phage, so may be an advantage for those carrying SaPIs.

Some virulence factors encoded by the SaPIs are the toxic shock syndrome toxin (TSST) (Lindsay *et al.*, 1998) and enterotoxins B, K and Q, which are one of the most important causes of food poisoning (Yarwood *et al.*, 2002). The protein Bap, related to the formation of the biofilm, is found in the SaPIbov2 (Ubeda *et al.*, 2003) and the vWbp protein, which is a host-specific coagulase, is encoded by the SaPIbov4 (Viana *et al.*, 2010).

1.1.8. MGE and evolution

It has been observed that rabbit strains ST121 did not present characteristic bacteriophages or pathogenicity islands that gave them capacity of adaptation to the host, unlike the human ST121 strains (Figure I1-5). The ST121 clone was adapted to the rabbit host from a human strain due to a single nucleotide mutation only 40 years ago (Viana *et al.*, 2015a). This

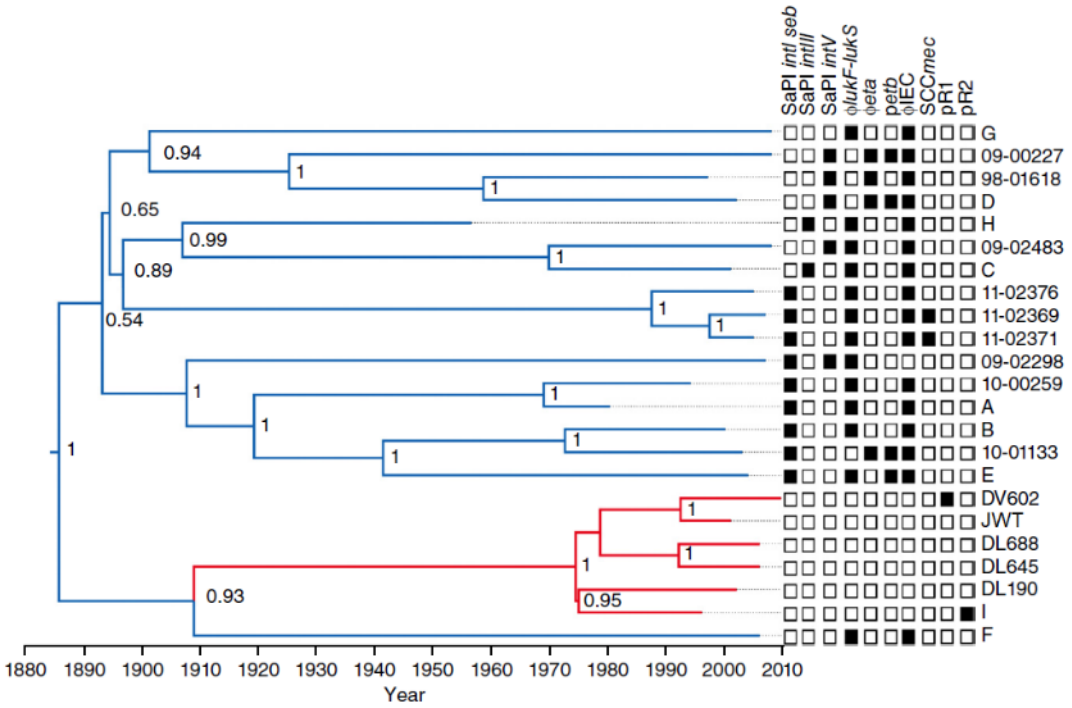

Figure I1-5. Evolution of ST121 strains and mobile genetic elements present in the different strains. Red lines indicate rabbit strains and blue lines indicate human strains (Adapted from Viana *et al.*, 2015).

mutation occurred in the *dltB* gene, which codifies an integral membrane protein, and it is involved in the regulation of positive charges essential for resistance to cationic peptides and for developing virulence (Collins, L. V. *et al.*, 2002). In addition to this single mutation, the rabbit ST121 strains lack the phage with the human IEC that truncates the *hlb*, so the β-haemolysin is expressed. The expression of β-haemolysin, together with the loss of the IEC and the mutation of single nucleotides were considered adaptive changes (Viana *et al.*, 2015a).

Due to the high adaptability of *S. aureus*, our hypothesis is that the ST121 strains affecting rabbitries in the last years may have changed. For example, they could have acquired or lost mobile genetic elements, or they could have mutated some genes, making these strains more virulent. ST96 also could have changed or lost some MGE, turning this strain into a more virulent clone for rabbits. In addition, new strains may have emerged in rabbitries producing the recent outbreaks.

1.2. Welfare and housing in rabbit production

1.2.1. History and current situation of livestock welfare

Animal welfare is currently one of the main challenges facing livestock production as social and commercial demands are increasing. In fact, a European survey (Food Chain Evaluation Consortium, 2016) observed that 94% of the European population (the same percentage for Spanish population) considered important to protect the welfare of farmed animals and the 82% considered that it should be better protected. Moreover, 59% of the population would be willing to pay more for products sourced from animal welfare-friendly production systems.

But the concern about welfare in animal production comes for far. In 1991, the Farm Animal Welfare Council (FAWC) named for the first time “the five freedoms” as the basis of animal welfare. These freedoms, which all farmed animals should have, are free of pain, free of discomfort, disease and injury, free from hunger and thirst, free of fear and stress and free to express natural behaviour. However, these were recommendations and there was no law to protect production animals. Thus, in 1998, the European Union published the Directive 98/58/EC for the protection of all animals kept for farming purposes, which lays down general rules concerning the protection of farmed animals. But this was a basic guideline about the staff, medical recording, housing and freedom of movement, feeding, water and mutilations and it was necessary to improve even more the laws about animal welfare.

For this reason, the European Council began to develop more strict and specific directives for each animal species. The first directive was in 1999 (Council Directive 1999/74/EC) with the minimum standards for the protection of laying hens. They required that all hens must have a nest, perching space, litter to allow pecking and scratching and unrestricted access to a feed trough. Later, in 2007, they also established the minimum rules for the protection of chickens kept for meat production (Council

Directive 2007/43/EC). It set a maximum stocking density and ensured better animal welfare by specifying requirements such as lighting, litter, feeding, and ventilation. This was the first time that scientific assessment with 'Welfare Indicators' were included in animal welfare legislation. Subsequently, in 2008, both calves and pigs were also protected by Council Directive 2008/119/EC and Council Directive 2008/120/EC, respectively. Briefly, the Directive about calves set that they must not to be tethered, they have to be in collective pens after the age of eight weeks and it sets out the minimum dimensions for housing and the nutrient needs. Regarding to pigs, it was established that pregnant sows must be kept in groups instead of individual stalls during part of their pregnancy and they must have permanent access to enrichment materials.

Rabbit production is also affected by the social demands about welfare and sustainability. The European Food Safety Authority (EFSA) was concerned about the lack of welfare in rabbit farms, attending the high level of intensification, the high mortality and morbidity and the lack of enriched housings. For this reason, EFSA published in 2005 a scientific opinion about the impact of the current housing and husbandry systems on the health and welfare of farmed domestic rabbits. They reported a lack of scientific information and they demanded many changes to improve the welfare in rabbit production. However, it was not until 2016 when the European Parliament approved an initiative report to establish the minimum standards for the protection of farm rabbits (2016/2077 (INI)). It recommends the minimum characteristics of the cages (dimensions, platform implementation and even group accommodation) and insists on the need for further scientific research about rabbit farming, considering the demand for a transition to better welfare in production systems. This lack of scientific evidence is mainly in terms of the best housing from the viewpoint of welfare and the consequences on the health of rabbit does. But this report is not a Directive and therefore, there is still no law at the European Parliament for rabbit breeding as there is one for chickens, pigs or other species.

1.2.2. Factors affecting the welfare in rabbit production

Animal welfare must be the priority goal for farmers because it has a direct impact on farm profitability. But sometimes, improving welfare can compromise farm profitability. It is essential to know and understand factors affecting welfare. There are several stressors but the most important are grouped in physical and psychological (Verga *et al.*, 2007).

First of all, health and diseases, included within physical stressors, influences greatly the welfare, since it is widely demonstrated that sick rabbits show higher infertility rates, more abortions and weight loss, and therefore, low productivity (Marai *et al.*, 2002; Rosell and De La Fuente, 2008). The presence of diseases in a farm, such as staphylococcal infections, affects greatly the welfare because sick animals are not free from pain, disease, stress and even hunger and thirst and sometimes they stop doing natural behaviour (Sánchez *et al.*, 2012). *S. aureus*, as explained in section 1.1.2., produces two of the most common pathologies affecting rabbits: pododermatitis and mastitis (Figure I2-1). Other frequent diseases in female rabbits in Iberian farms are diseases of the respiratory system (particularly coryza), the reproductive tract (as pyometra) and subcutaneous abscesses (Segura *et al.*, 2007; Sánchez *et al.*, 2012). All these diseases impair the welfare of the animals and it is mandatory to try to minimize them as much as possible.

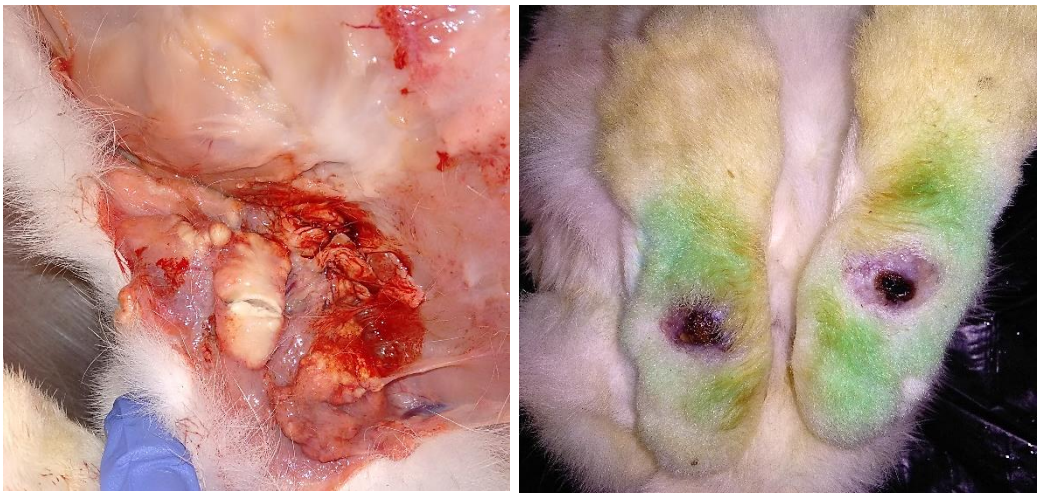

Figure I2-1. A: purulent mastitis with abscesses. B: ulcerative pododermatitis.

Housing conditions are another crucial factor affecting welfare since housing must guarantee the five freedoms and the integrity of the animals. For this reason, it must be taken into account that keeping animals in cages may favour the discomfort and the appearance of different injuries, which may be related to unsuitable cage characteristics. For example, the wire-mesh floor increases the prevalence of sore hocks and it is strongly recommended the use of footrests to avoid this disease which impairs greatly the welfare of adult rabbits (Rosell and de la Fuente, 2013). But information about the relationship between other common pathologies and cage type is scarce.

Lack of space, stimuli and social deprivation could generate the appearing of stereotypies, which are indicators of impaired welfare (Hoy and Verga, 2006; Verga *et al.*, 2007). Stereotypies are defined as unvarying, repetitive behaviour patterns that have no obvious goal or function (Odberg, 1978), such as gnawing the bars of the cage or excessive grooming.

1.2.3. Proposals to improve welfare

As welfare is a major concern in livestock production nowadays, there is a continuous research to find the best strategies to improve welfare. These strategies must cover all the needs of the animals previously explained together with the production performance. For example, a major surface area per animal and higher cages were recommended to improve welfare because they allow the animals resting with the body completely extended and to stand up (Hawkins *et al.*, 2008). However, Matics *et al.* (2004) observed that rabbits preferred one of the smallest cages and Princz *et al.* (2005) reported that they chose the higher cages when they were active and the lower cages when they were resting. Therefore, this should be taken in consideration to design a proper cage because if it is too large it could decrease the welfare of the animals and also the farm profitability because as larger cages, less quantity fits in the farm.

In addition to increasing the surface, several authors also recommend placing items for environmental enrichment purposes in cages. The lack of

stimuli generates frustration and environmental enrichment is designed to alleviate that frustration, which reduces stress and improves welfare (Buijs *et al.*, 2011). These elements are, for example, mirrors (Dalle Zotte *et al.*, 2009), shelters (Hawkins *et al.*, 2008) and platforms (Hansen and Berthelsen, 2000) (Figure I2-2).

The use of platforms has been studied in-depth and has shown advantages and disadvantages (Szendrő *et al.*, 2019). On one hand, it is an environmental enrichment element, which allows rabbits more space to move, jump, hide under and stand up in the highest area of the cage (Hansen and Berthelsen, 2000). In addition, if it is made of plastic, it prevents the sore hocks (Mikó *et al.*, 2014) (Figure I2-2). On the other hand, the use of the platform may produce hygienic problems due to retained faeces on the platform and faeces and urine falling onto animals located in the lower part of the cage (Alfonso Carrillo *et al.*, 2014; Cervera *et al.*, 2018). Regarding to females and kits performance, there is also controversy about the positive effect of platform inclusion, because some authors observed a little improvement (Mikó *et al.*, 2014) and others did not observed any effect (Cervera *et al.*, 2018). Finally, it is difficult to compare the results of different studies between them because of the different characteristics and comparisons used in each one.

In recent years, a further proposal has been the use of collective housing because it is a system to allow breeding female rabbits more mobility and social contact between them, and thereby improving their welfare (Figure I2-2). However, in the different studies about this type of housing it has been observed some inconveniences, as greater number of injured does as a result of aggressive interactions between animals (Rommers *et al.*, 2006; Zomeño *et al.*, 2017; Szendrő *et al.*, 2019), a deficient hygienic status and worse productive performance (Cervera *et al.*, 2017; Dal Bosco *et al.*, 2019). Nevertheless, new ways of solving these problems and implementing this system at the industrial level are still being studied, such as part-time grouping (Maertens and Buijs, 2016; Rommers and de Greef, 2018), hiding

places (Rommers *et al.*, 2014) (Figure I2-2) and the type of floor (Zomeño *et al.*, 2018). These strategies attempt to optimize collective housing, which covers the social nature of rabbits. But for now, these systems still produce important psychological stressors that impairs welfare and further investigation is needed.

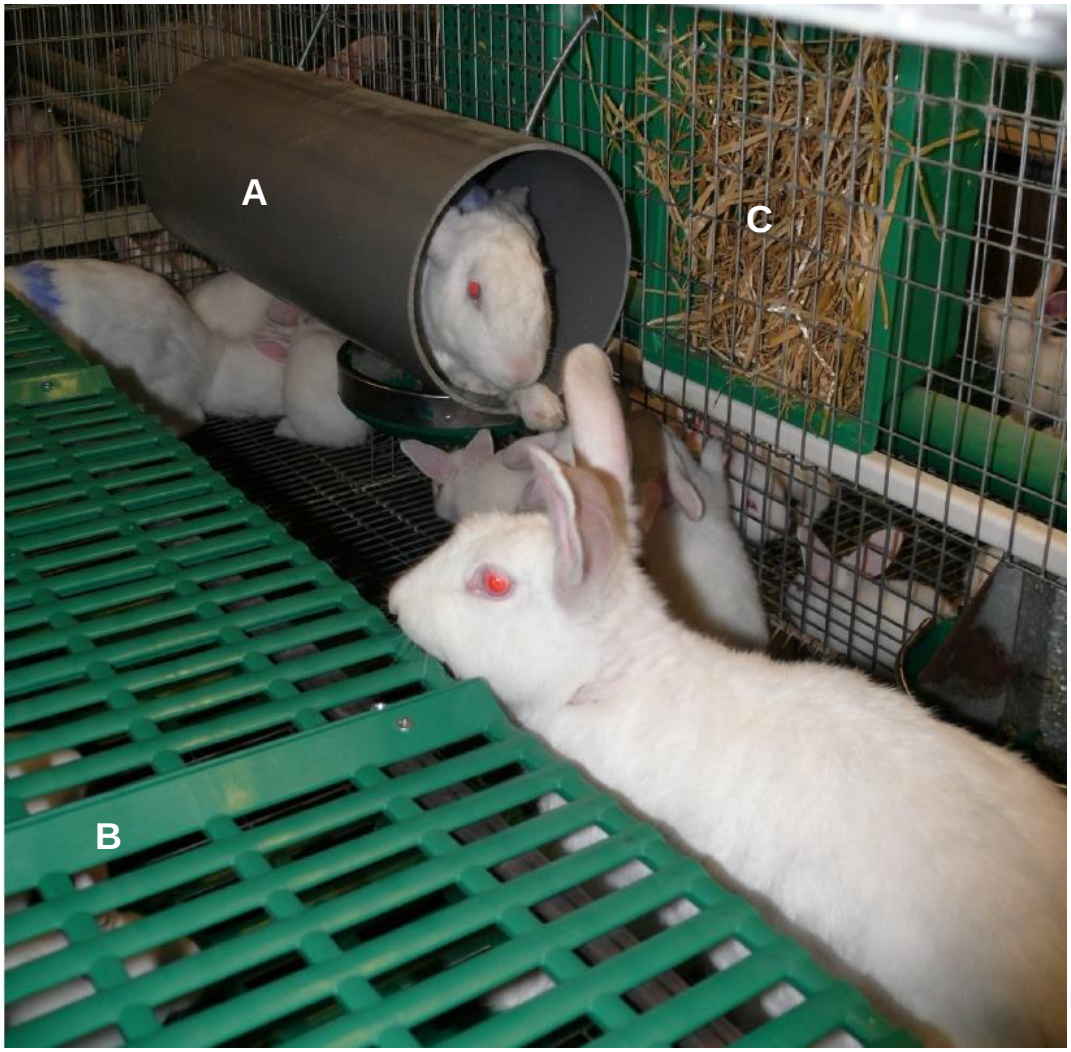

Figure I2-2. Collective housings for rabbit does. A: PVC tubes for hiding places. B: plastic platform. C: straw (Adapted from Rommers *et al.*, 2014).

In summary, there are several strategies to improve the welfare, such as more surface per animal, higher cages, platforms and collective housing, but there is not a conclusive study about the best housing type for rabbit does taking into account the health status together with the performance and behaviour.

1.2.4. Welfare indicators in rabbits

It is necessary to scientifically analyse different parameters to better know the degree of welfare that animals have. In recent years, numerous studies have been carried out to find the best welfare indicators in rabbits. Broom (1986) stated that any parameter (behaviour, litter survival, diseases, hormones, milk production, growth rate, fertility, etc.) is valid and reliable for the evaluation of welfare. However, other authors gave more importance to some indicators than others, such as body condition and morbidity (Sánchez *et al.*, 2012) or mortality and physiology (Hoy and Verga, 2006). In fact, a decrease in productivity associated with high morbidity, mortality, infertility and weight loss, for example, may indicate an important lack of welfare (Hoy and Verga, 2006).

Behaviour is the most common indicator used to assess welfare in the different studies. In some papers behaviour observations are the only indicators used to study the housing characteristics (Hansen and Berthelsen, 2000; López *et al.*, 2004; Mugnai *et al.*, 2009; Alfonso Carrillo *et al.*, 2014; Zomeño *et al.*, 2017). Other studies assess the welfare evaluating the injures between rabbit does together with behaviour patterns (Graf *et al.*, 2011; Rommers *et al.*, 2014; Buijs *et al.*, 2015; Maertens and Buijs, 2016) and other ones also take into account productive performance (Mugnai *et al.*, 2009; Mikó *et al.*, 2014; Zomeño *et al.*, 2018; Dal Bosco *et al.*, 2019). Less frequent are papers assessing welfare using only performance and mortality (Mirabito *et al.*, 2000), health and body conditions (Sánchez *et al.*, 2012) or just injuries (Rosell and De la Fuente, 2009; Buijs *et al.*, 2014; Masthoff and Hoy, 2019). One of the most complete studies about housing and welfare was published by Szendrő *et al.* (2013).

These authors used several indicators: behaviour, productive performance, skin lesions, mortality and faecal corticosterone concentrations. But they only compared one single cage with one collective cage.

Although it is widely demonstrated that stress activates the hypothalamic–pituitary–adrenal (HPA) axis with the consequent release of cortisol and corticosterone to blood (Mormède *et al.*, 2007), this molecule has not been used often to assess rabbit welfare. In other species, common biological samples for the analysis of cortisol are blood, saliva, urine and faeces but in these materials, the levels represent only a timespan of a few minutes up to one or two days (Heimbuerge *et al.*, 2019). In rabbits, serum cortisol levels change with the circadian cycle and with social stress (Szeto *et al.*, 2004), heat stress (De la Fuente *et al.*, 2004) and also vary with the productive stage (Argente *et al.*, 2014). In addition, obtaining the animal's blood can itself cause stress and the increase of cortisol in the blood. Cortisol from faeces was used to confirm the transport as a stressful moment for rabbits, because cortisol levels were higher 16 h after transport (Buijs *et al.*, 2011). Therefore, measuring cortisol from plasma or faeces, provides information about the stress in a specific moment but not about a long-term retrospective cortisol level. The measurement of cortisol from hair may solve these problems, since systemic cortisol incorporates into the growing hair from blood vessels via passive diffusion (Figure I2-3), being a slow accumulation process (Pragst and Balikova, 2006). Comin *et al.* (2012) described the concentration of hair cortisol in the New Zealand White Rabbit at different body sites and now, it is widely accepted that hair cortisol is a good biomarker for chronic stress assessment in animals (Heimbuerge *et al.*, 2019).

Other biomarkers of stress and health are the acute phase proteins (APP). They are a group of blood proteins involved in innate immune response to external or internal challenges, such as infection, inflammation, traumas or stress. They can be used for the diagnosis, prognosis and monitoring of therapies and health status (Eckersall and Bell, 2010). The most important

APP in rabbits are Haptoglobin and Serum Amyloid A because they are very sensitive (Petersen *et al.*, 2004). Haptoglobin significantly raises between the first day and the 7th day after the inoculation of *S. aureus* in rabbits (Dishlyanova *et al.*, 2011), thus it can be used to assess the health status and the presence of inflammation and infection in this species. In addition, Argente *et al.* (2014) reported a positive correlation between cortisol and haptoglobin in lactating females. Therefore, both molecules could be analysed together to evaluate the health and stress status of the female rabbits.

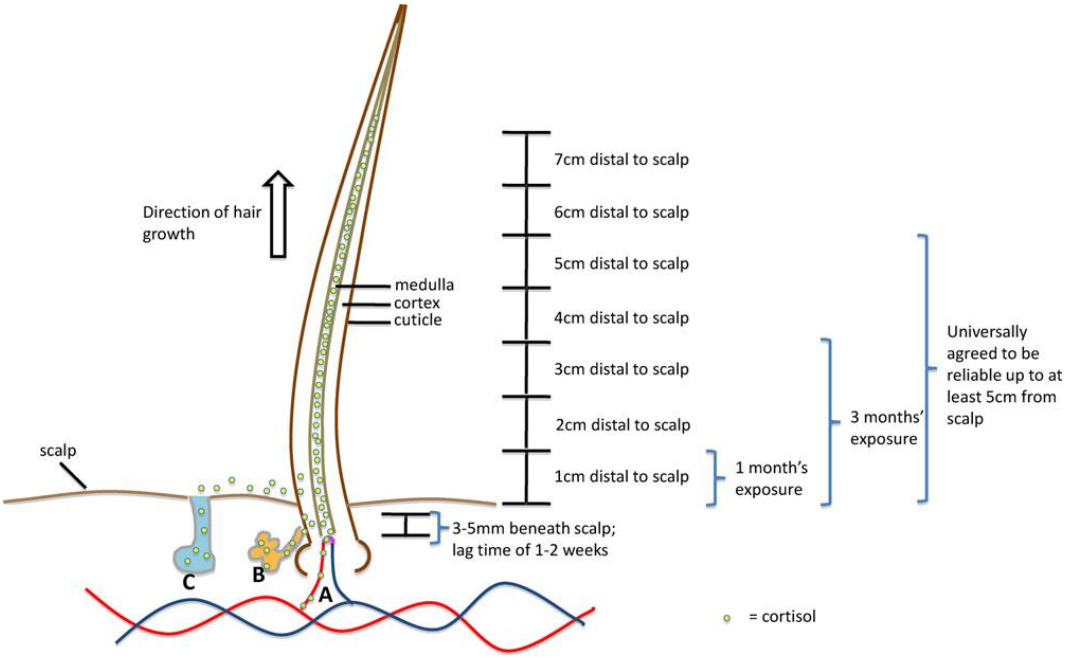

Figure I2-3. Cortisol accumulation into hair (Russell *et al.*, 2012).

Regarding to cellular components of the blood, neutrophils are the most important leukocytes in the innate immune response. In rabbits, neutrophils are called heterophils because they contain eosinophilic granules (Weiss and Wardrop, 2011). On the contrary, lymphocytes are most important leukocytes in the adaptive immune response. Stress and the consequent release of cortisol affects the relative quantity of these two immune cells, resulting in the so-called stress leukogram. This produces an increased heterophils/lymphocyte (H/L) ratio which is easy to determine, and all this makes H/L ratio a good indicator of stress (Davis *et al.*, 2008). However, in

rabbits the range considered normal for neutrophils and heterophils is wider than other mammal species and leukogram must be analysed carefully (Weiss and Wardrop, 2011).

In addition to the number of heterophils, it is also important the functionality, since an increase in glucocorticoids affects the phagocytic activity of heterophils (Goulding *et al.*, 1998; Jeklova *et al.*, 2008). As stress leads to the release of glucocorticoids, the evaluation of phagocytic activity could be a good biomarker of the animal welfare.

In summary, there are several indicators and biomarkers that can be used together to evaluate the stress and health status of the animals in different common housings. Some of them are the behaviour, the mortality and morbidity, the presence of diseases and injuries, the productive performance, the hair cortisol and plasma haptoglobin concentrations, the leukogram and also the heterophil activity. Several housing types and characteristics have already been studied from the point of view of the behaviour, but there is scarce information from the health point of view. According to the results obtained by other groups, the hypothesis of this study was that female rabbits housed in a collective housing have worse health status, while those housed in individual cages have better health status. The comparison of more different housings with more welfare indicators and biomarkers than in previous studies would allow to establish properly the best housing for rabbit does from the point of view of health, stress and productive performance.

II- OBJECTIVES

II- OBJECTIVES

Staphylococcus aureus is a big concern in the current rabbit breeding, mainly by the large number of affected animals and the difficult treatment, which causes great economic losses. Farmers and veterinarians have also warned us about an increase in the frequency and virulence of staphylococcal infections in the rabbit farms in the last years. The population of *S. aureus* is very heterogeneous, and it adapts easily to new environments and hosts, so the knowledge of the strains that are currently affecting these animals is crucial to understand the evolution of this bacterium and find an effective treatment. Therefore, the objectives of the first part of this thesis were the following:

1. To characterize genetically the *S. aureus* isolates obtained from Spanish and Portuguese farms to find new clones or mutations.
2. To analyse the evolution of geographical distribution of *S. aureus* clones.
3. To test *in vitro* and *in vivo* these strains to know whether genetic differences can justify the increase in the frequency and virulence of outbreaks.

The welfare is also another important concern in rabbit production, but there is scarce information and data to assess the best housing system from a health and immunological point of view. For this reason, the objectives of the second part of the thesis were:

1. To characterize the evolution of haematological values and hair cortisol concentrations of commercial rabbit does between the first insemination and the fifth parturition in four different single-housing systems and one group-housing system.
2. To compare the impact of these five housing systems at fifth parturition over rabbit does haematological parameters and hair cortisol concentrations and over kit mortality before weaning.

3. To evaluate morbidity and mortality causes observed in these five housings, including pathological and microbiological studies of the rabbit does.

III- MATERIAL AND METHODS

III- MATERIAL AND METHODS

3.1. Characterization of *S. aureus* isolates from rabbit farms

3.1.1. Sampling

A total of 498 isolates were analysed between 2012 and 2019 (Figure III1-1). Samples were collected by veterinarians from 137 rabbit farms with different staphylococcal problems located in 32 provinces of Spain and Portugal (Table III1-1). Samples were taken with a sterile cotton swab and they were sent to our laboratory. Samples were taken from *S. aureus* compatible lesions, such as suppurative wounds or pododermatitis, from females, males and kits. Animals without lesions were also sampled to check the carrier status. These samples were obtained from nares and ears.

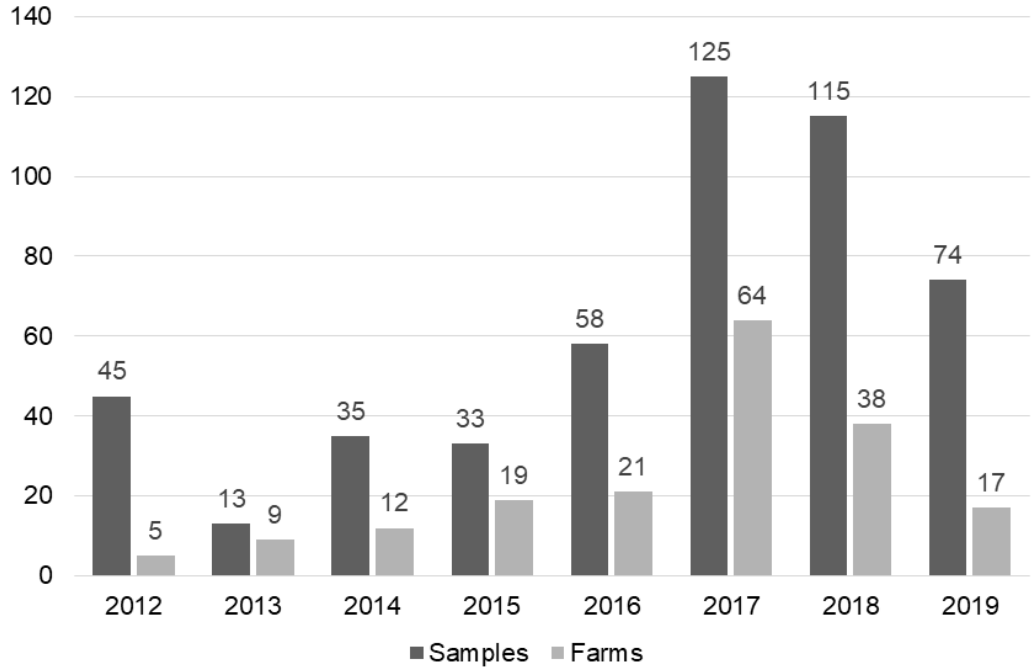

Figure III1-1. Number of isolates and farms sampled from 2012 to 2019.

Table III1-1. Number of samples and farms in each province.

Province	Samples	Farms
A Coruña	20	8
Albacete	26	4
Alicante	3	2
Asturias	5	3
Barcelona	39	16
Burgos	3	2
Castellón	13	6
Córdoba	1	1
Cuenca	14	3
Gerona	3	1
Huesca	2	1
Jaén	2	1
León	51	12
Lérida	24	4
Logroño	2	1
Lugo	3	1
Murcia	1	1
Navarra	4	1
Ourense	6	3
Palencia	6	3
Pontevedra	6	4
Salamanca	2	1
Segovia	6	3
Soria	3	1
Tarragona	61	10
Teruel	9	2
Toledo	1	1
Valencia	126	15
Valladolid	25	9
Vizcaya	4	1
Zamora	3	2
Zaragoza	7	3
Portugal	17	11
Total	**498**	**137**

The samples arrived at our laboratory as briefly as possible and they were plated on blood agar plates and incubated at 37ºC for 24h. Subsequently, colonies compatible with *S. aureus* were cultured in tryptone and soybean broth (TSB) and incubated 24 hours at 37ºC with shaking. These cultures were kept with 75% glycerol at -80ºC and DNA extraction was also carried out from the liquid culture using the Genelute Bacterial Genomic DNA (SIGMA) kit following the manufacturer's protocol, previously lysing the cell wall with 12.5 µg/mL of lysostaphin for one hour at 37ºC. Once the DNA was obtained, different typing techniques were performed.

3.1.2. Molecular typing

The polymorphic regions of the genes *coa*, *spa* and *clfB* were amplified by polymerase chain reaction (PCR) and subsequently the products obtained from *coa* and *spa* were digested to analyse its polymorphism in the restriction fragments (RFLP) (Viana *et al.*, 2007).

A 2720 thermal cycler (Life Technologies) was used to perform the PCRs. The reaction mixtures contained 2.5 µL of 10X DreamTaq Buffer, 200 µM of each of the nucleotides (dATP, dCTP, dGTP and dTTP), 200 nM of each of the primers, 0.5 U of DreamTaq® Polymerase (ThermoFisher), 40 to 80 ng of purified DNA and water up to a final volume of 25 µL.

PCR amplification of the *coa* gene was carried out according to the procedure described by Hookey *et al.* (1998), with some modifications. For this, the oligonucleotides coa-1m and coa-2c (Table III1-2) were used at a concentration 5 times higher than usual. The PCR conditions consisted of an initial denaturation step at 94ºC for 3 minutes, followed by 35 cycles at 94ºC for 30 s, 52ºC for 30 s and 72ºC for 60 s, with a final step at 72ºC for 10 minutes.

PCR amplification of the *spa* gene was performed according to the procedure described by Viana *et al.* (2007), but with modifications. The oligonucleotides spa-1m and spa-2c (Table III1-2) were used. The PCR conditions consisted of an initial denaturation step at 94ºC for 3 minutes,

followed by 35 cycles at 94ºC for 30 s, 50ºC for 30 s and 72ºC for 90 s, with a final step at 72ºC for 10 minutes.

Finally, the variable region of *clfB* gene was performed using the procedure described by Koreen *et al.* (2005), employing oligonucleotides clfB-1m and clfB-2c (Table III1-2). The PCR conditions consisted of an initial denaturation step at 94ºC for 3 minutes, followed by 35 cycles at 94ºC for 30 s, 56ºC for 30 s and 72ºC for 60 s, with a final step at 72ºC for 10 minutes.

An aliquot of 5 µL of the PCR product of each gene was analysed by electrophoresis on a 2% agarose gel in presence of Red Safe (Intron) and the gels were photographed with ImageQuantTM LAS 4000 under UV light. The size of the bands was compared with the controls and the markers in each side of the gel. Therefore, each band was named as described by Viana *et al.* (2007).

Subsequently, an analysis of the polymorphism of the restriction fragments of the *coa* and *spa* genes was carried out (Hookey *et al.*, 1998). For this, the *coa* and *spa* PCR products were purified with the GenElute™ PCR Clean-Up Kit (Sigma) and were digested with 5 U of the Hin6I restriction enzyme (ThermoFisher) at 37ºC for 1.5 hours. The result of the digestion was analysed by electrophoresis in a 2% agarose gel in the presence of Red Safe (Intron) and the gels were photographed with ImageQuantTM LAS 4000 under UV light.

3.1.3. Multilocus Sequence Typing (MLST).

With the same DNA obtained from the strains, another typing method called Multilocus Sequence Typing (MLST) was performed according to Enright *et al.* (2000). The purpose of this was to be able to compare our strains with the rest of the strains in other studies. The amplification was carried out by PCR of seven genes that code for metabolic enzymes: *arc* (Carbamate kinase), *aro* (Shikimate dehydrogenase), *glp* (Glycerol kinase), *gmk* (Guanylate kinase), *pta* (Phosphate acetyltransferase), *tpi* (Triose-phosphate isomerase), and *yqi* (Acetyle coenzyme A acetyltransferase).

The PCR program consisted of an initial denaturation step at 94ºC for 3 minutes, followed by 35 cycles at 94ºC for 30 s, 54ºC for 30 s and 72ºC for 60 s, with a final step at 72ºC for 10 minutes. As in the previous technique, an aliquot of 5 µL of the PCR product of each gene was analysed by electrophoresis on a 2% agarose gel in the presence of Red Safe and the gels were photographed with ImageQuantTM LAS 4000 under UV light. The PCR of the seven loci of MLST can be observed in Figure III1-2, where there is an isolate, the positive control and the negative control for each gene.

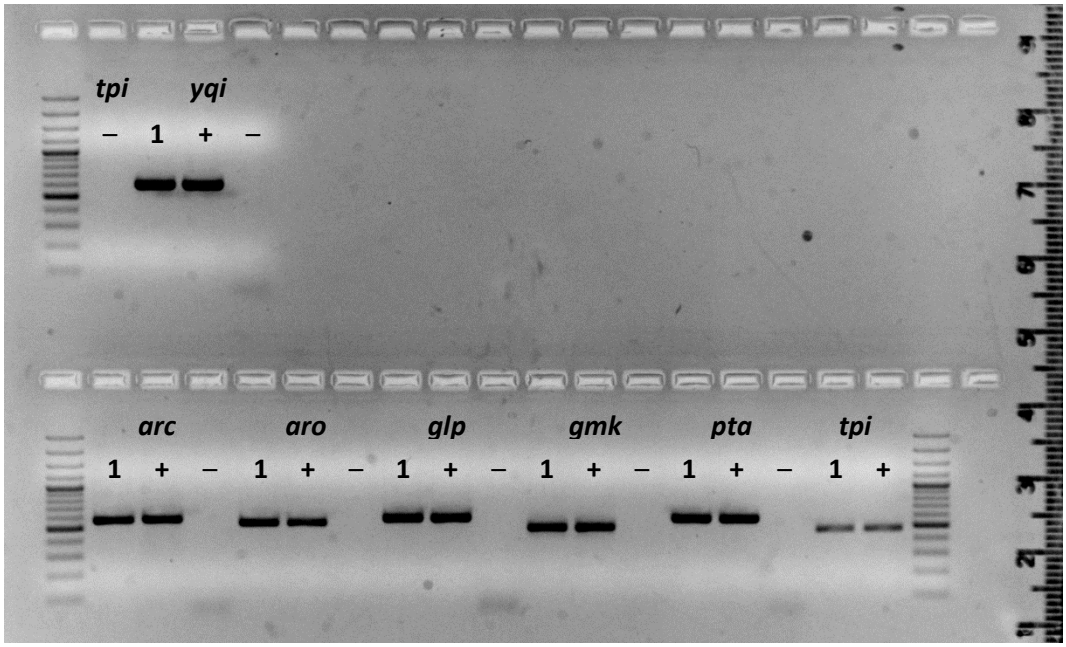

Figure III1-2. Gel electrophoresis of the seven PCRs of the MLST typing. (1): sample; (+): positive control; (–): negative control.

The PCR products were purified and sequenced by the classical Sanger sequencing of the Sequencing Service of the de Biología Molecular y Celular de Plantas de la Universidad Politécnica de Valencia (IBMCP-UPV). The obtained loci sequences were compared with those already existing in the centralized database "https://pubmlst.org/saureus/", obtaining an allelic profile or Sequence Type (ST).

3.1.4. Complete genome sequencing

Thirty representative strains with different genotypes and ST were selected to perform the complete genome sequencing. First of all, the strains were

streaked in TSA (tryptone and soy agar) plates from the stock tube and incubated at 37ºC for 24 hours to obtain an isolated colony. An isolated colony was again streaked in TSA and incubated at 37ºC for 24 hours and then all the bacteria in the second plate were collected with a loop and homogenised in the specific tubes previously sent by the sequencing laboratory. In addition, information on the strains was filled out in the laboratory's website (https://microbesng.com/).

Once the sequences were obtained, the genetic characteristics of the MGE were studied, as well as the presence of different virulence factors, toxins and host adaptation genes, using different informatics tools: the RAST (Rapid Annotation using Subsystem Technology, http://rast.theseed.org/), NCBI's BLAST tool (https://blast.ncbi.nlm.nih.gov/) and computer programs to visualize genomes. The genome information was divided into different fragments, called contigs, then some PCRs were performed in order to join few of them (Table III1-2). This PCR products were sent to sequence to Eurofins Genomics.

3.1.5. Prophage induction and titre

The induction of the prophages was performed to check whether they were functional and able to lyse the bacteria. To perform this, the different strains were grown in TSB o/n at 37ºC with shaking and then 1/50 dilutions were made in TSB and they were grown at 37ºC until an OD_{540}=0.3. Subsequently, 2 µg/mL mitomycin C (Sigma) were added to each culture and they were grown in slow agitation (80 rpm) at 32ºC. After 4 hours, the cultures were removed from the incubator and they were kept at room temperature until the next day, when the obtained lysates were filtered with 0.2 µm filters to eliminate bacterial debris. All cultures were filtered regardless of whether they had been lysed or not (Figure III1-3). Finally, these lysates were stored at 4ºC until use. The strain Jwt (Vancraeynest *et al.*, 2006) was used as negative control and phage 80α (Úbeda *et al.*, 2009) as positive control.

As there were some phages that were not able to infect the RN4220 strain and, therefore, the title could not be compared among all the strains, another strain sensitive to most phages was used to perform the titre, the Sa1039 (Goerke *et al.*, 2009). The Sa1039 was grown in TSB o/n at 37ºC with shaking and then 1/50 dilutions were made in TSB and they were grown until an OD_{540}=0.34. While the culture was growing, the different lysates were diluted in phage buffer (1 mM $MgSO_4$ + 100 mM NaCl + 0.05 Tris pH 7.8 + 4 mM $CaCL_2$) up to 10^{-8}. Subsequently a 10 mL tube and a base agar plate (20 g/L Nutrient Broth nº2 (Oxoid) + 7 g/L agar + 10mM $CaCl_2$) for each phage and dilution were prepared.

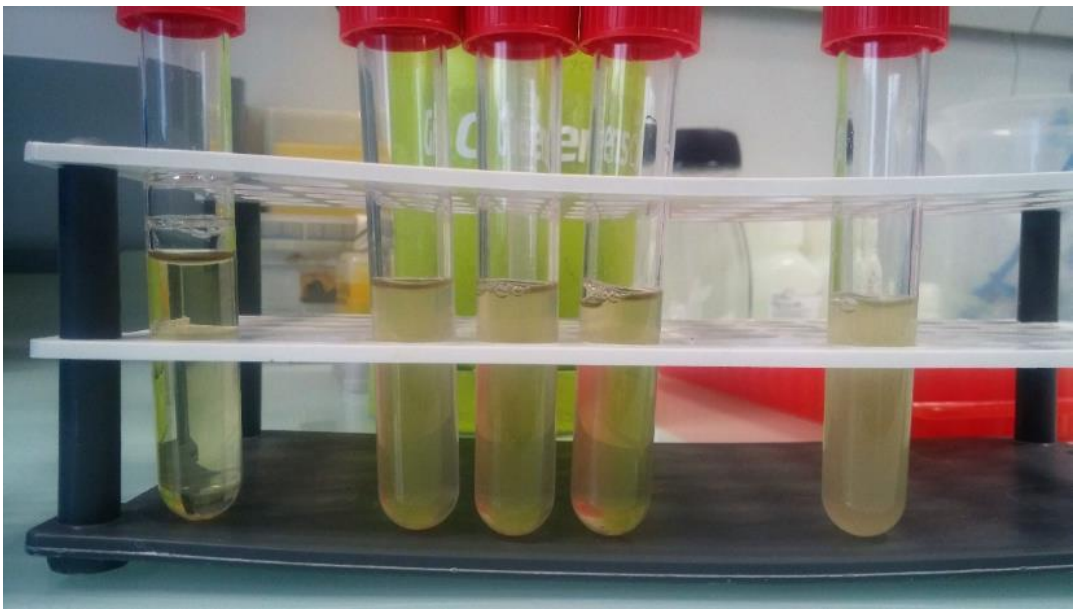

Figure III1-3. Bacterial cultures after induction with Mitomycin C. Left: completely lysed. Centre: partially lysed. Right: not lysed.

Once the culture was grown, 50 µL of it were put together in each tube with 100 µL of the lysates and incubated 10 min at room temperature. After this, 3 mL of Phage Top Agar (20 g/L Nutrient Broth nº2 (Oxoid) + 3.5 g/L agar + 10 mM $CaCl_2$) at 55ºC were added to each tube and the mixture was immediately pour over the surface of the phage base plate. The plates were kept about 30 minutes without flipping at room temperature until the phage top agar was solidified and then they were put inside the 37ºC incubator 24h. The next day the number of lysis spots produced by the phage were

quantified and multiplied by the dilution factor to know the number of viral particles.

3.1.6. Detection of prophages by PCR

In addition to seeking mobile genetic elements through genetic sequencing, a multiplex PCR was also carried out, as described by Goerke *et al.* (2009) to identify prophages with the most common integrases, i.e.1, 2, 3, 4, 5, 6 and 7. The multiplex PCR was done in 121 isolates with different ST. These 121 isolates were different to those used for complete genome sequencing. Thus, the presence of bacteriophages was studied in a total of 151 isolates. The primers used are listed in Table III1-2 (Goerke *et al.*, 2009).

3.1.7. *In vitro* tests

- Haemolysis in blood agar plates: this test was performed to know which haemolysins the sequenced strains were expressing. It was following the protocol described by Traber *et al.* (2008). Briefly, an isolated colony of the strain RN4220 was inoculated on Columbia agar forming a line in the centre of the plate. Then, the strains to be tested were inoculated forming perpendicular lines to the RN4220 without touching each other. These plates were cultivated 24 hours at 37ºC. Finally, they were left 24 hours at 4ºC to better observe the produced haemolysis and therefore the result was read (Figure III1-4).
- Coagulase assay: this test was performed to check the coagulation capacity of the strains. Rabbit, and human plasma were obtained in tubes with EDTA by centrifugation 10 min at 2500 rpm. The *S. aureus* inoculum was obtained from an overnight culture by centrifugation and then diluted in TSB to achieve 10^8 bacteria inoculum. Coagulase assay was performed in glass tubes by mixing 300 µL of plasma with the *S. aureus* inoculum. The tubes were incubated at 37ºC, and the coagulation was verified by tilting the tubes each half an hour. A positive test resulted in a formation of a clot before 24 h of incubation.

Table III1-2. Primers used in this study

Name	Sequence (5´-3´)	Reference
coa-1m	ATAGAGATGCTGGTACAGG	Hookey *et al.*, 1998
coa-2c	GCTTCCGATTGTTCGATGC	Hookey *et al.*, 1998
spa-1m	GATTTTAGTATTGCAATACATAATTCG	Viana *et al.*, 2007
spa-2c	CCACCAAATACAGTTGTACCG	Viana *et al.*, 2007
clfB-1m	CAGCAGTAAATCCGAAAGACCC	Koreen *et al.*, 2005
clfB-2c	CACCTTTAGGATTTGATGGTGC	Koreen *et al.*, 2005
arc-1m	TTGATTCACCAGCGCGTATTGTC	Enright *et al.*, 2000
arc-2c	AGGTATCTGCTTCAATCAGCG	Enright *et al.*, 2000
aro-1m	ATCGGAAATCCTATTTCACATTC	Enright *et al.*, 2000
aro-2c	GGTGTTGTATTAATAACGATATC	Enright *et al.*, 2000
glp-1m	CTAGGAACTGCAATCTTAATCC	Enright *et al.*, 2000
glp-2c	TGGTAAAATCGCATGTCCAATTC	Enright *et al.*, 2000
gmk-1m	ATCGTTTTATCGGGACCATC	Enright *et al.*, 2000
gmk-2c	TCATTAACTACAACGTAATCGTA	Enright *et al.*, 2000
pta-1m	GTTAAAATCGTATTACCTGAAGG	Enright *et al.*, 2000
pta-2c	GACCCTTTTGTTGAAAAGCTTAA	Enright *et al.*, 2000
tpi-1m	TCGTTCATTCTGAACGTCGTGAA	Enright *et al.*, 2000
tpi-2c	TTTGCACCTTCTAACAATTGTAC	Enright *et al.*, 2000
yqi-1m	CAGCATACAGGACACCTATTGGC	Enright *et al.*, 2000
yqi-2c	CGTTGAGGAATCGATACTGGAAC	Enright *et al.*, 2000
Protint7-1m	AGGATCTCCGACATTGACC	This study
sakint7-2c	ATGAGAATAATAACAATAAAACAG	This study
sakint7-3m	GGATTCAACTTAATTACAAAGG	This study
lsdB-4c	CATTCGTATTACCTAGAAAACG	This study
Holinint3-1m	AAGCTTTAGAATATGAAGAACC	This study
Lysinint3-5m	AGTTTTGGTAAGTTTAGTGC	This study
scnint3-6c	TAAAACGATTGCTAAAGTTCC	This study
int1-F	AAGCTAAGTTCGGGCACA	Goerke, 2009
int1-R	GTAATGTTTGGGAGCCAT	Goerke, 2009
int2-F	TCAAGTAACCCGTCAACTC	Goerke, 2009
int2-R	ATGTCTAAATGTGTGCGTG	Goerke, 2009
int3-F	GAAAAACAAACGGTGCTAT	Goerke, 2009
int3-R	TTATTGACTCTACAGGCTGA	Goerke, 2009
int4-F	ATTGATATTAACGGAACTC	Goerke, 2009
int4-R	TAAACTTATATGCGTGTGT	Goerke, 2009
int5-F	AAAGATGCCAAACTAGCTG	Goerke, 2009
int5-R	CTTGTGGTTTTGTTCTGG	Goerke, 2009
int6-F	GCCATCAATTCAAGGATAG	Goerke, 2009
int6-R	TCTGCAGCTGAGGACAAT	Goerke, 2009
int7-F	GTCCGGTAGCTAGAGGTC	Goerke, 2009
int7-R	GGCGTATGCTTGACTGTGT	Goerke, 2009

Figure III1-4. Haemolysis produced by two strains of *S. aureus* in Columbia agar after 24 hours at 4ºC.

3.1.8. *In vivo* characterization

An intradermal infection was performed to know the infectivity of 6 clinical isolates. These strains were a recent ST96 isolate, two ST2855 isolates from two different areas, two ST3764 also from two different regions and one ST398 isolate (Table III1-3). Jwt was used as positive control and DL9 as negative control.

Sixty-four 2-month-old New Zealand rabbits (*Oryctolagus cuniculus*) were used, each one inoculated with the two controls and one tested strain. Animals were housed under conventional conditions in individual housings and they were fed with a white commercial diet *ad libitum*. The handling and care of the animals were carried out following the international regulations of the European Economic Community ("Order 86/609/CEE"). The experimental tests carried out were approved by the ethical committee of the Universidad CEU Cardenal Herrera and the Consellería d'Agricultura, Pesca i Alimentació, Generalitat Valenciana (permit number 2011/010).

Table III1-3. Strains and inoculum used for infections.

Strain	Lesion	Region	MLST	Genotype	Inoculum (CFU/100 µL)	Reference
Jwt	Unknown	France	121	A1 II1 δ	640	Vancraeynest *et al.*, 2006
766	Mastitis	León	3764	A1 II1 κ	710	This study
1151	Abscess	Valencia	3764	A1 II1 κ	850	This study
DL9	Mastitis	Valencia	96	B1 IV2 α	630	This study
908	Pododermatitis	Valencia	96	B1 I1 λ	900	This study
862	Dermatitis	León	2855	B1 IV2 α	400	This study
991	Metritis	Portugal	2855	B1 IV2 α	660	This study
842	Mastitis	Barcelona	398	F4 IV3 -	330	This study

To perform the experimental procedure, animals were sedated with a combination of ketamine (Imalgene®, 100 mg/mL, Merial) and xylazine (Xilagesic®, 200 mg/mL, Calier). After that, a 20x15 cm area of the dorsal-lumbar region was shaved, disinfected with ethanol and inoculation points were drawn with permanent marker (Figure III1-5). Each strain was intradermally inoculated in duplicate with a 25G needle. The inoculum ranged between 300 and 900 CFU in 100 µL of DPBS (D8537, Sigma). The general status of the animals and the size and characteristics of the lesions (presence of erythema, skin elevation, nodules, dermo-necrosis and ulceration) were recorded daily for 7 days.

Animals were euthanized at the 7th day post inoculation by an intravenous injection of T-61® (MSD Animal Health) and a complete necropsy was carried out. Skin samples from the inoculation site were taken with a scalpel after sterilization of the area with alcohol to perform the microbiological studies. Then, samples were weighed, crushed and homogenized in 1 mL of sterile DPBS (D8537, Sigma). Serial dilutions were made and plated on Columbia agar. These plates were incubated 24 h at 37ºC and the CFU were counted in those plates that had between 30 and 300 colonies.

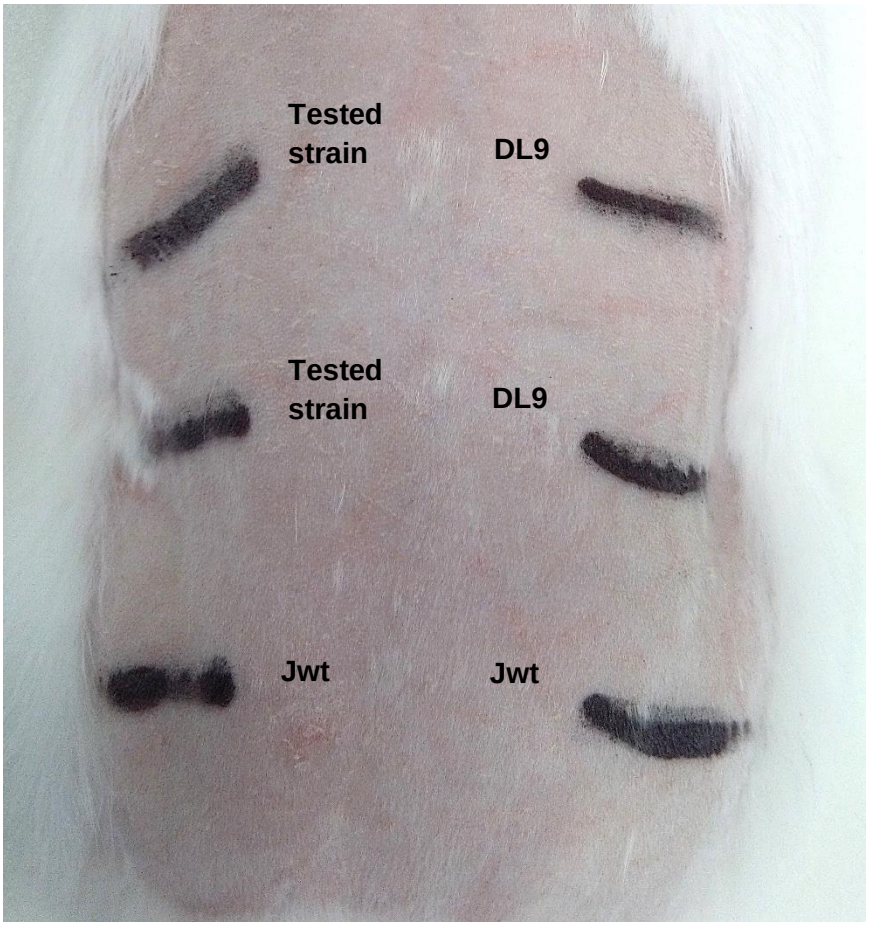

Figure III1-5. Rabbit dorsal-lumbar region shaved and marked prior to inoculation.

3.1.9. Statistical analysis

First, the percentage of infected animals with each strain was compared using a Chi Square test. Subsequently, a General Lineal Model (GLM) analysis was carried out including the strain as fixed effect to compare the lesion size and the CFU/g between the different strains. To perform this, those inoculation points that had not developed an injury after 7 days were considered an absent data.

3.2. Effect of different housing systems on health and welfare traits of commercial female rabbits

3.2.1. Animals and housings

One hundred and fifty 19-week-old breeding female rabbits were used. They were placed in five different cage types one week before the first artificial insemination (1AI). These cages were located in the facilities of the Universitat Politècnica de València (UPV) and the study was carried out in 2016 and 2017 in collaboration with the Animal Science Department of UPV.

All the cages had wire floors and footrests were placed inside all the cage types. The detailed characteristics of the five cage types are shown in Table III2-1. The five housing systems were three single-housing systems with different total floor surface areas and cage heights: [24 polyvalent (Figure III2-1) (PV; 3,920 cm^2 and 38 cm high); 24 higher and deeper (Figure III2-1) (HD; 5,250 cm^2 and 50 cm high); 24 traditional (Figure III2-2) (TR; 4,270 cm^2 and 38 cm high)]; 24 single-housing with wire platform (Figure III2-2) (PF; 4,960 cm^2 and 57 cm high); 4 collective systems for six females (Figure III2-3) (COL; 4,980 cm^2 and 65 cm high). In the collective system, female rabbits were placed in groups of six which, between 28 days of gestation and 18 days post-partum (dpp), were separated into individual compartments (41 cm wide; Figure III2-3).

Female rabbits and kits were monitored throughout five reproductive cycles or until they died or were culled. When a female rabbit died or was culled, another 19-week-old animal was placed in its cage to achieve 30 animals per group. A reproductive management rhythm of 42 days was used, with insemination at 11 dpp and weaning at 28 dpp.

The average temperatures were between 16ºC and 21ºC.The lighting programme was 16 h of light and 8 h of dark, and the building was artificially ventilated. Animals had free access to fresh water through automatic drinkers and were fed with a commercial pelleted diet *ad libitum* (chemical

composition: 91% dry matter (DM) and 8% ash; 17% crude protein, 34% neutral detergent fibre, 17% acid detergent fibre and 3.2% lignin).

The experimental proceedings were approved by the competent authority (Generalitat Valenciana, Spain) as set out in Spanish Royal Decree 53/2013 on protection and use of animals for experiments.

Table III2-1. Characteristics of the 5 types of housings.

	Polyvalent (PV)	**Higher and deeper (HD)**	**Traditional (TR)**	**With platform (PF)**	**Collective (COL)**
Width (cm)	40	50	50	40	41*
Depth (cm)	98	85	70	98	100
Height (cm)	38	50	38	57	65
Nest (cm)	40x25x47	40x25x37	35x22x37	40x22x39	40x22x37
Type of nest	Internal	External	External	Internal	Internal
Platform	No	No	No	40x26x35	40x22x30
Area (cm^2)	3920	5250	4270	4960	4980

* Six compartments with 41cm width that joined together between 18 days post-partum and 28 days of gestation.

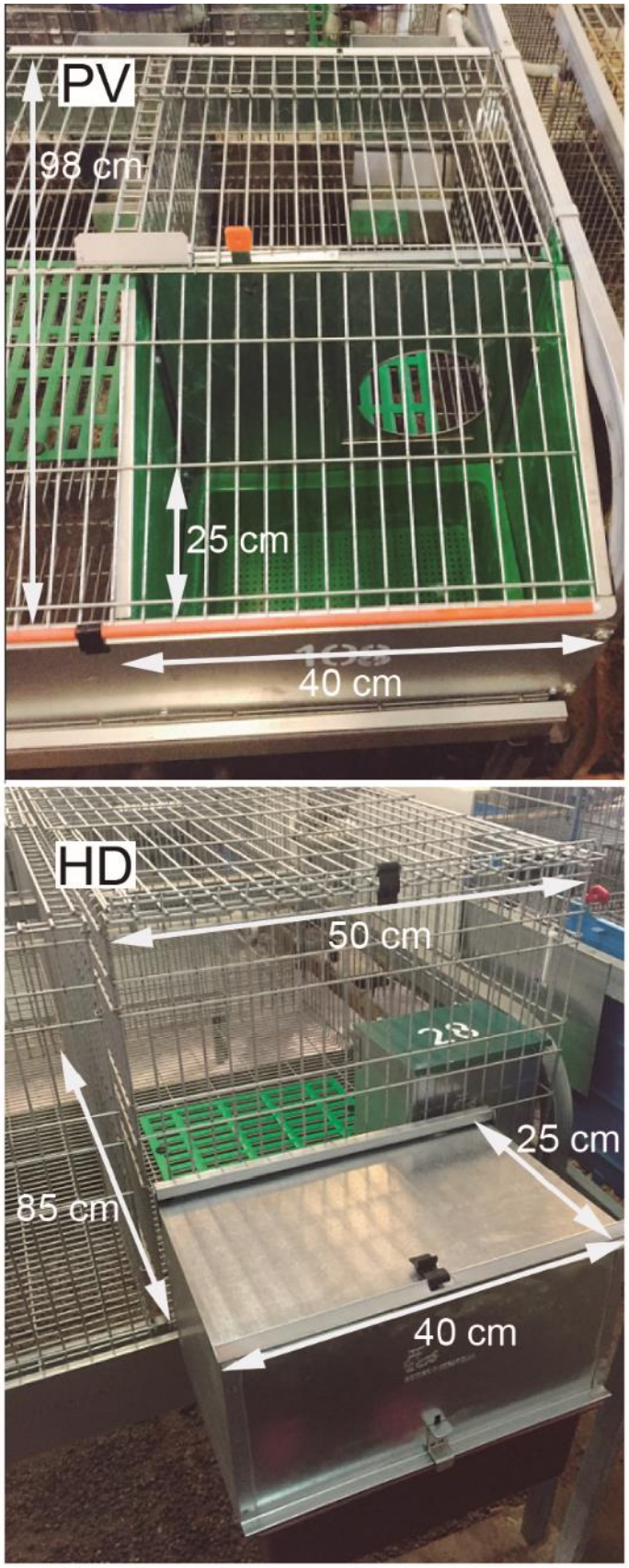

Figure III2-1. Pictures of Polyvalent (PV) and Higher and deeper (HD) cages.

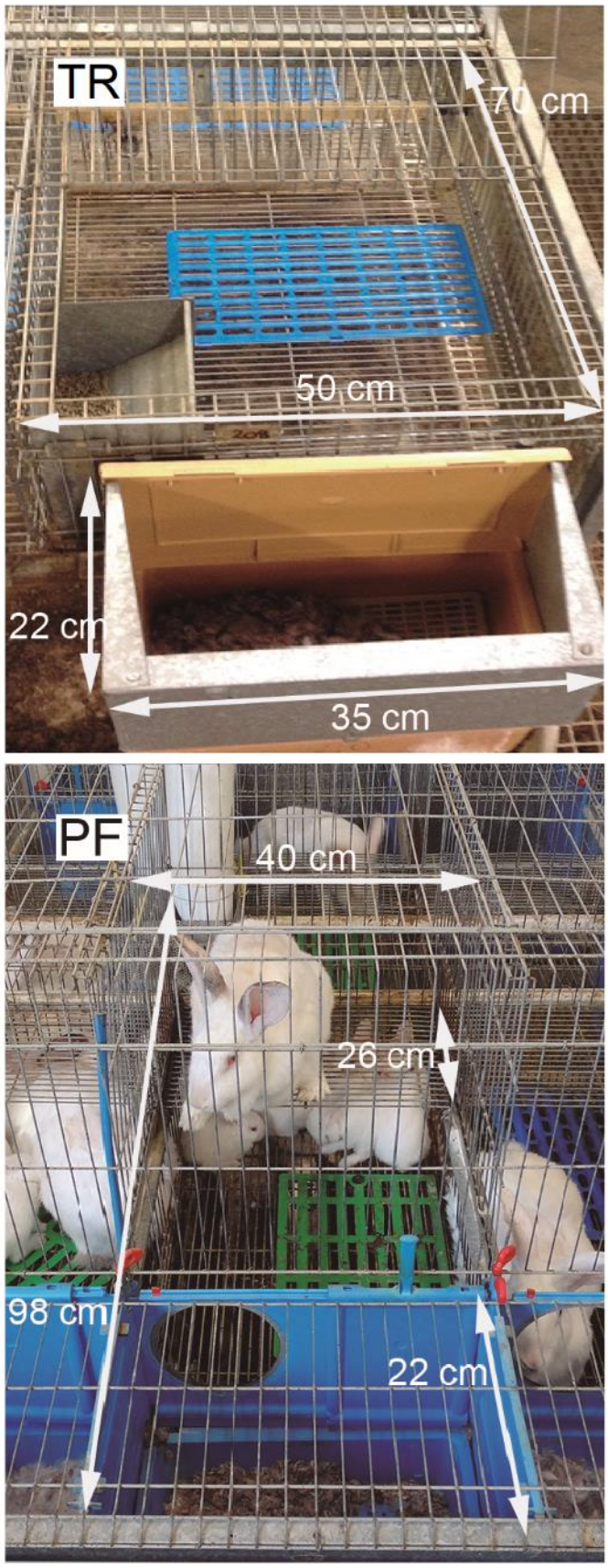

Figure III2-2. Pictures of Traditional (TR) and Platform (PF) cages.

Figure III2-3. Pictures of Collective housing without separations (COL-1) and separated between 28 gestation days and 18 days post-partum (COL-2).

3.2.2. Blood and Immune system assessments

Between 19 and 22 female rabbits were sampled per group at first insemination (1AI) and between 16 and 17 females at fifth parturition (5P) to analyse the haematological parameters. Nine ml of blood were drawn from the median artery of each animal's ear using tubes with EDTA (Figure III2-4A) to carry out different analyses:

- Blood cell counts (erythrocytes, platelets and white blood cells), haematocrit and haemoglobin were analysed by a haematology analyser cell counter (MEK-6410, Nihon Kohden, Japan). Blood tubes were gently turned 5 times before analysis. The intra-assay CV of haematology analyser was 5.3%.
- The differential leukocyte counts were obtained from Giemsa-stained blood extensions over a slide glass by counting 200 leukocytes for each animal with an optical microscope (Figure III2-5).
- Haptoglobin concentration was determined from blood plasma, which was extracted from whole blood by centrifugation at 2,500G. Subsequently, plasma was sent to an external laboratory and there it was analysed by a colorimetric assay (Phase Range, Tridelta Developments Ltd, Ireland) according to manufacturer's protocol. Intra- and inter-assay CV were 1.0% and 2.1%, respectively. In the detection limit tests, the samples containing 0.10 mg/ml were significantly different from the 0- samples (n=12; $P<0.001$).
- *In vitro* cell function test: the phagocytic capacity of heterophils, as a representative cellular type of innate immunity, was evaluated. Heterophils purification was carried out according to the protocol described before by Siemsen *et al.* (2014) with some modifications. Briefly, 5 mL of whole blood were lysed with 45 mL of ammonium chloride lysing solution for 6 min at room temperature in the dark. Tubes were centrifuged at 400 g for 5 min at room temperature without brake. The supernatant was eliminated, and the pellet was carefully resuspended in 5 mL of Rabbit Neutrophil Buffer (RNB). The suspension was transferred carefully with a Pasteur pipette over 7 mL of

Histopaque® 1077 (Sigma) in a 50-mL tube and different washes were performed as described in this protocol. Once the heterophils were purified (Figure III2-4B), the number of cells was calculated by counting with a Neubauer chamber. Then they were subjected to FITC-stained *Staphylococcus aureus* for 30 min. The final bacteria:neutrophils ratio was 20:1. After 30 min, phagocytosis was stopped by adding 1 mL of cold DPBS (D8537, Sigma). Then tubes were centrifuged at 420 g for 10 min at 4ºC. The supernatant was eliminated, and the pellet was resuspended in 200 µL of DPBS (D8537, Sigma). Ten µL of this suspension were placed over a slide and fixed with 4% paraformaldehyde. The cell cytoplasm was stained with Rhodamine Phalloidin® (Thermo Fisher) and the nucleus with DAPI (P36941, Invitrogen) (Figure III2-6). The phagocytosis ratio was calculated by fluorescence microscopy (Leica) by counting 100 cells.

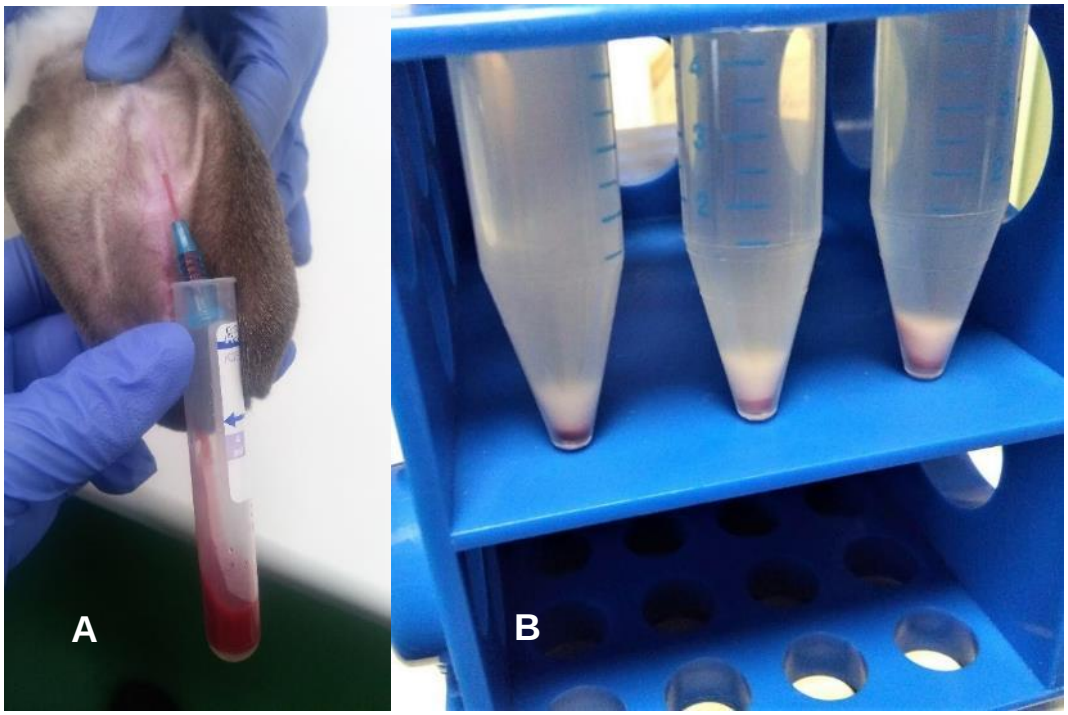

Figure III2-4. A: collection of blood from ear central artery. B: purified neutrophils after washings and centrifugations.

Cortisol concentration was also measured from the hair to evaluate the chronic stress according to Tallo-Parra *et al.* (2015). Briefly, at first insemination hair was shaved from the front of the head of all the does and

then again at fifth weaning. Samples were processed to extract the cortisol and finally, an ELISA assay (Ref: 402710, Neogen) was performed to know the concentration of cortisol. This determination was carried out at the laboratory of the Institute of Animal Science and Technology of the Universitat Politècnica de València.

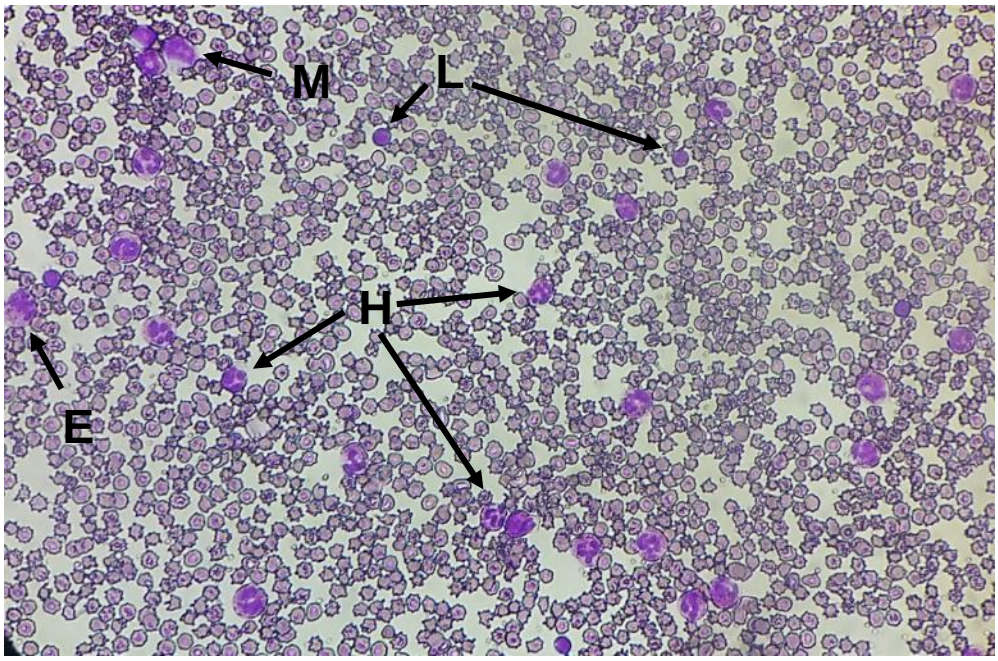

Figure III2-5. Microscopic image of the blood extension with Giemsa staining. L: lymphocyte; H: heterophil; M: monocyte; E: eosinophil.

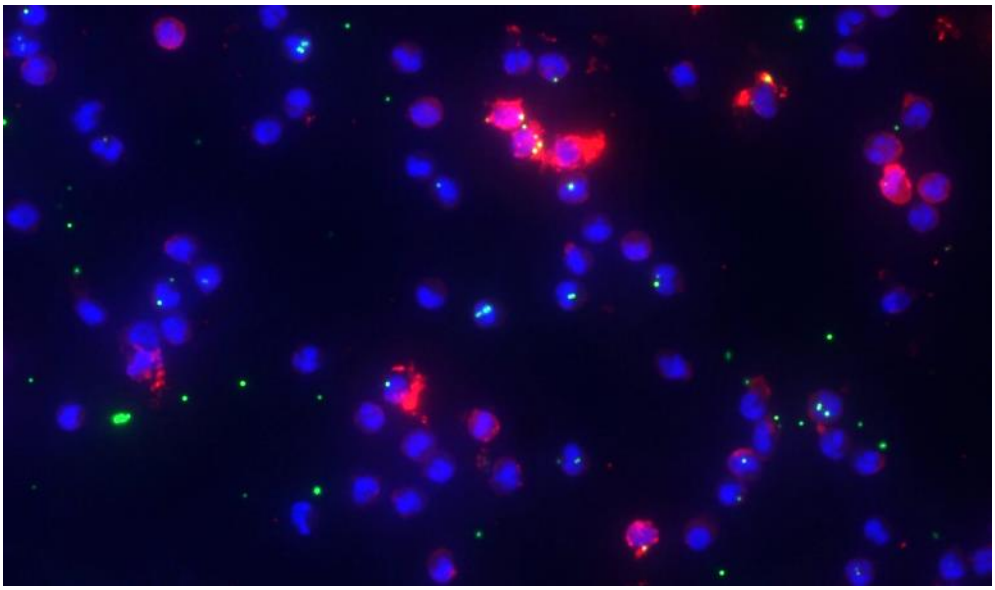

Figure III2-6. Microscopic image under fluorescence light of heterophils (blue) against *S. aureus* (green).

3.2.3. Pathological studies

All the culled and dead female rabbits during the study were evaluated. Females were culled for either evident lesions that seriously affected animal welfare (e.g. severe pododermatitis, mastitis, abscesses and bleeding injuries) or clinical reproductive problems, such as three negative artificial inseminations, dystocia or abortions. Animals were euthanized by an intravenous injection of T-61® (MSD Animal Health) and a complete necropsy was carried out. Samples from all organs showing lesions were taken both for histopathological and microbiological studies.

For histopathological studies, a piece of the affected tissue was fixed in 10% buffered formalin for two days. Then they were processed routinely and stained with haematoxylin-eosin. Gram and Red Congo stains were also performed whenever necessary. Stained samples were evaluated under an optical microscope (Nikon Eclipse E600) and photographs were taken with a digital camera (Nikon DXM 1200). The percentage of kit mortality during lactation was also recorded in each group.

3.2.4. Microbiological studies

Samples from the observed lesions were taken using sterile swabs. These swabs were immediately sown in Columbia blood agar plates and Salt Manitol agar plates and they were incubated at 37ºC for 24 h. The presence of *S. aureus* and *Pasteurella multocida* was studied based on the morphological appearance of colonies (Figure III2-7). Those compatible with *S. aureus* were sown in tryptone and soya broth (TSB) and were incubated 24 h at 37°C with shaking. Then DNA was extracted using the Genelute Bacterial Genomic DNA kit (Sigma) according to the manufacturer's protocol, except that bacteria were lysed by lysostaphin (12.5 mg/mL, Sigma) at 37ºC for 1 h before DNA purification.

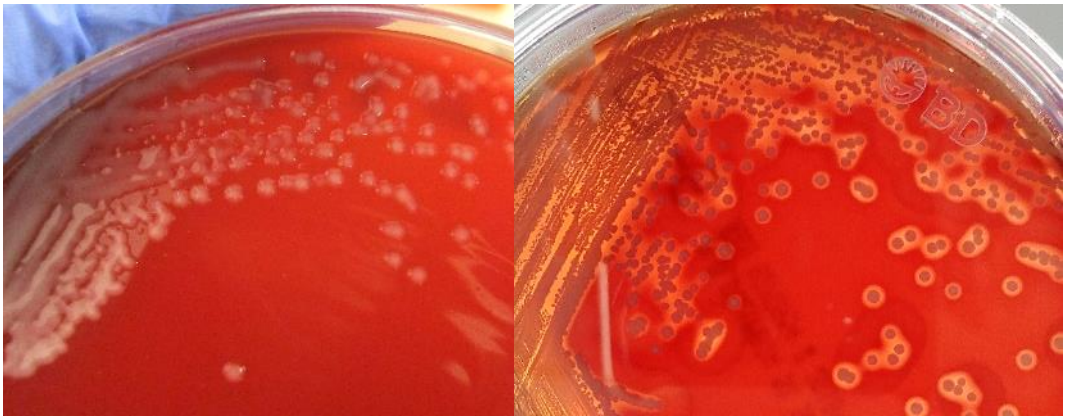

Figure III2-7. Bacterial cultures in blood agar plates. Left: *Pasteurella multocida*. Right: *Staphylococcus aureus* with different haemolysis.

Molecular typing was performed as previously described by Viana *et al.* (2007). Briefly, PCR of genes *coa*, *spa* and *clfB* (Table III1-2) and digestion of *coa* and *spa* products with Hin6I were performed. The PCR and digestion products were run into a 2% agarose gel in presence of Red Safe (Intron) and gels were photographed with ImageQuantTM LAS 4000 under UV. The MLST was also performed as explained in section 3.1.3. Then allelic profiles and the corresponding sequence types (ST) were determined from the MLST database.

To confirm any other microorganisms that differed from *S. aureus*, such as *P. multocida* and others frequently isolated, they were sent to a microbiological analysis laboratory. Isolates were analysed with Gram staining and biochemical tests: catalase, oxidase, indole, urea, citrate, triple sugar iron agar, Slanetz Barley, esculin and the CAMP test.

3.2.5. Statistical analysis

The leukocyte and haptoglobin values were normalised by applying logarithms due to the asymmetrical distribution of the original data, except for the heterophils/lymphocytes ratio (H/L), which was obtained directly from counts. These data were analysed by the MIXED procedure (SAS Institute, 2002), in a repeated measure design which allows variance among animals and the intra-animal covariance to be considered. Random terms included the permanent effect of each animal (*p*) and the error term (*e*), both

assumed to have an average of zero and a variance of σ^2 and σ^2, respectively. The model included the cage type and sampling time as fixed effects, as well as the permanent effect of the animal and the random effect.

$$Y = cage_i + time_j + cage_i \times time_j + p_k + e_{ijk}$$

Where “cage” is the type of experimental cage tested (5 different cage types), “time” the sampling time (first insemination or fifth parturition), “anim” the identification of the animal, and “Y” the trait studied.

The binomial data such as causes of culling and death, kit mortality, lesions and microbiology were analysed using a generalized linear model (GENMOD procedure) following a binomial distribution with a logistic regression. A least square means comparison test (LSMEANS) was carried out to determine differences between treatments.

IV- RESULTS

IV- RESULTS

4.1. Characterization of *S. aureus* isolates from rabbit farms

4.1.1. Genotypic characterization

A total of 498 samples were analysed. These samples were isolated from a wide diversity of lesions (Table IV1-1). The most frequent lesions were mastitis (n=209; 41.97), the second were pododermatitis (n=65; 13.05%) and the third were abscesses (n=46; 9.24%).

Table IV1-1. Origin of the samples

Source	Total	Percentage
Mastitis	209	41.97
Pododermatitis	65	13.05
Abscesses	46	9.24
Pyoderma	30	6.02
Pneumonia	19	3.82
Conjunctivitis	18	3.61
Metritis	16	3.21
Otitis	15	3.01
Hepatitis	4	0.80
Necrosis	4	0.80
Rhinitis	3	0.60
Other lesions	10	2.01
Carriers	59	11.85
Total	498	

After molecular genotyping by the method described previously by Viana *et al.* (2007), 45 different genotypes were obtained. Some of them had been previously described, while others were new. The most common genotype was the A1 II1 κ, corresponding to the 21.29% of the strains and the second was A1 II1 δ, reaching the 18.47% of the isolates (Table IV1-2).

Table IV1-2. Genotypes found in this study, sorted by the most frequent to the less frequent, and the ST obtained from each genotype.

Genotype	Number of isolates	Percentage of isolates	ST (nº of isolates)
A1 II1 κ	106	21,29	3764 (93), 121 (10), SLV121 (3)
A1 II1 δ	92	18,47	121 (90), 5000 (1), SLV121 (1)
A1 II1 η	52	10,44	121 (49), 3761 (3)
B1 IV2 α	42	8,43	2855
B1 I1 α	26	5,22	96
B1 IV2 β	24	4,82	5001 (20), 2855 (3), 4998 (1)
B1 IV1 α	21	4,22	96 (13), 2855 (8)
B3 IV2 γ	17	3,41	146
A1 III1 δ	16	3,21	121 (15), 5000 (1)
D2 V1 β	11	2,21	1
F4 I6 -	10	2,01	398 (9), 4999 (1)
B1 I1 λ	9	1,81	96
B1 I2 α	8	1,61	2855
C1 I1 β	7	1,41	1
B1 IV2 γ	4	0,80	2855 (3), 96 (1)
B3 I1 ι	4	0,80	5
D2 V1 δ	4	0,80	1
A1 III3 δ	3	0,60	121
B4 I2 κ	3	0,60	4774
C2 V1 θ	3	0,60	1
A1 II2 δ	2	0,40	121
B1 II1 α	2	0,40	96
B2 I2 -	2	0,40	45
B3 I1 γ	2	0,40	5 (1), 3759 (1)
B3 I2 γ	2	0,40	146
B3 IV1 γ	2	0,40	146
B4 I1 κ	2	0,40	4774
C1 I1 γ	2	0,40	1
D2 V1 γ	2	0,40	1
F4 I8 -	2	0,40	398
F4 IV4 -	2	0,40	4999
A1 II1 β	1	0,20	121
A1 III2 κ	1	0,20	121
A2 II1 κ	1	0,20	121
A3 I2 β	1	0,20	1
A3 III2 δ	1	0,20	121
B1 I2 β	1	0,20	2855
B3 - -	1	0,20	398
B4 I2 α	1	0,20	1945
D1 IV1 α	1	0,20	407
D1 IV1 β	1	0,20	407
D1 IV2 α	1	0,20	407
D2 IV2 γ	1	0,20	4773
D2 V1 α	1	0,20	1
F4 IV3 -	1	0,20	398
TOTAL	498		

Once the genotype was obtained, on the basis that each one would correspond to the same MLST (Viana *et al.*, 2011), 127 isolates were selected to perform the complete MLST genotyping to have at least one complete MLST from the most common genotypes. This was done to check if the ST had changed compared to those previously described, since one of the hypotheses was that the staphylococcal outbreaks could have been produced by new strains. Several Single Locus Variants (SLV) were found among the different ST, so it was necessary to assign new ST numbers. The ST obtained from the different genotypes are shown in Table IV1-2.

Table IV1-3 shows the allele combinations found in the different ST. ST3764 clone was identified for the first time in 2014 and it differed from ST121 in a nucleotide of the *glp* gene. The ST2855 clone differed from the ST96 in a nucleotide of the *yqi* gen and the ST5001 was a variant of the ST2855 in the *arc* gene. Therefore, nineteen different ST were found, and they belonged to 8 different clonal complexes (CC) (Table IV1-3). The genetic relationships between the different ST are shown in the Figure IV1-1.

Table IV1-3. Combination of alleles of the different ST found in this study.

arc	aro	glp	gmk	pta	tpi	yqi	ST	CC
1	1	1	1	1	1	1	1	1
562	1	1	1	1	514	1	4773	
1	4	1	4	12	1	10	5	5
1	43	1	4	12	1	10	146	
1	4	1	4	472	1	10	3759	
3	3	57	1	4	4	67	407	8
10	14	8	6	10	3	2	45	45
12	1	1	15	11	1	40	96	96
12	1	1	15	11	1	338	2855	
586	1	1	15	11	1	338	4998	
587	1	1	15	11	1	338	5001	
6	5	6	2	7	14	5	121	121
6	5	6	2	93	14	5	3761	
6	5	527	2	7	14	5	3764	
6	5	671	2	7	14	5	5000	
6	57	45	2	215	58	52	1945	130
6	57	45	2	7	542	52	4774	
3	35	19	2	20	26	39	398	398
3	35	19	2	611	26	39	4999	

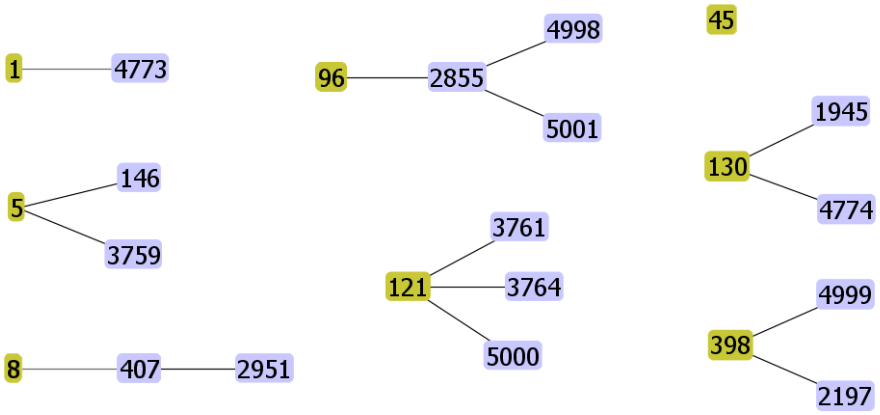

Figure IV1-1. Genetic relationships between the different ST. The group founders, which are the CC number, are coloured in yellow and the ST derived from the founder are coloured in blue.

Due to the SLV and DLV and the variability of ST belonging to the same genotype, it was not possible to associate all the strains to a ST. For this reason, it was necessary to check by PCR the genes with possible SLV in each farm and each genotype, according to previously observed. A total of 309 genes from 238 strains were evaluated and later, the ST of each genotype was extrapolated to the rest of the samples of the same farm. In this way, it was possible to assign the ST to each sample. The Table IV1-4 shows the number and the percentage of samples belonging to each ST. The most frequent ST was the ST121 with 173 isolates, representing the 34.74% of the total. The second most frequent ST was the ST3764, the third one was ST2855 and the fourth ST was ST96. Regarding to the clonal complexes, CC121 was the most frequent (275 isolates; 55.22%) followed by CC96 (137 isolates; 27.51%) (Figure IV1-2).

Table IV1-4. Number and percentage of samples belonging to each ST and CC.

ST	CC	Number of isolates	Percentage of isolates
121	121	173	34,74
3764	121	93	18,67
2855	96	65	13,05
96	96	51	10,24
1	1	31	6,22
146	5	21	4,22
5001	96	20	4,02
398	398	13	2,61
5	5	5	1,00
4774	130	5	1,00
SLV121	121	4	0,80
3761	121	3	0,60
4999	398	3	0,60
407	8	3	0,60
5000	121	2	0,40
45	45	2	0,40
4773	1	1	0,20
3759	5	1	0,20
4998	96	1	0,20
1945	130	1	0,20
TOTAL		**498**	**100**

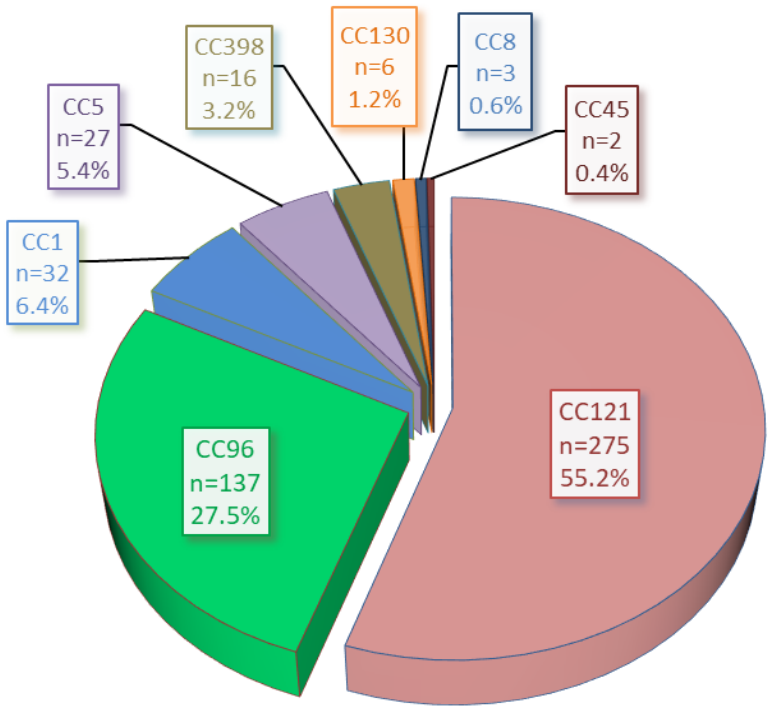

Figure IV1-2. Clonal complexes, number and percentage of each one.

Due to the detection of these new strains and alleles, it was necessary to check if the strains previously isolated in the Iberian Peninsula with the same genotypes belonged to the new ST. For this reason, 29 strains with genotypes A1 II1 k, A1 II1 n and A1 II1 δ were tested with the PCR of *glp* (CC121). Forty-two strains with genotypes B1 IV1 α, B1 IV1 β, B1 IV2 α and B1 IV2 β were tested with PCR of *yqi* (CC96). All the CC121 strains belonged to ST121 while there were two CC96 strains that belonged to ST2855 instead of ST96 (years 2006 and 2011).

The isolated clones from the different lesions and carrier animals were also studied (Table IV-5). The most common lesions were produced by the most common clones. Carrier animals had mainly ST121 strains. Less frequent lesions were produced by different strains.

Table IV1-5. Clones isolated from each lesion and carriers.

Source	Total	%	ST121	ST3764	ST96	ST2855	ST1	ST146	ST398	Others
Mastitis	209	41,97	75	50	5	35	18	6	5	15
Pododermatitis	65	13,05	10	10	22	1	7	3	1	11
Carriers	59	7,23	36	5	6	1	0	0	2	9
Abscesses	46	9,24	15	14	6	4	3	2	0	2
Pyoderma	30	6,02	16	2	4	3	0	3	0	2
Pneumonia	19	3,82	6	2	1	6	0	3	0	1
Conjunctivitis	18	3,61	6	3	1	1	0	0	2	5
Metritis	16	3,21	0	0	3	7	1	1	0	4
Otitis	15	3,01	2	2	0	2	2	3	2	2
Hepatitis	4	0,80	0	0	0	4	0	0	0	0
Necrosis	4	0,80	4	0	0	0	0	0	0	0
Rhinitis	3	0,60	0	2	0	0	0	0	1	0
Other lesions	10	2,01	3	3	3	1	0	0	0	0
Total	498		173	93	51	65	31	21	13	51

Figure IV1-3 shows the evolution of the most common clones from 2012 to 2019. It can be observed the emergence of ST3764 in 2014 and the decreasing trend of ST121 mainly due to the presence of ST3764 and ST2855.

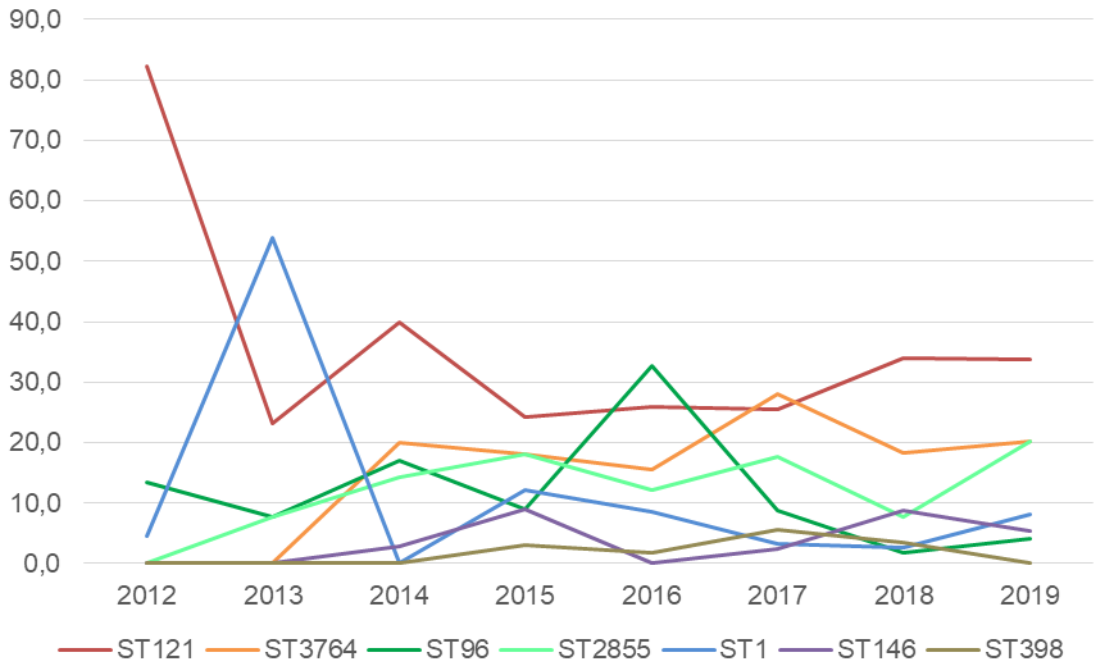

Figure IV1-3. Evolution of the most common clones (ST) from 2012 to 2019. Data shown in percentage.

4.1.2. Geographical distribution

The geographical distribution was studied to know the possible origin of the new strains and the extent of the dissemination of each clone. The table IV1-6 shows the isolated clones from each province, which is represented in percentages in Figure IV1-4. Only the most common ST are shown. ST121 and ST2855 were the most widespread. ST3764 was first isolated in the north-west of Iberian Peninsula in 2014 and then in the east in 2017.

Table IV1-6. Number of samples of each ST in the different provinces.

Province	ST121	ST3764	ST96	ST2855	ST1	ST146	ST398	Others	Samples	Farms
A Coruña	4	10	1	2	0	0	0	3	20	8
Albacete	23	1	0	2	0	0	0	0	26	4
Alicante	3	0	0	0	0	0	0	0	3	2
Asturias	0	0	0	3	0	0	0	2	5	3
Barcelona	19	0	0	0	7	0	7	6	39	16
Burgos	0	1	0	1	0	0	1	0	3	2
Castellón	4	0	2	1	2	0	0	4	13	6
Córdoba	0	0	0	0	0	1	0	0	1	1
Cuenca	8	0	0	1	0	5	0	0	14	3
Gerona	0	0	0	0	0	0	0	3	3	1
Huesca	0	0	0	2	0	0	0	0	2	1
Jaén	2	0	0	0	0	0	0	0	2	1
León	3	29	6	9	0	0	0	4	51	12
Lérida	18	0	3	0	1	2	0	0	24	4
Logroño	2	0	0	0	0	0	0	0	2	1
Lugo	3	0	0	0	0	0	0	0	3	1
Murcia	1	0	0	0	0	0	0	0	1	1
Navarra	1	0	0	0	3	0	0	0	4	1
Ourense	0	3	0	0	0	0	0	3	6	3
Palencia	3	3	0	0	0	0	0	0	6	3
Pontevedra	0	3	0	2	0	0	0	1	6	4
Salamanca	0	0	0	2	0	0	0	0	2	1
Segovia	1	5	0	0	0	0	0	0	6	3
Soria	3	0	0	0	0	0	0	0	3	1
Tarragona	24	0	0	1	6	4	5	21	61	10
Teruel	1	0	0	5	0	3	0	0	9	2
Toledo	0	0	0	0	0	1	0	0	1	1
Valencia	41	21	39	9	11	3	0	2	126	15
Valladolid	3	11	0	8	0	2	0	1	25	9
Vizcaya	0	3	0	1	0	0	0	0	4	1
Zamora	0	3	0	0	0	0	0	0	3	2
Zaragoza	6	0	0	0	1	0	0	0	7	3
Portugal	0	0	0	16	0	0	0	1	17	11

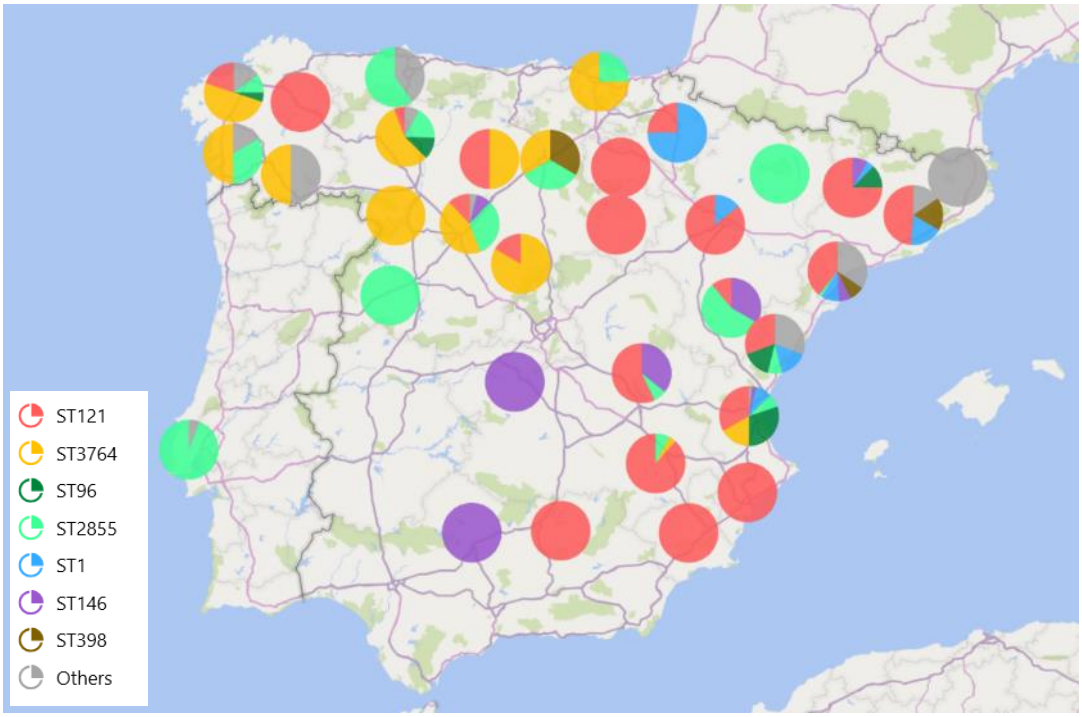

Figure IV1-4. Geographical distribution of the most common clones (ST) in the Iberian Peninsula. The circles represent the percentage of each clone over the total isolates of its specific province.

It can be observed that the clone ST3764 was located mainly in the north-west of Spain while the ST121 was located mainly in the east of Spain. Regarding to the CC96, ST96 and ST2855 clones, they have a more homogenous distribution. It should be noted that there were some provinces with very low number of isolates (1, 2 or 3), mostly in the south of Spain. For this reason, the 100% of the isolates in those provinces corresponded to ST121 or ST146.

4.1.3. Complete genome sequencing

It has been observed a rising and dissemination of new and less common clones in rabbit farms, such ST3764 and ST2855, respectively. However, the MLST and genotyping is not enough to know the reason of the increasing outbreaks and further investigation was needed. In order to detect other genetic variations between the new STs and the ancestors, the complete genome sequencing was performed in 30 strains, representing a selection of different ST and genotypes. They were compared with two well-

studied strains obtained from rabbits by our group: the Jwt as ST121 strain (high virulence) and DL9 as ST96 strain (low virulence).

Several differences were found between all the strains (Table IV1-7). In general, it was found that only strains belonging to CC121 and ST146 carried the enterotoxin gene cluster called *egc* (*seg, sei, sem, sen, seo* and *seu*). All the CC121 strains had *bbp*, a homologous gene of *sdrE*, except Jwt strain, which only carried *sdrC* and *sdrD* genes. This also occurs to CC96 strains, ST146 strains and one ST398 strain (1006). ST4774 strains and two ST398 strains carried the three *sdr* genes. All the strains harboured both *lukAB* and *lukED* leukocidins, except ST398 strains, that had only lukAB leukocidin. Strains belonging to CC96 were the only ones with the CRISPR-cas system. All strains contained the three subunits of the *hlgABC* locus, but none carried *tst*, *bap* and *eta* genes.

Variations in the *dltB* gene were also studied. It was observed that *dltB* remained invariable within the same ST, with the exception of strains ST398, which presented changes in amino acids 2, 227 and 405. All strains belonging to CC96 had the same *dltB* with the sole exception of amino acid 227, being an Isoleucine in ST96 strains and a Threonine in the rest of them. All ST121 and ST3764 strains (CC121) had the same amino acid sequence, this is, ST3764 strains have not changed their *dltB* with respect to ST121 strains. A ST398 strain had the same amino acid sequence than the ST2855, ST4998 and ST5001 strains (Table IV1-8). The other two ST398 strains had two different sequences.

Regarding to the mobile genetic elements, it was observed that all the sequenced strains carried at least one bacteriophage within the genome (Table IV1-9). Due to this unexpected result and the fragmentation of the phage sequences in different contigs, it was necessary to perform several PCRs to join the contigs and to be able to compare the phage sequences. However, because of the lack of time it was only possible to join the contigs of phages with type 3 integrase (Sa3int) and type 7 integrase (Sa7int). As

some of the phages were not divided in different contigs, they could be studied directly.

Table IV1-7. Presence of toxins and CRISPR in the sequenced strains and controls.

Strain	Province	Typing	MLST	Enterotoxins	*sdr*	*bbp*	Luks	CRISPR
DL9	Valencia	B1 IV2 α	96	-	CD	-	AB/ED	Yes
797	Valencia	B1 IV1 α	96	-	CD	-	AB/ED	Yes
860	Valencia	B1 IV1 α	96	-	CD	-	AB/ED	Yes
1031	Castellón	B1 IV2 γ	96	-	CD	-	AB/ED	Yes
862	León	B1 IV2 α	2855	-	CD	-	AB/ED	Yes
985	Portugal	B1 IV2 α	2855	-	CD	-	AB/ED	Yes
991	Portugal	B1 IV2 α	2855	-	CD	-	AB/ED	Yes
1000	Asturias	B1 IV2 α	2855	-	CD	-	AB/ED	Yes
1001	Asturias	B1 IV2 α	2855	-	CD	-	AB/ED	Yes
1002	Asturias	B1 IV2 α	2855	-	CD	-	AB/ED	Yes
1009	Portugal	B1 IV2 α	2855	-	CD	-	AB/ED	Yes
1014	Castellón	B1 IV2 α	2855	-	CD	-	AB/ED	Yes
993	Tarragona	B1 IV2 β	4998	-	CD	-	AB/ED	Yes
1303	Tarragona	B1 IV2 B	5001	-	CD	-	AB/ED	Yes
Jwt	France	A1 II1 δ	121	GIMNOU	CD	-	AB/ED	No
737	Tarragona	A1 II1 δ	121	GIMNOU	CD	+	AB/ED	No
1198	A Coruña	A1 II1 δ	121	GIMNOU	CD	+	AB/ED	No
706	León	A1 II1 κ	3764	GIMNOU	CD	+	AB/ED	No
766	León	A1 II1 κ	3764	GIMNOU	CD	+	AB/ED	No
880	Palencia	A1 II1 κ	3764	GIMNOU	CD	+	AB/ED	No
912	León	A1 II1 κ	3764	GIMNOU	CD	+	AB/ED	No
1094	Valladolid	A1 II1 κ	3764	GIMNOU	CD	+	AB/ED	No
1123	Burgos	A1 II1 κ	3764	GIMNOU	CD	+	AB/ED	No
1151	Valencia	A1 II1 κ	3764	GIMNOU	CD	+	AB/ED	No
1197	León	A1 II1 κ	3764	GIMNOU	CD	+	AB/ED	No
795	Lleida	B3 IV1 γ	146	GIMNOU	CD	-	AB/ED	No
986	Valladolid	B3 IV2 γ	146	GIMNOU	CD	-	AB/ED	No
1006	Tarragona	F4 I6 -	398	-	CD	-	AB	No
1032	Barcelona	F4 I6 -	398	-	CDE	-	AB	No
1310	Barcelona	F4 I6 -	398	-	CDE	-	AB	No
987	Valladolid	B4 I1 κ	4774	-	CDE	-	AB/ED	No
999	Ourense	B4 I1 κ	4774	-	CDE	-	AB/ED	No

Table IV1-8. Polymorphisms identified in the amino acid sequence of *dltB* locus in the different ST.

ST	2	113	227	250	346	401	405
96	I	T	I	Y	Y	G	Q
2855	I	T	T	Y	Y	G	Q
4998	I	T	T	Y	Y	G	Q
5001	I	T	T	Y	Y	G	Q
121	I	K	I	H	Y	G	Y
3764	I	K	I	H	Y	G	Y
146	I	T	T	Y	C	G	-
398	I	T	I	Y	Y	G	-
398	I	T	T	Y	Y	G	Q
398	T	T	I	Y	Y	G	Q
4774	I	T	T	Y	H	D	-

Orange cells: amino acid found in human-origin strains.
C: Cysteine; D: Aspartic Acid; G: Glycine; H: Histidine; I: isoleucine; K: Lysine; Q: Glutamine; T: Threonine; Y: Tyrosine; –: no amino acid.

Strains carrying a prophage with Sa3int had the β-haemolysin sequence truncated. Interestingly, all ST96 strains had this phage, while ST2855, ST4998 and 5001 strains, belonging to CC96, did not carry this phage and therefore, the β-haemolysin sequence was intact (except strain 1014). ST96 also carried Sa7int phage. Five of the ST2855 carried Sa2int and Sa7int, one only Sa7int, other Sa2int, Sa6int and Sa7int and another Sa2int, Sa3int and Sa7int. Both ST4998 and 5001 carried only Sa7int. All the Sa3int from CC96 had a 99.09% of homology between them (Table IV1-10).

The Sa7int from strains 797 and 860 were exactly the same and they had an 86.08% of homology with strain 1031 (the three were ST96). Strains belonging to ST2855 (except 1014), ST4998 and ST5001 had very similar Sa7int phages, with a homology of 99.09 to 100%. Sa7int from ST96 strains differed with the rest of CC96 strains, having between an 82 to 94.09% homology. Sa7int from strain 1014 had around the 90% of homology with the rest of CC96 strains (Table IV1-11). The amino acid sequence of type 2 integrases from strains 862, 985 and 1014 differed by one amino acid from strains 1000, 1001, 1002 and 1009.

Table IV1-9. Presence of prophages, *hlb*, and virulence factors within the phages in the sequenced strains and controls.

Strain	Province	Typing	ST	Phage int	*hlb*	*sak*	*chp*	*scn*
DL9	Valencia	B1 IV2 α	96	3,7	≠	+	+	+
797	Valencia	B1 IV1 α	96	3,7	≠	+	+	+
860	Valencia	B1 IV1 α	96	3,7	≠	+	+	+
1031	Castellón	B1 IV2 γ	96	3,7	≠	+	+	+
862	León	B1 IV2 α	2855	2,7	+	+	-	-
985	Portugal	B1 IV2 α	2855	2,7	+	+	-	-
991	Portugal	B1 IV2 α	2855	7	+	+	-	-
1000	Asturias	B1 IV2 α	2855	2,6,7	+	+	-	-
1001	Asturias	B1 IV2 α	2855	2,7	+	+	-	-
1002	Asturias	B1 IV2 α	2855	2,7	+	+	-	-
1009	Portugal	B1 IV2 α	2855	2,7	+	+	-	-
1014	Castellón	B1 IV2 α	2855	2,3,7	≠	+	+	+
993	Tarragona	B1 IV2 β	4998	7	+	+	-	-
1303	Tarragona	B1 IV2 B	5001	7	+	+	-	-
Jwt	Valencia	A1 II1 δ	121	12	+	-	-	-
737	Tarragona	A1 II1 δ	121	1,6,12	+	-	-	-
1198	A Coruña	A1 II1 δ	121	1,6,12	+	-	-	-
706	León	A1 II1 κ	3764	1,6,12	+	-	-	-
766	León	A1 II1 κ	3764	1,6,12	+	-	-	-
880	Palencia	A1 II1 κ	3764	1,6,12	+	-	-	-
912	León	A1 II1 κ	3764	1,6,12	+	-	-	-
1094	Valladolid	A1 II1 κ	3764	1,6,12	+	-	-	-
1123	Burgos	A1 II1 κ	3764	1,6,12	+	-	-	-
1151	Valencia	A1 II1 κ	3764	1,6,12	+	-	-	-
1197	León	A1 II1 κ	3764	1,6,12	+	-	-	-
795	Lleida	B3 IV1 γ	146	3,5	≠	+	-	+
986	Valladolid	B3 IV2 γ	146	1,3	≠	+	-	+
1006	Tarragona	F4 I6 -	398	2,9	+	-	-	-
1032	Barcelona	F4 I6 -	398	2	+	-	-	-
1310	Barcelona	F4 I6 -	398	2,9	+	-	-	-
987	Valladolid	B4 I1 κ	4774	New	+	-	-	-
999	Ourense	B4 I1 κ	4774	New	+	-	-	-

≠: truncated gene.

Table IV1-10. Percentage of homology of the nucleotide sequence of Sa3int phages.

ST	Strain	797	860	1031	1014	795	986
96	**797**		99,9	99,9	99,9	86,9	87,0
96	**860**			99,9	99,9	86,9	87,1
96	**1031**				99,9	87,1	87,0
2855	**1014**					87,0	87,0
146	**795**						99,9
146	**986**						

Phages sharing the same colour were identical or very similar.

All the CC121 strains carried 3 different phages: type 1, 6 and 12, but they did not carry Sa3int phage, therefore they had the complete β-haemolysin sequence (Table IV1-9). It was only possible to compare the complete sequence of Sa6int phages from strains 860 and 1198 because the sequences of the rest strains were divided into several contigs. Thus, only the amino acid sequences of integrases were compared in the rest of Sa6int phages from CC121 strains. The result was that Sa6int phage from strain 860 had only a 14.9% of homology with the Sa6int phage from 1198.

Regarding to all the amino acid sequences of integrases type 6, all of them had the same amino acid sequence except one, the strain 1198, which had up to 6 amino acid changes. That is, the Sa6int phage of strain 1198 was different from the rest of phages from CC121. With respect to Sa1int phages, it was only possible to compare the AA sequences of the integrases and all of them were identical.

Comparison of the complete sequences of Sa12int phages was only possible in 7 of the 10 CC121 strains. All of them were identical except the strain 737 (ST121), which had 99.7% homology with the rest of Sa12int phages (Table IV1-12). The amino acid sequences of integrases type 12 were identical between the 10 strains. This indicates that Sa12int phage is conserved among the CC121 strains.

Table IV1-11. Percentage of homology of the nucleotide sequence of Sa7int phages.

ST	Strain	797	860	1031	862	985	991	1000	1001	1002	1009	1014	993	1303
96	**797**		100	86,8	82,0	82,7	82,7	82,7	82,7	82,7	82,7	89,9	82,7	82,7
96	**860**			86,8	82,0	82,7	82,7	82,7	82,7	82,7	82,7	89,9	82,7	82,7
96	**1031**				95,0	94,9	94,9	94,9	94,9	94,9	94,9	92,6	94,7	94,7
2855	**862**					99,9	99,9	99,9	99,9	99,9	99,9	90,8	99,9	99,9
2855	**985**						100	99,9	100	100	99,9	90,8	99,9	99,9
2855	**991**							99,9	100	100	99,9	90,8	99,9	99,9
2855	**1000**								99,9	99,9	99,9	90,8	99,9	99,9
2855	**1001**									100	99,9	90,8	99,9	99,9
2855	**1002**										99,9	90,8	99,9	99,9
2855	**1009**											90,8	99,9	99,9
2855	**1014**												90,8	90,8
4998	**993**													99,9
5001	**1303**													

Phages sharing the same colour were identical or very similar.

Table IV1-12. Percentage of homology of the nucleotide sequence of Sa12int phages. All of them were identical or very similar.

ST	Strain	737	706	766	912	1123	1151	1197
121	**737**		99,7	99,7	99,7	99,7	99,7	99,7
3764	**706**			100	100	100	100	100
3764	**766**				100	100	100	100
3764	**912**					100	100	100
3764	**1123**						100	100
3764	**1151**							100
3764	**1197**							

One ST146 strain (795) carried integrase 3 and 5 phages and the other ST146 (986) carried integrase 1 and 3 phages (Table IV1-9). The sequences of this two Sa3int phages were almost identical between them, while they had only an 87% homology with Sa3int phages from CC96 (Table IV1-10). This difference with CC96 phages was also found in their integrases, since the amino acid sequence differs by 4 amino acids with CC96 Sa3int phages. The amino acid sequence of the integrase type 1 from strain 986 differed only in one amino acid from the rest of CC121 strains.

Two ST398 strains (1006 and 1310) carried Sa2int and Sa9int phages while other ST398 strain (1032) carried only a Sa2int phage. The amino acid sequence of type 2 integrase from 1006 differed from 1032 and 1310 by one amino acid. At the same time, these type 2 integrases differed from those of ST2855 strains by 2 or 3 amino acids. The three ST398 carried a SaPI with integrase type II while the rest of the strains did not carry any SaPI. Finally, ST4774 strains carried a new type of phage, it had an integrase with a homology of 87% with the Sa3int and it was inserted in a different place.

The presence of virulence factors within the prophages was also studied. Any virulence factors were detected within the phages from CC121 strains. On the contrary, it was observed than all the phages with Sa7int carried the *sak* gene. All the Sa3int from CC96 carried *sak*, *chp* and *scn* genes while the Sa3int from ST146 carried only *sak* and *scn* genes (Table IV1-9).

In summary, several genetic differences were found between the different ST and even within the same ST. The strains belonging to ST2855 were quite heterogenic while strains belonging to ST96, ST121 and ST3764 were almost identical between the strains belonging to the same ST. CC121 and ST146 carried enterotoxins and CC96 carried CRISPR-cas system. There were also differences in *dltB* gene between the different ST. In addition, 9 different prophages (according to integrase type), including a new one, were found along the different strains. Some of these prophages differed between and within the different ST. SaPIs were only found in ST398 strains, which carried an integrase type II SaPI. The most important result of the complete genome sequencing, as far as it has been possible to get with basic tools and knowledge, is that the CC96 has undergone different genetic changes, while the strains belonging to CC121 have remained practically the same.

4.1.4. Prophages in other isolates

In order to increase the information of the different prophages in the population of *S. aureus* that affects rabbits, multiplex PCR of the integrases of 129 strains of different STs was performed. It was observed that the majority of ST121 strains did not carry any prophage, while almost all (32 of 35 isolates) of the ST3764 carried prophages type 1 and 6. Differences between ST96 strains and ST2855 strains were also observed. The most frequent combination of prophages in ST2855 strains was 2,7 (10 isolates), followed by the phage 7 alone (8 isolates). On the contrary, ST96 strains carried the combination of 3 and 7 phages (13 isolates), though phage 3 (2 isolates) and phage 7 (2 isolates) were also found separately (Table IV1-13). Regarding to the less common clones, ST1 strains carried predominantly Sa2int phages and ST146 isolates had Sa3int phages. ST398 carried different combinations of type 1, 2, 3, 5, 6 and 7 phages. ST398 was the clone with a wider variety of phages, while ST3764, ST96 and ST1 were the most homogeny clones, carrying only 2 different phages each one.

Table IV1-13. Presence of prophages in the different ST from not sequenced isolates.

ST	1	1,6	1,7	2	2,3	2,6	2,7	2,3,7	3	3,5	3,7	5	6	7	None	n
121	1	2	2	2	0	0	0	0	0	0	0	0	4	0	20	31
3764	3	32	0	0	0	0	0	0	0	0	0	0	0	0	0	35
2855	0	0	0	0	0	0	10	2	0	0	0	0	0	8	0	20
96	0	0	0	0	0	0	0	0	2	0	13	0	0	2	0	17
1	0	0	0	7	0	1	0	0	0	0	0	0	0	0	0	8
146	1	0	0	0	0	0	0	0	6	1	0	1	0	0	0	9
398	0	0	1	2	1	0	0	0	0	0	0	0	2	0	0	6
4999	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2
5000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1

4.1.5. Prophage activity

The functionality of the prophages was evaluated because of the finding of several prophages in all the strains. First, all the sequenced strains were induced with Mitomycin C and then lysates were tested in Sa1039 strain over phage base agar plates (Figure IV1-5). The titres of the different strains are shown in Table IV1-14.

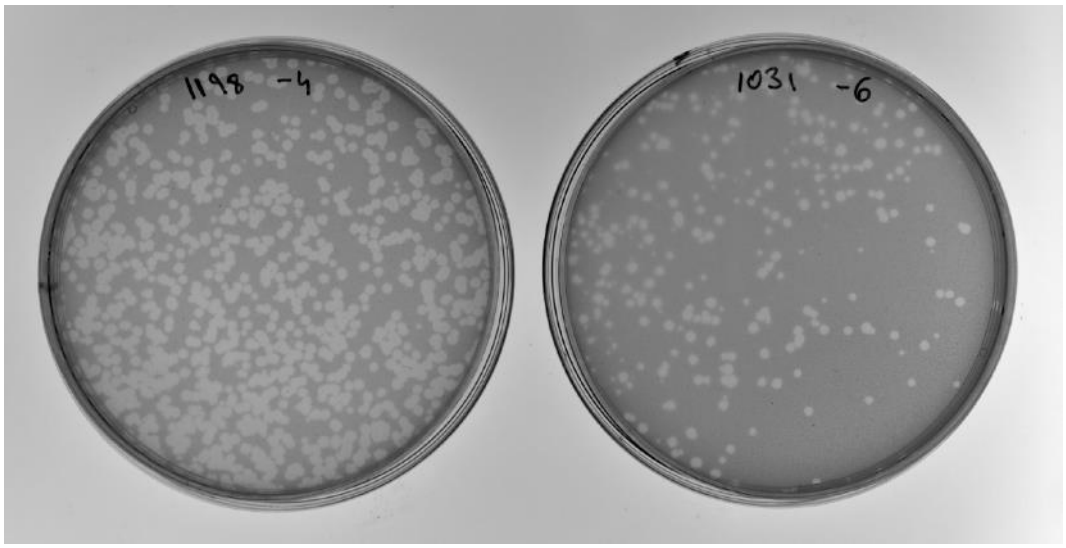

Figure IV1-5. Base plates with plaques made by lytic phages to Sa1039.

It was observed that all strains were lysed with the exception of 8 of them (1000, 993, 1303, 737, 795, 986, 987 and 999). Of these 8 strains, 3 were able to infect Sa1039 and thus it was obtained a titre. In addition, the titre was high in strains 795 and 986 while it was low in strain 1000.

Titres found in ST3764 strains were lower than the rest of the strains, except strain 1000 and those unable to infect Sa1039. It should be noted than the titres correspond to all the phages together instead of each phage separately and different phages within the same strain could interfere in the lysis and infection of other phages.

Table IV1-14. Lytic activity and titre of prophages in sequenced isolates.

Strain	ST	CC	Phage int	Bacteria lysis	Titre per mL
797	96	96	3,7	Yes	26600000000
860	96	96	3,7	Yes	19800000000
1031	96	96	3,7	Yes	3600000000
862	2855	96	2,7	Yes	260000000
985	2855	96	2,7	Yes	100000000
991	2855	96	7	Yes	1650000000
1000	2855	96	2,6,7	No	8900
1001	2855	96	2,7	Yes	13500000
1002	2855	96	2,7	Yes	17400000
1009	2855	96	2,7	Yes	13500000
1014	2855	96	2,3,7	Yes	257000000
993	4998	96	7	No	0
1303	5001	96	7	No	0
737	121	121	1,6,12	No	0
1198	121	121	1,6,12	Yes	250000000
706	3764	121	1,6,12	Yes	494000
766	3764	121	1,6,12	Yes	55600
880	3764	121	1,6,12	Yes	3900000
912	3764	121	1,6,12	Yes	396000
1094	3764	121	1,6,12	Yes	700000
1123	3764	121	1,6,12	Yes	158000
1151	3764	121	1,6,12	Yes	120000
1197	3764	121	1,6,12	Yes	198000
795	146	5	3,5	No	2860000000
986	146	5	1,3	No	182000000
1006	398	398	2,9	Yes	1670000000
1032	398	398	2	Yes	5800000000
1310	398	398	2,9	Yes	4800000000
987	4774	130	New	No	0
999	4774	130	New	No	0

Blue colour: not lysed strains with phage titre. Pink colour: not lysed strains without titre.

4.1.6. Haemolysis, coagulation and regulator genes

Haemolysis and coagulation tests were performed to check two of the most important characteristics of *S. aureus*. It was observed that the three sequenced ST96 strains only showed δ-haemolysin pattern, while all the strains ST2855 without Sa3int phage showed the three-haemolysis pattern, i.e. α, β and δ, with the exception of strains 1009 and 1014. ST3764 expressed the three haemolysins, showing a large area of total haemolysis, the same as Jwt. On the contrary, the two ST121 strains only showed β-haemolysis. The three ST398 strains showed the three haemolysins pattern but with a smaller area of total haemolysis. The different haemolysis patterns are showed in **Figure IV1-6** and detailed for each strain in **Table IV1-15**.

The presence of haemolysin genes was studied to check whether the phenotypic patterns in blood agar correlated with the presence of the genes. All of the strains carried the *hla* gene, while the *hlb* was only complete in the strains without Sa3int phage **(Table IV1-15)**. Regarding to δ-haemolysin, differences in *hld* genes were observed. CC121 strains had a smaller Hld due the insertion of a thymine in the nucleotide sequence. In addition, an amino acid (aa) switch was found in the protein sequence. Therefore, 3 different δ-haemolysins were found in the sequenced strains **(Figure IV1-7)**.

Coagulation of rabbit plasma was studied, and it was observed that all the strains were able to coagulate the plasma at 4 h with the exception of strain 737. However, strains 1031, 1151 and 1198 coagulated later than the rest of the strains (1 h and 0.5 h, respectively) **(Table IV1-16)**.

Figure IV1-6. Haemolysis patterns of 7 sequenced strains and controls Jwt (rabbit ST121), Fwt (human ST121) (Viana *et al.*, 2015) and DL9 (rabbit ST96).

Table IV1-15. Haemolytic phenotype in blood agar plates, presence of haemolysin genes (protein sequence) and regulator genes in the sequenced strains.

CEU	Typing	ST	CC	Int phages	Blood	*hla*	*hlb*	*hld*	*rot (aa length)*	*saeRS*
DL9	B1 IV2 α	96	96	3,7	δ	+	≠	+	133	OK
797	B1 IV1 α	96	96	3,7	δ	+	≠	+	166	OK
860	B1 IV1 α	96	96	3,7	δ	+	≠	+	166	OK
1031	B1 IV2 γ	96	96	3,7	δ	+	≠	+	133	OK
862	B1 IV2 α	2855	96	2,7	α-β-δ	+	+	+	133	OK
985	B1 IV2 α	2855	96	2,7	α-β-δ	+	+	+	133	OK
991	B1 IV2 α	2855	96	7	α-β-δ	+	+	+	133	OK
1000	B1 IV2 α	2855	96	2,6,7	α-β-δ	+	+	+	133	OK
1001	B1 IV2 α	2855	96	2,7	α-β-δ	+	+	+	133	OK
1002	B1 IV2 α	2855	96	2,7	α-β-δ	+	+	+	133	OK
1009	B1 IV2 α	2855	96	2,7	β	+	+	+	133	OK
1014	B1 IV2 α	2855	96	2,3,7	δ	+	≠	+	133	OK
993	B1 IV2 β	4998	96	7	α-β-δ	+	+	+	133	OK
1303	B1 IV2 B	5001	96	7	α-β-δ	+	+	+	133	OK
Jwt	A1 II1 δ	121	121	12	α-β-δ	+	+	≠	82	OK
737	A1 II1 δ	121	121	1,6,12	β	+	+	≠	82	OK
1198	A1 II1 δ	121	121	1,6,12	β	+	+	≠	82	≠
706	A1 II1 κ	3764	121	1,6,12	α-β-δ	+	+	≠	82	≠
766	A1 II1 κ	3764	121	1,6,12	α-β-δ	+	+	≠	82	≠
880	A1 II1 κ	3764	121	1,6,12	α-β-δ	+	+	≠	82	OK
912	A1 II1 κ	3764	121	1,6,12	α-β-δ	+	+	≠	82	≠
1094	A1 II1 κ	3764	121	1,6,12	α-β-δ	+	+	≠	82	≠
1123	A1 II1 κ	3764	121	1,6,12	α-β-δ	+	+	≠	82	≠
1151	A1 II1 κ	3764	121	1,6,12	α-β-δ	+	+	≠	82	OK
1197	A1 II1 κ	3764	121	1,6,12	α-β-δ	+	+	≠	82	≠
795	B3 IV1 γ	146	5	3,5	α-δ	+	≠	+	133	OK
986	B3 IV2 γ	146	5	1,3	α-δ	+	≠	+	133	≠
1006	F4 I6 -	398	398	2,9	α-β-δ	+	+	+	133	OK
1032	F4 I6 -	398	398	2	α-β-δ	+	+	+	133	OK
1310	F4 I6 -	398	398	2,9	α-β-δ	+	+	+	133	OK
987	B4 I1 κ	4774	130	New	β	+	+	+	133	OK
999	B4 I1 κ	4774	130	New	α-β-δ	+	+	+	133	≠

≠: truncated gene.

Figure IV1-7. Hld protein sequence in the different clones. Blue box indicates the common region between the clones. Red letters indicate the aa switch.

Genes *rot* and *saeRS* were also studied because they regulate haemolysin genes and also other virulence factors. *rot* acts as a repressor of toxins while *saeRS* is a promotor of virulence factors. Three different *rot* genes were observed. CC121 strains had a shorter rot gene with only 82 aa. ST2855, ST4998, ST5001, ST146, ST398 and ST4774 carried a *rot* gene with 133 aa length. Finally, two ST96 strains carried a larger rot gene with 166 aa while one ST96 strains carried the 133 aa length *rot* (Table IV1-15).

Regarding to the *saeRS* loci, it was intact in CC96 strains and ST98 strains. However, 7 of the CC121 strains, one ST146 and one ST4774 had a truncated *saeR*, lacking 6 aa in the N-terminus (Table IV1-15).

Table IV1-16. Coagulation test of different strains at different moments.

Strain	ST	0.5 h	1 h	1.5 h	2 h	2.5 h	3 h	3.5 h	4 h	24 h
DL9	96	Y	Y	Y	Y	Y	Y	Y	Y	Y
860	96	Y	Y	Y	Y	Y	Y	Y	Y	Y
1031	96	N	Y	Y	Y	Y	Y	Y	Y	Y
862	2855	Y	Y	Y	Y	Y	Y	Y	Y	Y
991	2855	Y	Y	Y	Y	Y	Y	Y	Y	Y
1009	2855	Y	Y	Y	Y	Y	Y	Y	Y	Y
1014	2855	Y	Y	Y	Y	Y	Y	Y	Y	Y
Jwt	121	N	Y	Y	Y	Y	Y	Y	Y	Y
737	121	N	F	F	F	F	F	F	F	P
1198	121	N	Y	Y	Y	Y	Y	Y	Y	Y
766	3764	Y	Y	Y	Y	Y	Y	Y	Y	Y
1151	3764	N	Y	Y	Y	Y	Y	Y	Y	Y
795	146	Y	Y	Y	Y	Y	Y	Y	Y	Y
986	146	Y	Y	Y	Y	Y	Y	Y	Y	Y
842	398	Y	Y	Y	Y	Y	Y	Y	Y	Y
1006	398	Y	Y	Y	Y	Y	Y	Y	Y	Y
1032	398	Y	Y	Y	Y	Y	Y	Y	Y	Y
987	4774	Y	Y	Y	Y	Y	Y	Y	Y	Y
999	4774	Y	Y	Y	Y	Y	Y	Y	Y	Y

Y: coagulated; N: not coagulated; F: fibrin at the bottom of the tube; P: partially coagulated.

4.1.7. Analysis *in vivo* of the virulence

To know the real infectivity of the strains, intradermal infections were performed in rabbits using low doses of inoculum. Six strains with different STs and genotypes were selected and strains Jwt and DL9 were used as controls.

It was observed that strains belonging to ST96, ST2855 and ST398 were not able to infect and no significant CFU were recovered from the inoculation point. On the opposite, the two ST3764 strains were able to infect and produce lesions to a larger number of animals (70%-87.5%) than Jwt (58.7%), but no significant differences were found (Table IV1-17).

Table IV1-17. Results of intradermal infection.

Strain	MLST	Genotype	Infected animals (%)	Type of lesion	Lesion size (cm^2)	CFU/g
Jwt	121	A1 II1 δ	58.7	Abscesses and necrosis	1.74	2.26E+07
766	3764	A1 II1 κ	87.5	Abscesses and necrosis	1.26	3.28E+06
1151	3764	A1 II1 κ	70	Abscesses and necrosis	2.08	6.40E+07
DL9	96	B1 IV2 α	2.4	None	0.57	20
908	96	B1 I1 λ	0	None	-	0
862	2855	B1 IV2 α	0	None	-	0
991	2855	B1 IV2 α	0	None	-	0
842	398	F4 IV3 -	0	None	-	640

The observed lesions were abscesses and some of them also developed necrosis, which opened the following day. Despite of the differences in the number of animals with lesions, there were no differences in the type of lesion produced by Jwt, 766 and 1151 (CC121 strains). Figure IV1-8 shows the typical lesions produced by *S. aureus* when inoculated intradermally after 1, 3, 5 and 7 days. A starting necrosis area can be observed in the centre of the nodule at 7 dpi.

The CFU/g obtained from the lesions and the lesion size produced by strain 766 were lower than the Jwt while the CFU and the lesion size produced by strain 1151 were greater than Jwt. However, there were no significant differences either.

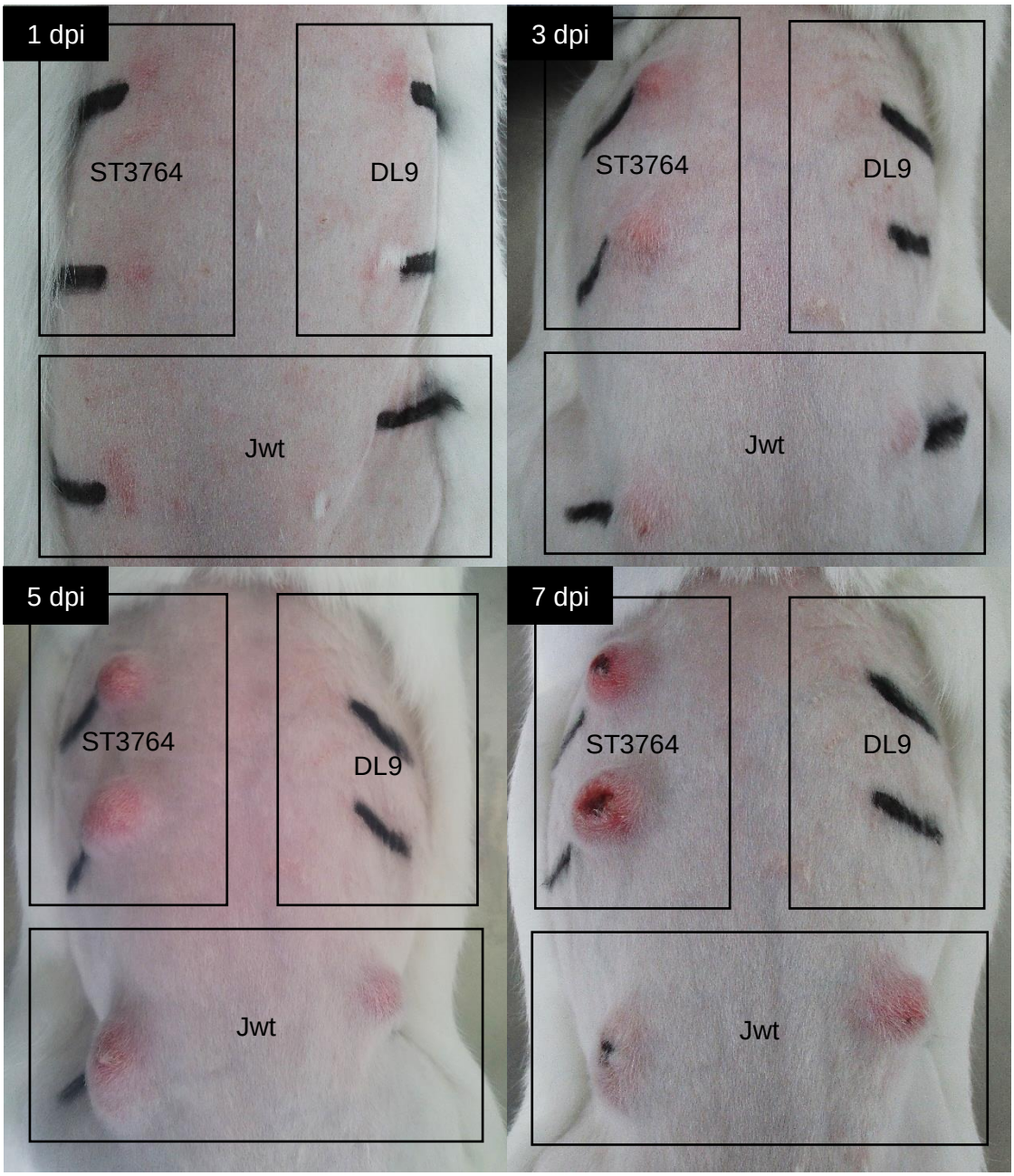

Figure IV1-7. Evolution of the lesions caused by different strains of *S. aureus*.

4.2. Effect of different housing systems on health and welfare traits of commercial female rabbits

4.2.1. Haematological parameters and hair cortisol results

The aim of this study was to assess the effect of the different housing types on health status of female rabbits from the first insemination to the fifth parturition. For this reason, different immune parameters were evaluated at two different production moments. First of all, parameters of all animals were compared between 1AI and 5P (Table IV2-1). Values at 1AI could be considered as the baseline values for these females at the age of 19 weeks old, as they were not under any housing group effect until first insemination.

Therefore, in Table IV2-1 it can be observed that the total number of erythrocytes, haemoglobin and haematocrit decreased from 1AI to 5P (-12%, -17% and -16%, respectively; $P<0.001$), while the number platelets increased (+20%; $P<0.001$). The number of leukocytes were greater at 5P (+14%; $P<0.001$), mainly due to an increase in the number of total heterophils (+3%; $P<0.01$). Haptoglobin and cortisol concentrations also augmented at 5P (+4% and +94%; $P<0.05$), whereas phagocytic capacity of the heterophils was reduced compared to 1AI (-31%; $P<0.001$).

Table IV2-1. Blood parameters and cortisol concentration in hair at first insemination (1AI) and fifth parturition (5P).

	1AI	**5P**	**SEM**	***P-value***
Erythrocytes (x10^{12} cel/L)	6.19^{b}	5.43^{a}	0.046	<0.001
Haemoglobin (g/L)	133^{b}	111^{a}	1.179	<0.001
Haematocrit	40.5^{b}	34.0^{a}	0.342	<0.001
Platelets (x10^{9} cel/L)	267^{a}	320^{b}	9.171	<0.001
Leukocytes (log_{10} 10^{9} cel/L)	0.88^{a}	1.00^{b}	0.017	<0.001
Heterophils (log_{10} 10^{6} cel/L)	3.51^{a}	3.62^{b}	0.024	0.004
Lymphocytes (log_{10} 10^{6} cel/L)	3.56	3.61	0.026	0.199
Monocytes (log_{10} 10^{6} cel/L)	2.70	2.60	0.056	0.266
Eosinophils (log_{10} 10^{6} cel/L)	1.25	1.62	0.121	0.054
H/L ratio	0.95	1.66	0.241	0.057
Haptoglobin (log_{10} mg/L)	2.54^{a}	2.63^{b}	0.024	0.018
Cortisol (ng/g)	0.48^{a}	0.93^{b}	0.043	0.001
Phagocytosis (%)	24.4^{b}	16.8^{a}	1.378	0.001

SEM: standard error. $^{a, b}$: average values in the same group of significantly different data ($P<0.05$).

After the first comparison between 1AI and 5P, only the parameters at 5P were compared between the 5 groups to assess the effect of the different housing systems on the health and stress of rabbit does at an advanced time of their productive lives, i.e. the fifth parturition (Table IV2-2). The female rabbits placed inside the PF cages had higher haemoglobin values than those in cages HD and TR (on av. +7%; $P<0.05$). The females housed in the COL cages had more platelets than those in cages HD and PF (on av. +35%; $P<0.05$). The females in the COL housing had higher haptoglobin concentrations than those in cages HD, PF and TR (on av. +8%; $P<0.05$). The PF and COL groups had higher cortisol concentrations than the PV and TR groups (on av. +136%; $P<0.05$), while the HD group had the lowest cortisol concentrations (on av. -578% vs. COL and PF; $P<0.05$) There were no significant differences in erythrocytes, haematocrit, leukocytes, H/L ratio and phagocytic capacity.

Table IV2-2. Blood parameters and cortisol concentration in hair in the animals located in each housing type at fifth parturition (5P).

	PV	HD	TR	PF	COL
Erythrocytes ($x10^{12}$ cel/L)	5.55±0.13	5.35±0.11	5.42±0.11	5.52±0.11	5.31±0.13
Haemoglobin (g/L)	116±3.38^{ab}	108±2.74^{a}	108±2.70^{a}	115±2.71^{b}	107±3.27^{ab}
Haematocrit	35.3±0.89	33.1±0.80	33.7±0.78	34.9±0.78	32.8±0.95
Platelets ($x10^{9}$ cel/L)	312±26.3^{ab}	281±21.3^{a}	335±20.7^{ab}	272±21.0^{a}	369±25.4^{b}
Leukocytes ($\log_{10} 10^{9}$ cel/L)	1.04±0.05	0.96±0.04	0.99±0.04	0.97±0.04	1.04±0.05
Heterophils ($\log_{10} 10^{6}$ cel/L)	3.65±0.07	3.59±0.06	3.66±0.06	3.60±0.06	3.63±0.07
Lymphocytes ($\log_{10} 10^{6}$ cel/L)	3.63±0.08	3.63±0.06	3.53±0.06	3.62±0.06	3.65±0.07
Monocytes ($\log_{10} 10^{6}$ cel/L)	2.62±0.16	2.51±0.13	2.67±0.13	2.64±0.13	2.57±0.15
Eosinophils ($\log_{10} 10^{6}$ cel/L)	1.68±0.35^{ab}	1.12±0.28^{a}	2.00±0.28^{b}	1.57±0.28^{ab}	1.72±0.33^{ab}
H/L ratio	1.38±0.28	1.13±0.56	2.68±0.55	1.25±0.55	1.87±0.66
Haptoglobin ($\log_{10}$ mg/L)	2.62±0.06^{ab}	2.57±0.06^{a}	2.57±0.06^{a}	2.59±0.06^{a}	2.78±0.07^{b}
Cortisol (ng/g)	0.61±0.10^{b}	0.23±0.09^{a}	0.71±0.10^{b}	1.67±0.09^{c}	1.45±0.10^{c}
Phagocytosis (%)	13.4±3.37	16.2±2.91	18.7±2.66	16.5±3.04	19.0±3.24

Type of cages: polyvalent (PV), higher and deeper (HD), traditional (TR), with platform (PF), collective (COL). $^{a, b, c}$: average values in the same group of significantly different data ($P<0.05$).

4.2.2. Pathological studies

Sixty-two rabbit does were removed during the study (Table IV2-3). Thirty-five were culled for different reasons and 27 died. Necropsy was performed on 55 animals and 52 had at least one evident lesion. More animals were removed from the COL group (n=18, 60% of animals; $P<0.05$) because there were more culled does (n=13, 43.3%; $P<0.05$) than in the individual cages (Table IV2-3).

Taking into account all the groups together, the most frequent causes of culling were reproductive problems (n=21) and presence of abscesses (n=10), being the COL group the one with the highest percentage of such problems. However, there was only a significant difference in reproductive problems between COL and HD groups ($P<0.05$) (Table IV2-3). Other reasons of culling were mastitis in one female (PV group) and pododermatitis in another one (COL group). The culling cause remained unknown in two females (PV and COL).

The observed kit mortality rates during lactation oscillated between 6.4 and 13.1. It was significantly higher in the COL group than in groups HD and PF (on av. +6.0%; $P<0.05$) (Table IV2-3).

Table IV2-3. Number and percentage of dead and culled females in each housing system type and kit mortality rate.

	PV		HD		TR		PF		COL		Total	
	n	%	n	%	n	%	n	%	n	%	n	*P*-value
Removed	12^{ab}	40.0	10^{a}	33.3	13^{ab}	43.3	9^{a}	30.0	18^{b}	60.0	62	0.0410
Dead	5	16.7	7	23.3	7	23.3	3	10.0	5	16.7	27	0.1769
Culled	7^{ab}	23.3	3^{a}	10.0	6^{ab}	20.0	6^{ab}	20.0	13^{b}	43.3	35	0.0067
Reproductive problems	5^{ab}	16.7	1^{a}	3.3	3^{ab}	10.0	5^{ab}	17.7	7^{b}	23.3	21	0.0487
Abscesses	0	0.0	2	6.7	3	10.0	1	3.3	4	13.3	10	0.1386
Others	2	6.7	0	0	0	0	0	0	2	6.7	4	0.1119
Kit mortality	-	9.3^{ab}	-	6.4^{a}	-	8.2^{ab}	-	7.8^{a}	-	13.1^{b}	-	0.0382

Type of cages: polyvalent (PV), higher and deeper (HD), traditional (TR), with platform (PF), collective (COL). Values in percentages over the total of animals in each group. $^{a, b}$: average values in the same group of significantly different data ($P<0.05$).

Regarding to the causes of death, no significant inter-group differences were found. The main causes of death were infectious or inflammatory processes (n=19), especially metritis (n=7), pneumonias (n=6), peritonitis (n=4) and pericarditis (n=2). Other causes of death were liver necrosis (n=1), uterine torsion (n=1) and bone fracture (n=1). The cause of death of 5 animals was unknown (Table IV2-4).

Table IV2-4: death causes in each housing type.

	PV	HD	TR	PF	COL	Total
Metritis	0	3	1	1	2	7
Pneumonia	3	0	1	1	1	6
Peritonitis	0	2	0	1	1	4
Pericarditis	1	0	1	0	0	2
Liver necrosis	0	0	1	0	0	1
Uterine torsion	0	0	1	0	0	1
Bone fracture	0	0	0	0	1	1
Unknown	1	2	2	0	0	5
Total	5	7	7	3	5	27

Regarding to all the lesions observed during the necropsies, the most frequently found were those related to reproductive system (n=38) (Figure IV2-1) and respiratory system (n=33) (Figure IV2-2). Other common lesions were abscesses (n=14) (Figure IV2-3), peritonitis (n=13) (Figure IV2-4A) and pericarditis (n=7) (Figure IV2-4B). All lesions observed in each group are detailed and separated into 7 categories in Table IV2-5. It should be noted that the same animal could present different lesions, and these lesions did not necessarily cause death or euthanasia. The group with more observed lesions in the necropsies was the COL group (n=50), although this group had also more discarded animals than the other groups. The groups with less lesions were PV and PF (n=24 and 28, respectively). The PV group had smaller number of reproductive lesions (n=2) than females in groups PF, TR and COL (n=10, 10 and 9, respectively; $P<0.05$). More females with abscesses were detected in the COL group (n=7) than in groups PV and HD (n=0 and 1, respectively; $P<0.05$). Finally, there were 4 females without any evident lesion HD (n=0 and 1, respectively; $P<0.05$).

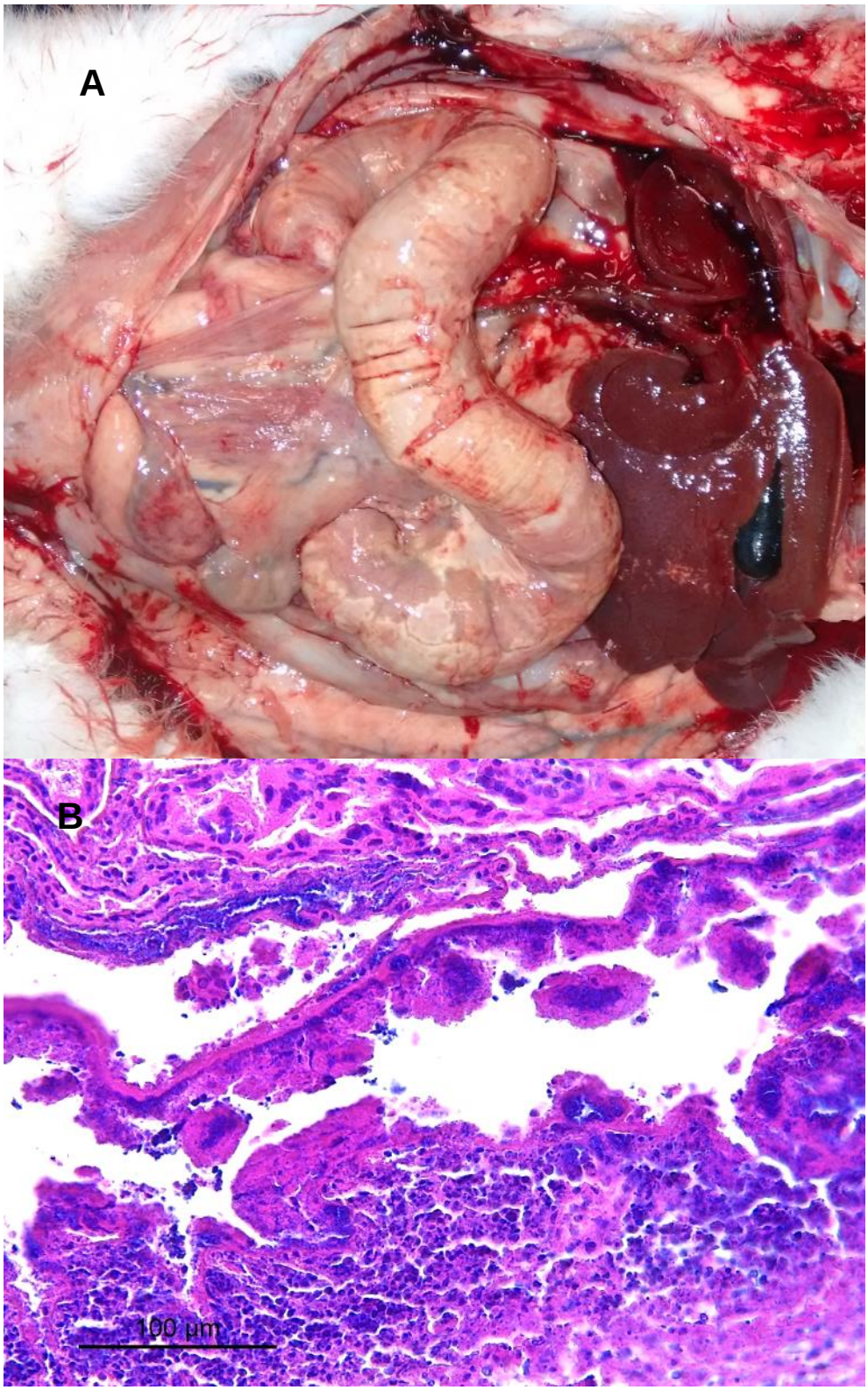

Figure IV2-1. A: macroscopic image of suppurative metritis. Uterine dilation due to the presence of pus. B: microscopic image of purulent metritis with numerous bacteria (Haematoxylin-eosin).

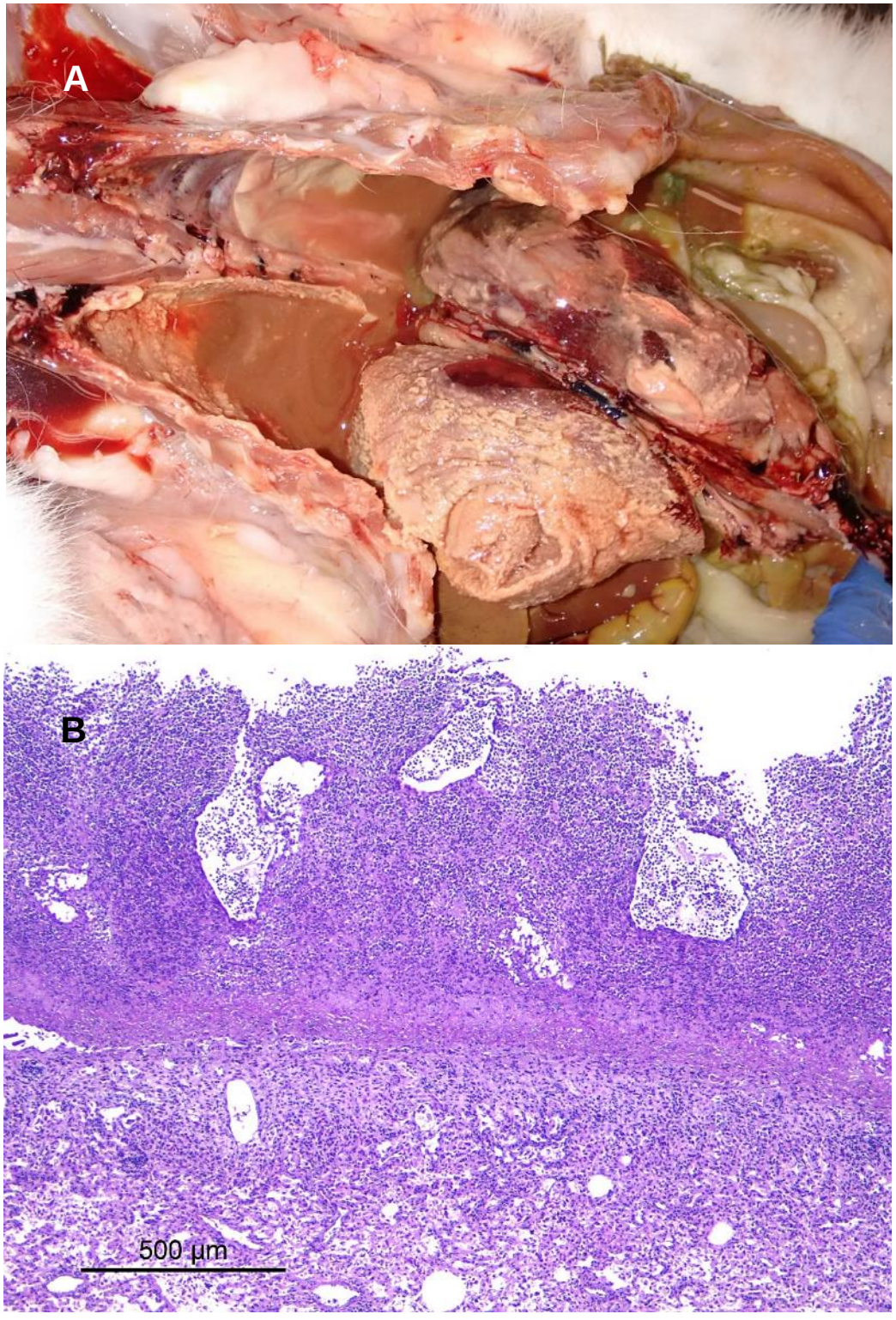

Figure IV2-2. A: macroscopic image of fibrinous pneumonia (abundant fibrinous exudate in the thoracic cavity and lungs surface). B: microscopic image of fibrinous pneumonia with severe inflammatory infiltrate affecting pleura and alveoli (Haematoxylin-eosin).

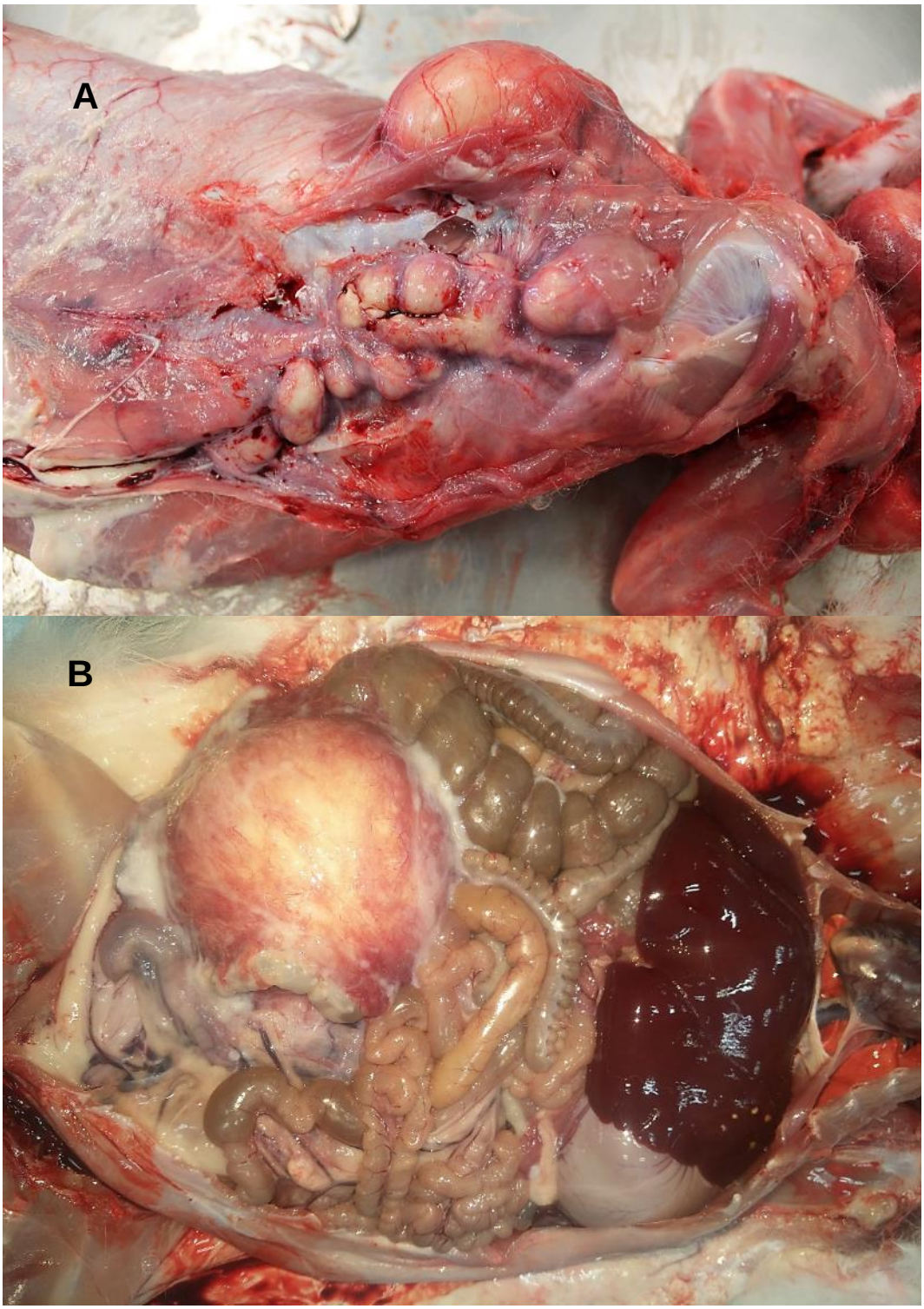

Figure IV2-3. A: macroscopic image of subcutaneous abscesses. B: macroscopic image of an abdominal abscess.

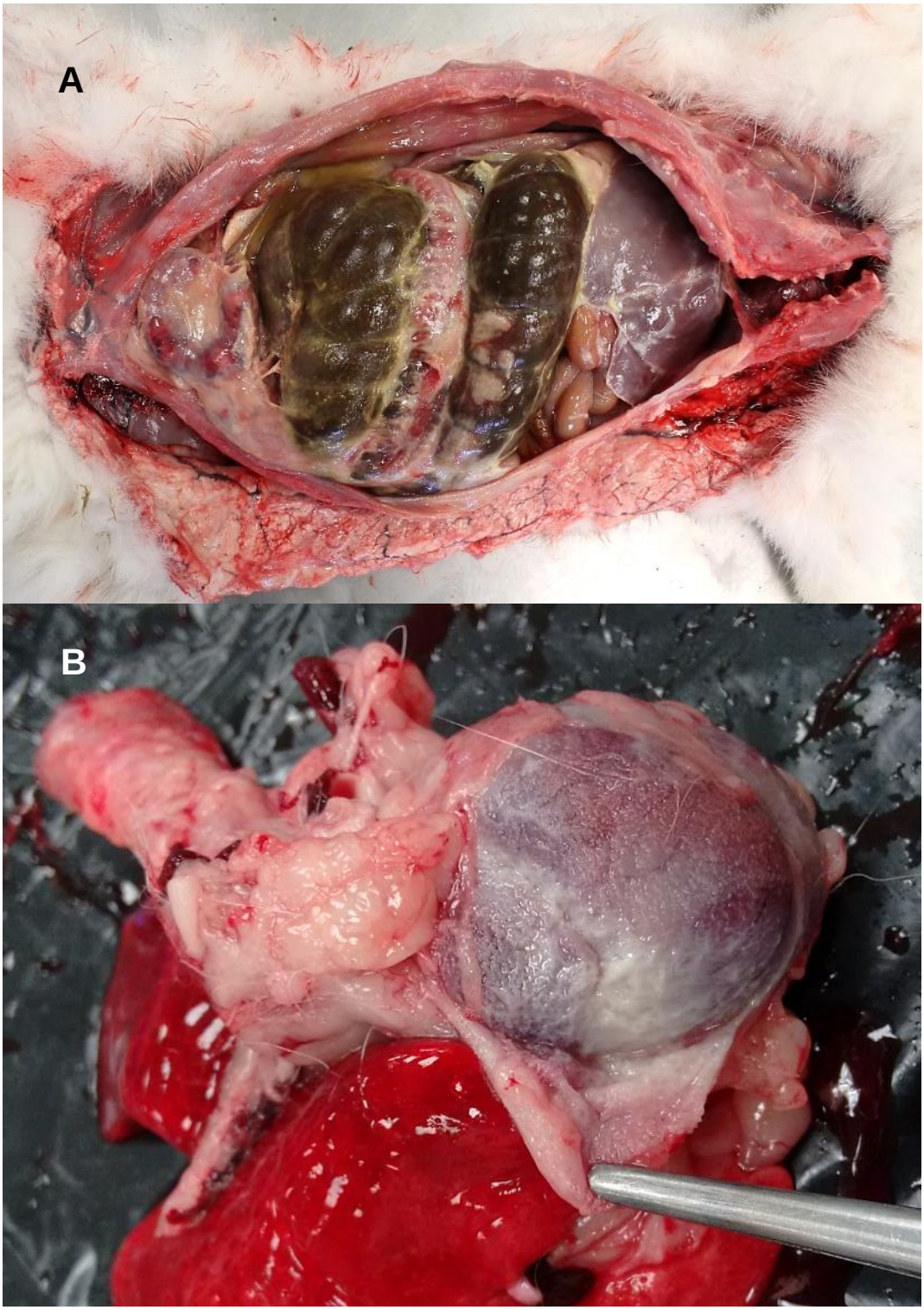

Figure IV2-4. A: macroscopic image of fibrinous peritonitis. B: macroscopic image of fibrinous pericarditis.

Table IV2-5. Removed animals and lesions found in necropsies in each group.

	CAT	PV	HD	TR	PF	COL	Total
Removed animals		12	10	13	9	18	62
Fibrinous metritis		1	3	2	2	5	13
Purulent metritis		1	1	4	3	2	11
Dystocia	1	0	3	2	2	1	8
Salpingitis		0	0	1	3	1	5
Uterine torsion		0	0	1	0	0	1
Congestion and alveolar oedema		3	3	2	1	5	14
Catarrhal-purulent pneumonia		3	1	3	1	2	10
Embolic-metastatic pneumonia	2	2	1	2	0	0	5
Fibrinous pneumonia		1	0	1	1	0	3
Interstitial pneumonia		0	0	0	1	0	1
Subcutaneous abscesses	3	0	1	3	0	4	8
Abdominal abscesses		0	0	1	2	3	6
Peritonitis		0	2	3	1	3	9
Pododermatitis		1	0	1	1	5	8
Pericarditis/Endocarditis		2	1	2	0	2	7
Nephritis		2	1	0	1	2	6
Gastric ulcers		0	3	1	0	0	4
Hepatic necrosis	4	0	1	2	0	1	4
Hepatitis		0	0	1	0	1	2
Enteritis		1	0	0	0	1	2
Cholecystitis		0	0	0	1	0	1
Mastitis		1	0	0	0	0	1
Tracheitis		0	0	1	0	0	1
Cardiac dilatation		2	3	1	2	5	13
Calcifications		0	2	3	1	0	6
Liver degeneration	5	2	3	1	0	0	6
Renal amyloidosis		0	1	1	0	3	5
Splenic Amyloidosis		0	1	0	0	2	3
Splenomegaly		0	0	0	1	0	1
Hydropericardium		1	2	0	3	0	6
Hydrothorax	6	1	1	0	0	0	2
Ascites		0	1	0	0	0	1
Fractures or injuries	7	0	0	0	1	2	3
Total		24	35	39	28	50	176

CAT: lesion category (1: reproductive; 2: respiratory; 3: abscesses; 4: other infectious/inflammatory; 5: metabolic and functional; 6: liquid accumulation; 7: fractures or injuries). Polyvalent (PV), higher and deeper (HD), traditional (TR), with platform (PF), collective (COL).

4.2.3. Microbiological analysis

After the identification of the bacteria isolated from lesions, the most frequent bacterium was *P. multocida* (55% of the necropsied animals), mainly isolated from metritis, pneumonia and abscesses, followed by *S. aureus* (22% of the necropsied animals), mainly isolated from pododermatitis and a wide range of different lesions. *Proteus* spp. was isolated from abdominal abscesses and other lesions (9% of the necropsied animals). No inter-group differences were found (Table IV2-6). Other isolated bacteria were *Escherichia coli*, *Enterococcus* spp., *Pseudomonas aeruginosa*, *Bordetella bronchiseptica* and one *Staphylococcus* coagulase negative.

Regarding to the *S. aureus* genotyping, one female carried two different strains: B1/I1/α and A1/II1/δ. Two other females carried only genotype B1/I1/α, while four carried B1/I1/λ. Finally, two rabbit does carried genotypes B1/I1/α and B1/I1/λ at the same time. Genotypes B1/I1/α and B1/I1/λ belonged to ST96, while A1/II1/δ belonged to ST121.

Table IV2-6: Results of microbiological analysis for each group.

	PV		**HD**		**TR**		**PF**		**COL**		**Total**	
	n	%*	n	%*	n	%*	n	%*	n	%*	n	%
P. multocida	3	30	4	50	9	81.8	6	66.7	8	47.1	30	54.5
S. aureus	3	30	1	12.5	1	9.1	1	11.1	6	35.3	12	21.8
Proteus spp.	2	20	0	0	0	0	1	11.1	2	11.8	5	9.1
Total necropsied	10		8		11		9		17		55	

* Percentage calculated over the necropsied animals of each group.
polyvalent (PV), higher and deeper (HD), traditional (TR), with platform (PF), collective (COL)

V- DISCUSSION

V- DISCUSSION

5.1. Characterization of *S. aureus* isolates from rabbit farms

5.1.1. Genotypic characterization

The present study was proposed to understand the increase of the number and severity of staphylococcal outbreaks in Iberian rabbit farms. Veterinarians and farmers are really worried because this pathogen is becoming more frequent, more persistent, more virulent and more difficult to treat. The first objective of this study was to characterize genetically the isolates of *Staphylococcus aureus* obtained from Iberian farms to find new genes or mutations which could explain the virulence increasing.

The most frequent lesion were mastitis, which are the most important lesion produced by *S. aureus* in rabbits, not only from the point of view of the number of affected animals (Segura *et al.*, 2007), but also from the viewpoint of the welfare and the economic losses (Rosell and De La Fuente, 2018). In fact, Segura *et al.* (2007) described *S. aureus* the main pathogen associated with mastitis, pododermatitis and abscesses. However, according to Sánchez *et al.* (2012), the main pathologies affecting female rabbits were respiratory diseases, while in this study pneumonia and rhinitis only represented the 4.42% of the *S. aureus* positive lesions. This could be due respiratory diseases may be produced by other bacteria, such as *Pasteurella multocida* (Segura *et al.*, 2007), fungi or viruses (Harcourt-Brown and Harcourt-Brown, 2002).

Surprisingly, there was found a high heterogeneity along the isolates and also new clones belonging to different clonal complexes. Previously, a high virulent clone, belonging to ST121, was described as the most common clone affecting rabbits worldwide (Vancraeynest *et al.*, 2006). After that, Viana *et al.* (2007) described the genotype A1 II1 δ, belonging also to ST121, as the most common clone affecting rabbitries in the east of Spain, corresponding to the 70% of the isolates. In fact, they also genotyped the

high virulent strains isolated by Hermans *et al.* (2000b) and confirmed that they also had the A1 II1 δ genotype. So this demonstrates that A1 II1 δ was the most common strain affecting rabbits worldwide. The second most common strain according to Viana *et al.* (2007) was C1 I1 β (7.3%) and the third was B1 IV1 α (5.0%). In the present study, from 2012 to 2019, percentages were greatly different, being A1 II1 δ only the 18.47% of the isolates. There is clearly a change in the prevalence of the different clones in rabbit farms compared to what was described a decade ago. However, it should be taken into account that Viana *et al.* (2007) did not sample the same areas than in the present study. As it has been observed now, strain A1 II1 δ (ST121), which was the most common a decade ago, would continue to be the most frequent in the east of the Iberian Peninsula (Figure IV1-4), the area sampled by Viana *et al.* (2007).

The most common genotype isolated in this study, considering the whole Iberian Peninsula, was the A1 II1 κ. These isolates belonged mainly to the new clone ST3764 (93 isolates out of 106 A1 II1 κ), a SLV of ST121 in the *glp* gene. This clone was found first in 2014 in the northwest of the Peninsula. In the east of the Iberian Peninsula, genotype A1 II1 κ did not possess the *glp* mutation and, therefore, it still corresponded to ST121, as checked with the sequencing of *glp* allele from older isolates. ST3764 clone was isolated in the east of the Iberian Peninsula (Valencia and Albacete) since 2017. However, due to the absence of previous data of the northwest of the Iberian Peninsula, it cannot be assured whether ST3764 was already present long time ago there without being spread or, on the contrary, it is a recent mutation of ST121, and it has been quickly disseminated to rest of the Peninsula.

In the present study, ST96 strains continued representing a low percentage of isolates (about 10%) although this clone represented only the 5% in a previous study about mastitis in rabbits (Guerrero *et al.*, 2015). The origin of ST96 is unknown, since it was described rarely in humans (Mendes *et*

al., 2012; Azis *et al.*, 2017). Smyth *et al.* (2009) studied the *S. aureus* clones and they only found the ST96 in rabbits.

Here, the ST2855 was described as the third most common clone in the Iberian Peninsula in the last years, reaching the 13% of the isolates and overcoming the ST96. This result is surprising because the ST2855 was not described in any publication before, with the exception of the article published by our group in 2018 (Moreno-Grua *et al.*, 2018). The only reference to this ST is in the MLST database and it was an isolate from rabbit meat in Italy in 2012. Although ST2855 strains belong to the CC96, a clonal complex characterized as low virulence clone, they may be causing problems on farms due to the methicillin resistance (Moreno-Grua *et al.*, 2018). Therefore, the clone ST2855, together with the clone ST3764, could be one of the causes of the increase of staphylococcal outbreaks. In addition, the clone ST2855 is widespread in the whole Iberian Peninsula, affecting 15 provinces and Portugal (Figure IV1-4).

Other ST isolated in this study but less common than the previous ones were ST1, ST146 and ST398. ST1 has been isolated from different hosts, as human, swine and cows, but rarely from rabbits (Viana *et al.*, 2011; Post *et al.*, 2017; Bonsaglia *et al.*, 2018; Parisi *et al.*, 2019). Here, it was found with a prevalence of 6.2% in rabbit farms, which is higher than described in previous articles, but it seems to be only in the north-west of the Peninsula. ST146 is even less frequent but also colonizes humans and it carries the *mecA* gene (Lozano *et al.*, 2015). This clone was not previously described in rabbits but in the present study it reached the 4.2% of the strains. In fact, our group reported for first time the clone ST146 in rabbits and those isolates harboured *mecA* gene (Moreno-Grua *et al.*, 2018). Therefore, it could be said that both ST1 and ST146 are clones that could be beginning to affect rabbits, although it cannot be assured if they come from farmers, veterinarians or other animals with whom they could have had direct or indirect contact.

On the contrary, ST398 clone is highly prevalent in both humans and animals, including rabbits, although swine is the main host (Aires-de-Sousa, 2017). ST398 have been isolated from commercial rabbits and companion rabbits (Agnoletti *et al.*, 2014; Loncaric *et al.*, 2014; Guerrero *et al.*, 2015). In fact, in 2014 there was the first report of ST398 methicillin resistant strains in a rabbit farm and also farmers were colonized (Agnoletti *et al.*, 2014). Here, 13 isolates (2.6%) belonged to ST398 clone, which could seem a small number but it is really remarkable because this ST is considered a multi-host clone, it can carry a variety of prophages with different virulence factors (for example PVL, Sak, Sea), it also possess resistance genes (as *mecA*) and therefore, it could be a potential risk for farmers and other people, as described before (Agnoletti *et al.*, 2014; Kraushaar *et al.*, 2017).

Nine different *clfB* alleles have also been detected in this study and it seems that δ allele is related with CC121 and CC1. Other alleles are in several CC, as described previously by Basset *et al.* (2009). They hypothesized that it could exist a convergent evolution and this, together with the fact that *clfB* is an important virulence factor for adhesion to host corneal surfaces. This could explain the spread of the genotype A1 II1 κ at the expense of the reduction of A1 II1 δ. It has already been shown that small changes in certain genes can change the specificity or virulence of the strains (Kurt *et al.*, 2013; Viana *et al.*, 2015a). Therefore, changes in the *clfB* and other genes analysed by genotyping could be producing changes in the behaviour of the strains and making them more virulent.

The identification of all these new strains coincides with the reports of farmers and veterinarians about a change in the clinical behaviour of *S. aureus* characterized by the appearance of especially virulent outbreaks. However, a relationship between the clones and the type of lesion could not be determined since a pure epidemiological sampling was not carried out. Contrary, samples mainly come from farms with staphylococcal problems. All these results were not enough to understand the current outbreaks and,

for this reason, the complete genome sequencing of 30 isolates was performed.

5.1.2. Genome sequencing

The whole genome sequencing is becoming a common and cheap technique to study the different organisms. But after the sequencing, it is needed the processing of the information by specific programs handled by experts and, finally, it is needed to interpret the results for the optimal analysis. Here, the complete genome of 30 strains was sequenced using an external laboratory and the annotation of the contigs was done with the RAST server. After that, basic tools were used to look for the different genes and to compare them between different strains. The results obtained in this study were very interesting, but the maximum information of the sequences could not be obtained. However, it is something that remains to continue working.

Regarding to the enterotoxins, they were only present in CC121 strains and ST146 (CC5) strains. These clones carried the *egc* cluster with the six toxins *seg*, *sei*, *sem*, *sen*, *seo* and *seu* while the rest of sequenced clones did not carry any enterotoxin. The presence of *egc* in CC121 in rabbit and human isolates have already been described (Rasigade *et al.*, 2010; Viana *et al.*, 2015b) and also in CC5 from humans, dogs, cats, horses and turkeys (Rasigade *et al.*, 2010; Feßler *et al.*, 2011; Loncaric *et al.*, 2019). These enterotoxins seem to be involved in infection and pathogenicity, since ST121 had greater number of enterotoxins than ST96 (Viana *et al.*, 2015b). However, these genes may not be involved in the dissemination of ST2855 clone since neither ST96 nor ST2855 had any enterotoxins and they are spreading.

Any sequenced strain carried lukS/F-PV, *tst*, *eta* nor *bap* genes, which could indicate that these strains do not need these genes to colonise and develop the infection in rabbits, as observed before (Viana *et al.*, 2015a). However, it has been recently detected a ST121 strain carrying the *pvl* gene affecting a rabbit farm in China (Wang *et al.*, 2019). This strain was causing severe

lung infections and it may indicate an adaptation of ST121 to lung conditions, since PVL produces necrotizing pneumonia (Diep *et al.*, 2010). Interestingly, CC121 strains here studied carried an allelic variant of *sdrE*, called *bbp*, differing from the control strain Jwt, which lacks *sdrE* and *bbp*. *SdrE* is an adhesin that functions as a 'clamp' to capture complement factor H on the surface of *S. aureus* for complement evasion (Zhang *et al.*, 2017). Bbp recognizes the bone sialoprotein and also binds human fibrinogen but not the fibrinogen from other mammals (Vazquez *et al.*, 2011), thus it seems to be host-specific. However, rabbit fibrinogen was not included in Vazquez *et al.* (2011) study, and consequently, our results may indicate than rabbit strains would have acquired recently *bbp* to increase the virulence towards rabbits. Therefore, the acquirement of *bbp* is an important change from the predecessors, but it is needed further investigation about the *bbp* and it would be necessary to construct different mutants to check the importance of this gene in rabbit infections.

Remarkably, although it have been demonstrated that *pvl* has an effect over rabbit neutrophils (Löffler *et al.*, 2010), any of our strains, even strains with Sa2int phages, lacked *pvl* (Goerke *et al.*, 2009). Interestingly, the lack of virulence factors and enterotoxins of ST96 and ST2855 could make them to survive in the host and produce later the infection, as demonstrated very recently by Tuchscherr *et al.* (2019). They observed that the low-cytotoxicity strains survived in higher numbers and were less efficiently cleared by the host than the highly cytotoxic strains, making them a source for chronic infections. Therefore, this may be one of the reasons why CC96 (low virulence) strains are increasing their presence in rabbit farms and also producing diseases. And this is in line with the fact that carrier animals are a source for future infections (Selva *et al.*, 2015).

Other important gene related with host adaptation and infection is the *dltB* gene. This gene encodes for a membrane protein involved in the transport of positive charges outside the bacteria (Collins, L. V. *et al.*, 2002). Here, different amino acid polymorphisms in this gene were observed, producing

different protein sequences in each ST and even within the same ST (Table IV1-8). All of these polymorphisms have been previously described (Viana *et al.*, 2015a), but some of them were present in other CC different from this study. Interestingly, while there was not any change in this gene between ST121 and ST3764 strains, there was a change in one amino acid (Ile227Thr) between ST96 strains and the other strains of this same CC. Furthermore, this change was also observed in the other sequenced clones. Therefore, as described previously, this change could be a convergent evolution towards rabbit host (Viana *et al.*, 2015a; Holmes *et al.*, 2016). However, the most important mutation that favours the adaptation demonstrated to date in *dltB* was only present in CC121 strains. Whether the change Ile227Thr observed in the rest of ST is involved in rabbit adaptation and in the increase of the virulence, remains to be determined.

In addition to all these findings, the presence of CRISPR-cas system was observed only in CC96 strains. CRISPR is widespread in bacteria as well as in archaea and protects them against viral infections. When the bacterium detects the presence of viral DNA, it integrates short fragments of viral DNA into the CRISPR locus. Then, the bacterium transcribes this sequence into short RNA, forming a complex with another RNA, which guides a protein called Cas9 to recognize viral DNA. Then, the Cas9 nuclease cleaves the viral DNA, thus combating the viral attack (Javed *et al.*, 2018). The recombination between phages and CRISPR-cas loci facilitates horizontal gene transfer in staphylococci (Varble *et al.*, 2019). The importance of this finding in our strains is unknown and it is needed further investigation to asses if there is any effect in the pathogenesis of CC96 strains.

5.1.3. Mobile genetic elements and adaptation to rabbit host

Regarding to mobile genetic elements observed in this study, there were several interesting findings. The MGE are an important source of genetic variability and transmission of virulence factors. The first studies about MGE in rabbit strains hypothesized that rabbit ST121 strains had lost the MGE

because they did not need them to develop infections (Viana *et al.*, 2015a). In fact, ST121 strains suffered different mutations, as commented above, to adapt rabbit host about 40 years ago. Although in this study the majority of ST121 strains did not carry any MGE (except the Sa12int phage), surprisingly, some of them have acquired Sa1int, Sa2int, Sa6int and Sa7int phages. Moreover, all the ST3764 strains (CC121) carried Sa1int and Sa6int phages at the same time (except 3 isolates). However, the effect of Sa1int and Sa6int phages in rabbit strains remains unknown, but it may be advantageous since this combination of phages is highly conserved in ST3764. Therefore, this result is really remarkable because the acquisition of phages by ST121 rabbit strains is a great change from previous studies (Viana *et al.*, 2015a).

Strains belonging to CC96 have also suffered changes regarding to the presence of phages, since ST96 strains carried mainly Sa3int phage together with Sa7int phage while almost none of ST2855 strains carried Sa3int phage. ST146 isolates also carried a Sa3int phage (9 of 11 isolates) while ST398 isolates did not carry Sa3int phages (only 1 out of 9 isolates).

Sa3int prophages are also called β-haemolysin converting bacteriophages and they are present mainly in human strains, but not in animal strains, regardless of the clonal complex (Sung *et al.*, 2008; McCarthy and Lindsay, 2013). These Sa3int phages carry different combinations of the IEC (*sak*, *chp* and *scn*) and it have been demonstrated that the genes included in IEC are human-specific (van Wamel *et al.*, 2006). Although ST96 rabbit strains carried the Sa3int phage and the IEC, it cannot be assured that ST96 strains had a human origin because there are very few ST96 strains isolated from humans (Mendes *et al.*, 2012; Azis *et al.*, 2017). The loss of Sa3int phage happened with ST121 when it became adapted to rabbit host and therefore it could be happening the same with the ST96 and ST2855. The loss of the Sa3int phage restores the β-haemolysin sequence and it could be an advantage for ST2855 strains.

The absence of a Sa3int phage in ST398 and ST1 strains could also indicate the rabbit adaptation of these isolates. ST398 strains lacking phage have also been observed in pigs and horses (Cuny *et al.*, 2015). This result is consistent with the finding of the intII SaPI found in ST398 strains. It was identical to SaPIbov5 previously described by Viana (2010). This SaPI carries a host-specific *vwb* allele, called vWbp^{Sbo5}, which can coagulate ruminant plasma but also rabbit plasma. Therefore, this may partially explain the percentage of strain belonging to ST398 isolated from rabbit lesions.

Regarding to CC5 (ST5, ST146 and ST3759) strains found in this study, they carried a Sa3int phage with an IEC. This could suggest that they were not adapted to rabbit host, as it was described previously in pigs (Hau *et al.*, 2015). However, a significant number of isolates belonging to this CC (n=27) were found from rabbit lesions and, therefore, they have the capacity to produce natural infections in rabbits despite of the presence of IEC.

In addition to genomic analysis, the functionality of the prophages was also studied, since some phages could be immobile or defective, like the Sa12int phage from ST121 strains (Viana, 2009). It is remarkably that almost all the strains carry at least one phage with the ability of lyse the recipient bacteria and infect a susceptible one. This could explain the variability observed in the phage profile carried by the different isolates. But there were some prophages unable to lyse the bacteria and to infect the Sa1039, which could indicate a lack of functionality of those prophages, including the new prophages found in strains 987 and 999 (ST4774, CC130).

On the one hand, the variability, the lytic activity and the ability of infection may be considered as health risk, since these bacteria, with the help of the phages, could transmit virulence factors and antibiotic resistance genes more easily. Furthermore, the acquisition of new phages could be a disadvantage for the phage therapy because the bacteria's own phages can prevent infection by other phages. But the further knowledge of the phages

could also be an advantage when seeking therapeutic alternatives against staphylococcal infections.

All these findings are worrisome, as it has been shown that LA-strains, such as ST1, ST146, ST398, and ST2855, could reacquire Sa3int phage and recover the infective capacity for humans (Kraushaar *et al.*, 2017). In addition, our group has described these clones from rabbit farms as MRSA, increasing the risk of infections with difficult treatment (Moreno-Grua *et al.*, 2018). Moreover, a new mechanism has been discovered through which some phages replicate and package before excision. This phenomenon was called “genome hypermobility by lateral transduction” and can promote the efficient transfer of several hundred kilobases of bacterial genome, including virulence factors and toxins (Chen *et al.*, 2018). Finally, it has recently been discovered that a Sa3int phage can excise temporally depending on growth conditions, such in response to hydrogen peroxide-induced oxidative stress and during biofilm growth. This excision restores the β-haemolysin expression, which could increase the pathogenicity of the strain when the bacteria need it (Tran *et al.*, 2019).

5.1.4. Virulence of the strains

As it has been explained before, this project was carried out to know the causes of more virulent and persistent outbreaks in rabbit farms in the Iberian Peninsula. Once the different strains were studied genetically and also geographically, it was confirmed that there has been a change in the isolated strains in these farms compared to previous studies. The dissemination of a clone described only once in Italy in 2012, the ST2855 (CC96), was observed throughout the Iberian Peninsula, reaching 13% of all isolates. A new variant of the ST121 clone, the ST3764, was also described. ST3764 clone seems to have its origin in the northwest area of Spain and it also began to be found in the east since 2017. Finally, it was found a relative high percentage of samples belonging to CC1, CC5 and CC398. The change in the percentage of the different strains could be due a change in the virulence. For this reason, different tests were carried out.

After performing the blood haemolysis test, differences between strains with the same ST were observed and therefore the presence of *hla*, *hlb* and *hld* genes was checked. The strains that did not show β-haemolysis were due to the presence of Sa3int phage, as previously mentioned. However, there were other phenotypes that did not correlate with the presence of the genes, such as strains 1009 and 1014 (ST2855) or strains 737 and 1198 (ST121) and 987 (ST4774). The variation in the length of Hld was not enough to explain the phenotype of 737 and 1198, since Jwt had the same genes and expressed the three haemolysins. It was reported that strains without *hdl* produced less α-haemolysin than strains with the *hld* gene, indicating a regulatory function of this gene (Morfeldt *et al.*, 1995). The smaller Hld present in CC121 strains may be functional since α-haemolysis area is bigger than the total haemolysis produced by ST2855 strains. Thus, the smaller Hld did not explain the lack of α-haemolysin in blood agar. Strains 1009 and 987 also carried the three haemolysin genes although they only showed β-haemolysis.

Regarding to coagulation tests, all the rabbit strains were able to coagulate rabbit plasma. It has been previously described that rabbit plasma is coagulated by strains from different hosts (Viana *et al.*, 2010). The ability to coagulate rabbit plasma may help the different clones to adapt to rabbit host and spread throughout the farms, although Coa activity is not essential to develop lesions (Muñoz-Silvestre, 2019). In fact, the only strains that produced lesions were those belonging to the CC121 and they coagulated rabbit plasma later than the rest of the strains that did not infect.

The altered haemolytic phenotype could be due to the complex regulation pathways between the different regulator genes, such as *rot* and *saeRS* (Li and Cheung, 2008). For this reason, the *rot* and *saeRS* regulatory genes were studied to try to explain the different observed phenotypes. However, neither the different *rot* alleles nor the truncated *saeR* gene explained the different observed phenotypes between the strains. This may indicate the presence of other regulatory mechanisms, such as *agr* mutations (Trabber

et al., 2008). These mutations were not found in these strains, although CC96 had a different *agr* than CC121 strains (type 3 vs type 4) (Moreno-Grúa *et al.*, 2018).

It was hypothesized that these strains could infect more rabbits than the ancestors did, as observed in previous studies (Viana *et al.*, 2015a; Penadés, 2017), and therefore intradermal infections with different strains were carried out to check if they were able to produce lesions under experimental conditions. It was observed that ST3764 strains were able to infect a greater number of animals than the Jwt (ST121) control strain, but this difference was no significant. On the contrary, neither ST96, ST2855 nor ST398 strains were able to infect although some CFU were recovered from ST398 lesions. This was an unexpected result, since our hypothesis was that ST2855 strains had lost the Sa3int phages and then, they would have acquired an infectious advantage over its ancestor ST96 getting adapted to rabbit host. Conversely, the infection capacity of ST2855 remained identical to ST96 at low doses and a similar result was obtained with the strain ST398.

In previous studies with ST96, similar results were obtained. Under experimental infections in mammary gland, ST96 produced a smaller number of infected animals and the lesions were less severe than ST121 (Penadés, 2017). In natural mastitis, ST96 produced less immune response than ST121 (Guerrero *et al.*, 2015). It was hypothesized that the different pathogenicity and immune response between ST121 and ST96 strains could be due the greater number of enterotoxins in ST121 compared to ST96 (Viana *et al.*, 2015b). When an intradermal infection was done with ST121 strains, they produced lesions in the 70% of the animals (Viana *et al.*, 2015a). This is consistent with the low percentage of ST96 strains observed in this study in rabbit farms compared to ST121 strains, which represented the 34.74%. However, despite the fact that under experimental conditions the ST96 strains were not able to infect as well as ST121, it is clear that they did cause problems on the farms, being isolated from all

types of lesions, such as mastitis, pododermatitis and abscesses, but no relationship between genotype and lesion was found (Viana *et al.*, 2007), neither in this study.

Nevertheless, it could be possible that these clones may infect with a greater number of bacteria. Unfortunately, there are scarce information about rabbit intradermal models using high doses of rabbit strains. Hermans *et al.* (2000a) carried out an intranasal infection with high and low virulence strains at high doses and observed that HV strains colonized more than LV strains. Moreover, some animals with HV strains developed purulent lesions at the end of the experiment. This indicated that those LV strains were unable to colonize and neither to develop lesions, but they were old LV strains and they cannot be compared them with the new ST2855 strains. Later, Meulemans *et al.*, (2007) carried out an intradermal infection with high doses using HV and LV strains and they observed that LV strains were able to infect but produced smaller lesions than the HV strain. Therefore, it is needed further investigation and experiments, especially because of the importance of these clones, which exhibit resistance to methicillin and represent a risk for rabbit to human transmission.

In the case of clone ST3764, this dissemination could be due to a greater virulence, but it was not possible to confirm with the statistical analysis. Regarding to ST2855, ST146 and ST398 clones, the spreading could be related to several aspects. First, the low number of affected animals or even the absence of injuries in some farms may difficult the diagnosis and the problem could be underestimated. In these farms sometimes they simply choose to eliminate the most affected animals or to treat with antibiotics. This links with the second aspect, which are the presence of antibiotic resistance in these clones. The treatments administered in the farms could be counterproductive, since on the one hand, the risk of developing more resistance increases and on the other hand, the bacteria may persist in the farm and can spread to other animals or other farms. The third aspect related to the spreading could be unknown genetic factors, since here only

few genes involved in pathogenesis have been analysed. Finally, it is difficult to know the flow of animals, people, resources (as feed and lorries) and sperm. All this could be a source of dissemination between farms and provinces.

In summary, it can be confirmed that the outbreaks of recent years are produced by different clones present in rabbit farms, both previously isolated clones and new ones. However, there are wide differences between them and therefore it is necessary to correctly identify the strains present in each farm for efficient management of the problem. Although an increase in the infective capacity of ST2855 and ST3764 have not been observed, it is obvious that these clones and others are spreading.

5.2. Effect of different housing systems on health and welfare traits of commercial female rabbits

5.2.1. Haematological parameters and hair cortisol concentration between 1AI and 5P

In this study, several parameters associated with the health status affecting the rabbits housed in different cages were evaluated as an important factor of animal welfare. First of all, the values between 1AI and 5P were compared to know the general status of the female rabbits. The reduction in erythrocytes, haemoglobin and haematocrit may indicate a productive senescence, as previously reported in other studies (Archetti *et al.*, 2008). The increase in leukocytes number, mainly due to an increase of heterophils, and the higher number of platelets between the 1AI and 5P is considered a normal increment because the female rabbits moved from about 5-months-old at 1AI to be between 1-year-old to 1.5-years-old at 5P and these parameters increase as the rabbits get older (Weiss and Wardrop, 2011). Therefore, despite these changes, the levels of erythrocytes, haemoglobin, haematocrit, platelets, leukocytes and heterophils remained within the normal range of values for female rabbits with these ages.

Regarding to the significant increase of 4% haptoglobin concentration from 1AI to 5P, it has been also reported previously by Argente *et al.* (2014). This rising may be related to the development of chronic diseases such as pododermatitis and abscesses in the female rabbits of this study (Dishlyanova *et al.*, 2011). The reproductive stress augments not only haptoglobin but also cortisol concentration (Argente *et al.*, 2014), which was also increased in hair of females at 5P (+94%; *P*=0.001). In addition, stress and high cortisol concentrations impairs phagocytic activity, which was also significantly decreased at 5P compared to 1AI (-31%; *P*=0.001). The reduced heterophil activity enhances the risk of development of bacterial and viral infections (Heller *et al.*, 2000).

The relationship between the increase of cortisol levels, the loss of functionality of the heterophils and the appearance of lesions can occur in the opposite direction, since if a disease or lesion develops, the level of stress and cortisol concentration will rise, which will reduce the phagocytic activity and may contribute to the chronicity of the lesions and the emergence of new infections (Stephan *et al.*, 2002).

Surprisingly, the H/L ratio had only a non-significant increasing trend between the 1AI and the 5P. This increase of the H/L ratio was mainly due to the increase in the number of total heterophils. Therefore, the alteration of the majority of the studied parameters (except the H:L ratio) would indicate a worse health and welfare status of the animals at 5P.

5.2.2. Haematological parameters and hair cortisol concentration at 5P

The most important analysis to assess the health and stress status of the females in the different housing types herein studied was performed by the comparison of the parameters at advanced moment of the productive life (5P). After comparing the five groups at 5P, significant differences were observed in haemoglobin, platelets, eosinophils, haptoglobin and hair cortisol concentration. Despite the significant differences in haemoglobin, platelets and eosinophils, the levels of these parameters remained within the normal range of values for these animals according to Weiss and

Wardrop (2011), as well as the rest of the blood parameters. Then, these three parameters should not be used alone to decide which housing is better, especially when there were no differences in erythrocytes and haematocrit.

Significant differences in haptoglobin concentrations were observed among groups, with higher values in the COL group than in HD, TR and PF. The increased haptoglobin in the COL group could be related to more animals showing lesions in this group because haptoglobin concentrations rise during several days when a wound or inflammation occurs (Dishlyanova *et al.*, 2011). In addition to the injuries, aggressive interactions between females located in COL could have produced an increase in haptoglobin concentration alongside the increasing of cortisol concentration (Argente *et al.*, 2014).

Regarding to hair cortisol concentration, there were also significant differences between groups. PF group (1.67±0.09) and COL group (1.45±0.10) had the greatest levels and HD group had the lowest concentration (0.23±0.09). These results were similar to those obtained in other studies in which faecal corticosterone concentrations were 3-fold higher in the group-housed does than in the single-caged ones (Szendrő *et al.*, 2013). Cortisol is a hormone of physiological response to stress and it accumulates in hair along the productive life (Pragst and Balikova, 2006). In fact, it is reported that acute ACTH-induced elevations of circulating cortisol do not affect hair cortisol concentrations in calves (Tallo-Parra *et al.*, 2017). Thus, the female rabbits placed in both PF and COL housing types could have been subjected to greater chronic stress conditions, which may suggest that they were housed under worse welfare conditions.

The high cortisol concentration in PF females is surprising because platforms in cages were incorporated as a form of environmental enrichment to reduce animal stress (Hansen and Berthelsen, 2000; Buijs *et al.*, 2011). However, it should be taken into account that the platform cages herein used were narrower than the other cages, and platforms were made of wire.

Therefore, these two characteristics of the PF cages could explain the high cortisol concentration in the hair of the PF does, but not the presence of the platform itself (Masthoff and Hoy, 2019). To solve these problems, this platform cage could be improved using a plastic platform instead of a wire one and increasing cage width, but this can impair farm productivity.

Although there were significant differences in haptoglobin and cortisol levels, other immunological indicators, such as the H/L ratio and phagocytic capacity, were not significantly affected by the type of housing. However, there was a trend in H/L, which could indicate more stress in TR group than HD group.

Taking into account the results of blood haptoglobin levels and hair cortisol concentrations, a higher stress level can be assumed in this COL housing system than in the others herein studied. This higher stress may be due to the aggressive interactions between the animals and also due to the higher number of lesions observed, as reported previously in other studies (Dal Bosco *et al.*, 2019; Szendrő *et al.*, 2019).

5.2.3. Pathological results

Morbidity and mortality of rabbit does were studied as two important parameters of welfare. Also kit mortality during lactation was evaluated. A greater kit mortality rate during lactation was observed in the present study for the COL group (13.1%) vs. groups HD (6.4%) and PF (7.8%). This could be due to the stress and aggressive behaviour of the females housed in this group, as observed by Szendrő *et al.* (2013), who reported 15.2% suckling mortality in single-caged does and 38.5% in group-caged does, being even higher than kit mortality herein observed in COL group.

More animals were discarded from the COL group (n=18), being significant compared to HD (n=10) and PF (n=9) groups. This significant difference was mainly due to the greater number of culled does (n=13). It was surprising that PF group had the lowest number of discarded animals while female rabbits in this housing had the highest hair cortisol concentration

together with the females in COL group. This suggests that the high cortisol level is not correlated with greater morbidity and mortality in this type of housing. And precisely because of this, it is mandatory to take into account not only one or two welfare indicators but several of each type, such as physiological, productive and behavioural ones.

Previous studies have shown that the survival rates of group-housed does were lower than single-caged does (Szendrő *et al.*, 2013). Regarding reproductive problems, which were herein found mainly in the COL group, have also been reported by Dal Bosco *et al.* (2019), who observed poorer reproductive performance (i.e. low kindling rate and pre-weaning kit mortality) in part-time housed rabbit does than individual housed ones. Nevertheless, part-time housing is clearly better than full-time housing regarding to injuries, productivity and behaviour (Dal Bosco *et al.*, 2019).

Reproductive problems, injuries and abscesses contributed to the greater number of discarded female rabbits in COL housing. But the high percentage of reproductive problems found in the present study does not match what Sánchez *et al.* (2012) observed, who described diseases of the respiratory system, mastitis and ulcerative pododermatitis as the commonest disorders in rabbit does on Iberian farms. However, it should be noted that Sánchez *et al.* (2012) studied diseases on several commercial rabbit farms, while this study was conducted on an experimental farm. The necropsied rabbit does in our study had very few cases of pododermatitis (n=8; 14.5%) and mastitis (n=1; 1.8%). The few pododermatitis cases could be due to the use of footrests in all the cages and the farm's high standard of cleanliness.

The infectious diseases, as the main causes of death in this study, seemed unrelated to the high level of stress and cortisol concentrations in PF and COL groups. However, it could not be confirmed because there were no significant differences between groups regarding to the causes of death, mainly because of the low number of dead animals. Rosell and de la Fuente (2016) reported the respiratory and digestive conditions as the most

common causes of death, while in this study digestive diseases were infrequent.

Regarding to all the lesions found in the necropsies, frequently involved reproductive and respiratory systems, but very few lesions affected digestive system This differed from lesions observed by Rosell and de la Fuente (2016), who described the digestive disorders as the second most common cause of death. In the present study, only four gastric ulcers and two enteritis were found. Although gastric ulceration has been related with stress (DeCubellis and Graham, 2013), no relationship could be demonstrated herein due the low number of animals.

In summary, there were no significant differences in mortality of the rabbit does between the five groups but there were significant differences in morbidity, since more females were culled in COL group than in HD group. This is consistent with the higher hair cortisol concentrations in COL group. On the contrary, PF group had also high cortisol levels but had a lower number of discarded females than COL group. There were significant differences in kit mortality, since COL group had higher rate than HD and PF groups. These data about culling and kit mortality may suggest that COL housing is worse than HD and PF housings.

5.2.4. Microbiological results

In breeding rabbits, the two most frequent bacteria producing purulent lesions are *S. aureus* and *P. multocida* (Segura *et al.*, 2007). In this study, *P. multocida* was the most frequently isolated bacterium from pneumonic lungs, metritis and abscesses. However, Segura *et al.* (2007) isolated *S. aureus* from abscesses more commonly than *P. multocida*. As explained above, this difference could be due to the distinct nature and management of the analysed farms. Other explanation to the high percentage of animals with *P. multocida* may be the presence of a high virulent strain of this bacterium (Massacci *et al.*, 2018; Peng *et al.*, 2018), but this could not be confirmed because the typing of isolated strains was not performed.

The *S. aureus* genotypes found in this study (mainly B1/I1/α and B1/I1/λ) differed from previous data because Viana *et al.* (2007) described A1/II1/δ as being the most frequent genotype in commercial rabbitries, with 70% of the isolates. B1/I1/α and B1/I1/λ, both isolated herein, belonged to the ST96 clone, which is considered a low-virulence *S. aureus* clone. This could explain the few staphylococcal lesions detected in this study. However, these genotypes have also been isolated from farms with high prevalence of staphylococcal infections, cause serious problems in those farms (Viana *et al.*, 2007).

The third most frequent bacteria isolated from lesions in this study were *Proteus* spp. This bacterium does not often produce lesions in rabbits, but it has been isolated sometimes from abscesses (Harcourt-Brown and Harcourt-Brown, 2002; Taylor *et al.*, 2010). *Pseudomonas aeruginosa* can also produce skin infections and can be involved in respiratory diseases but is less frequent than *S. aureus* or *P. multocida* (Rosell and de la Fuente, 2016). *Escherichia coli* is only present in small numbers in the intestinal flora of rabbits but pathogenic strains can proliferate under some circumstances and cause digestive diseases (Harcourt-Brown and Harcourt-Brown, 2002). However, in this study digestive diseases were not frequent, and the isolation of *E. coli* from lesions unrelated to digestive system could be due to cross contamination, since blood agar plates had not pure *E. coli* growth. Therefore, the finding of *E. coli* was considered not relevant. Finally, *Bordetella bronchiseptica* is usually found in respiratory tract without causing any disease but it can cause respiratory infections together with *P. multocida*, which makes the treatment more difficult (Harcourt-Brown and Harcourt-Brown, 2002).

The microbiological results must be analysed carefully because the isolation of a bacterium from a lesion could not mean that this bacterium is producing the disease. It could be a commensal bacterium or even a cross-contamination. In addition, some bacteria do not grow without specific conditions, thus a negative result could be obtained when analysing a

sample with conventional cultures. However, if a differential diagnosis is made previously based on the observed lesion, the microbiological diagnosis will be properly done.

5.2.5. Group-housing and wild rabbits

In recent decades, good group-housing designs for rabbit does have been sought to allow natural behaviour to develop. However, a large number of problems still need to be solved in group housing, like aggression between females even when they had escape routes (Rommers and de Greef, 2018), management problems (Machado *et al.*, 2016) or reduced productivity (Cervera *et al.*, 2017). Furthermore, no reduction in stress indicators was observed and a higher number of rabbit does had skin lesions (Maertens and Buijs, 2016; Zomeño *et al.*, 2018).

The aggressive interactions in collective systems also affects kits, increasing the mortality rate during lactation. In this study there was a kit mortality of 13.1% in the COL group and Szendrő *et al.* (2013) reported a suckling mortality of 38.5% in group-caged does. This problem could be related with the natural behaviour of wild rabbit does, as explained below.

Rödel *et al.* (2008) observed more aggressive interactions per hour between wild female rabbits in close proximity to their breeding burrows on the first 20 days of lactation. This behaviour was observed between female rabbits housed in collective systems (Szendrő *et al.*, 2013) and it could explain the high number of culled female rabbits observed in the COL group, mainly due to abscesses and reproductive problems. In wild rabbits, aggressive interactions also affect kits, producing a kit mortality up to 32.4% (Rödel *et al.*, 2009). In addition, aggressive behaviours have also been described in other animals, like horses (Vervaecke *et al.*, 2007) and mice (Van Loo *et al.*, 2002).

Unfortunately, making comparisons among several studies is not easy because they involve different cage characteristics. For this reason, further investigations are needed to understand the aggressive behaviour between

female rabbits to solve the problem of kit mortality, social stress, injuries and low productivity.

5.2.6. Choosing the best housing

This is one of the most extensive studies on the effect of different housings on the health of rabbit does. According to the parameters herein studied, higher and deeper single-housing (HD) had better welfare indicators, such as lower haptoglobin and cortisol concentrations, fewer culled rabbit does and lower kit mortality than the other housing types used in this study. Conversely, the COL group had higher haptoglobin and cortisol concentrations and kit mortality, and more culled rabbit does. These results suggest that the female rabbis housed in COL cages suffered more stress and were under worse health status compared to the individual housing systems herein studied. Thus, neither this part-time collective system (COL) nor this cage with a wire platform (PF) is recommended to guarantee the welfare of rabbit does.

Although the high kit mortality and large number of lesions observed in the group-housed rabbits in this study resemble those observed under natural conditions, the expression of typical aggressive behaviour should not prevail over animals' health and physical integrity when evaluating the best housing system. Finally, choice of the best system should also consider each farm's specific characteristics.

VI- CONCLUSIONS

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1- There has been a change of the *S. aureus* clones affecting rabbitries in Iberian Peninsula, mainly due the emergence and dissemination of ST3764 and ST2855 clones.

2- Recent ST121 strains and ST3764 strains (CC121) have acquired Sa1int and Sa6int prophages, which differs significantly in comparison with the ancestral rabbit ST121 from the year 2002.

3- ST2855 strains differed from ST96, both belonging to CC96. The majority of ST2855 strains have lost the Sa3int phage, losing the human IEC and recovering the β-haemolysin activity.

4- Other strains, such as ST1, ST146 and ST398, with different genetic characteristics, were also affecting rabbitries, although in a lower percentage.

5- The genetic differences observed between the old and the new strains did not explain the recent outbreaks, since ST2855 strains were not able to infect and ST3764 did but without significant differences with Jwt.

6- Female rabbits located in the higher and deeper housing had lower hair cortisol levels than females located in the rest of housings. Also culled females and kit mortality rate were the lowest in this housing than in the other housings.

7- Contrary, female rabbits located in collective housing had greater haptoglobin concentrations and kit mortality rate. In addition, more females were culled in collective housings.

8- Platform cages produced elevated hair cortisol concentrations although the rest of parameters were similar to the rest of cages.

9- The collective system here studied produced a worse health status in female rabbits than the higher and deeper housing.

VII- CONCLUSIONES

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1- Ha habido un cambio en los clones de *S. aureus* que afectan a los conejos en la Península Ibérica, principalmente debido a la aparición y diseminación de los clones ST3764 y ST2855.

2- Las cepas ST121 recientes y las cepas ST3764 (CC121) han adquirido profagos Sa1int y Sa6int, lo que difiere en comparación con la cepa ST121 de conejo del año 2002.

3- Las cepas ST2855 son diferentes de las ST96, ambas pertenecientes al CC96. La mayoría de cepas ST2855 han perdido el fago Sa3int, perdiendo el IEC humano y recuperando la actividad de la β-hemolisina.

4- Otras cepas, como la ST1, ST146 y ST398, con diferentes características genéticas, también se aislaron de lesiones en conejos, aunque en un porcentaje menor que ST121, ST3764, ST96 y ST2855.

5- Las diferencias genéticas observadas entre las cepas antiguas y nuevas no explicaron los brotes recientes, ya que las cepas ST2855 no pudieron infectar. Las cepas ST3764 sí lo hicieron pero sin diferencias significativas con la Jwt.

6- Las conejas ubicadas en la jaula más alta y más profunda tenían niveles más bajos de cortisol en el pelo que las hembras ubicadas en el resto de alojamientos. Las conejas eliminadas y la tasa de mortalidad de los gazapos fueron más bajas en esta jaula que en el resto.

7- Por el contrario, las conejas ubicadas en el alojamiento colectivo tenían mayores concentraciones de haptoglobina y mayor tasa de mortalidad de los gazapos. Además, un mayor número de conejas fueron eliminadas en este alojamiento colectivo.

8- Las jaulas con plataforma produjeron concentraciones elevadas de cortisol en pelo, aunque el resto de parámetros presentaron niveles similares a los observados en las otras jaulas individuales.

9- Las conejas alojadas colectivamente presentaron un peor estado de salud que aquellas alojadas en jaulas más altas y profundas.

VIII- REFERENCES

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