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EPIDEMIOLOGY, VIRULENCE FACTORS AND ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED IN SMALL RUMINANT DAIRY CHAIN

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ABSTRACT

Epidemiology, virulence factors and antibiotic resistance in *Staphylococcus aureus strains* isolated in small ruminants dairy chain.

The aim of the present thesis was to conduct an extensive characterization of *S. aureus* isolated from small ruminant milk and dairy products. *S. aureus* is considered a major public health concern because of its ability to give disease in human, after consumption of contaminated foods with enterotoxins. This pathogen can aquire resistance against several antibiotics used in human and veterinary practices. Farms and cheese-making plants can serve as a reservoir of *S. aureus* and are involved in the spread of the microorganism into environment. Concern exists on the possible acquisition of antibiotic resistance by human strains, as consequence of exchange of mobile genetic elements with strains of animal origin. The extent and impact of these strains on human health was also assessed.

In chapters 3, 4, and 5 are presented results on prevalence, virulence factors and antibiotic resitance of *S. aureus* strains isolated from goats with subclinical mastitis and from bulk tank milk. In chapters 6, 7 and 8 are reported results on *S. aureus* strains isolated from raw sheep's milk cheese. Prevalence, virulence daterminants and antibiotic resistance have been assessed. Additional informations have been provided on the genetic variability of the strains and their relationship with the host.

Abbreviations

AGP	antibiotic growth promoter
AM	ampicillin
AMOVA	Analysis of Molecular Variance
AMR	antimicrobial resistance
AMX	amoxicillin
BHI	brain hearth infusion
BP	baird-parker
BPM	baird parker medium
BPW	buffered peptone water
CAMHB	cation-adjusted Mueller-Hinton broth
CA-MRSA	community-associated methicillin resistant *Staphylococcus aureus*
CC	clonal complex
CF	cephalotin
CFP	cefoperazone
ClfA	clumping factor A
ClfB	clumping factor B
CLSI	clinical and laboratory standards institute
CoNS	coagulase negative staphylococci
CPS	coagulase positive staphylococci
CRO	ceftriaxone
DNA	deoxyribonucleic acid
E	erythromycin
EC	European commission
ECDC	European center of disease and prevention control
ECM	extracellular matrix
EFSA	European food safety authority
EMEA	European medicine agency

ETA	exfoliative toxin A
ETB	exfoliative toxin B
EYTE	egg yolk tellurite emulsion
FAO	food and agriculture organization
fnBPA	fibronectin-binding protein A
fnBPB	fibronectin-binding protein B
FSANZ	food standards Australia New Zealand
GHP	good hygiene practices
GLM	general linear model
GMP	good manufacturing practices
HACCP	hazard analysis and critical control points
HA-MRSA	health care-associated methicillin resistant *Staphylococcus aureus*
IMI	intramammary infection
K	kanamycin
KF	cephalothin
LAB	lactic acid bacteria
LA-MRSA	livestock-associated methicillin resistant *Staphylococcus aureus*
MGE	mobile genetic element
MIC	minimum inhibitory concentration
MRSA	methicillin resistant *Staphylococcus aureus*
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
MSSA	methicillin susceptible *Staphylococcus aureus*
NSW	new south Wales
NV	novobiocin
OB	cloxacillin
OFX	ofloxacin
OT	oxytetracycline
OX	oxacillin

P	penicillin
PABA	para-aminobenzoic acid
PBP	penicillin binding protein
PCR	polymerase chain reaction
PFGE	pulsed filed gel electrophoresis
PRP	penicilinase-resistant penicillin
PTAgs	pyrogenic toxin superantigens
PVL	panton-valentine leukocidin
RNA	ribonucleic acid
RPF	rabbit plasma fibrinogen
RPLA	reverse passive latex agglutination
rRNA	ribosomal ribonucleic acid
SAgs	superantigens
SaPI	staphylococcus aureus Pathogenicity Island
SBA	sheep blood agar
SCC	somatic cell count
SCC*mec*	staphylococcal cassette chromosome *mec*
SCM	subclinical mastitis
SD	standard deviation
SE	staphylococcal enterotoxin
SEA	staphylococcal enterotoxin A
SED	staphylococcal enterotoxin D
SEE	staphylococcal enterotoxin E
spA	staphylococcal protein A
SSSS	staphylococcal scalded-skin syndrome
ST	sequence type
TCS	two component system
TE	tetracycline

Tn	trasposon
TSST-1	toxic shock syndrome toxin-1
VA	vancomycin
VISA	vancomycin-intermediate *Staphylococcus aureus*
VRE	vancomycin resistance enterococci
VRSA	vancomycin resistance *Staphylococcus aureus*
WHO	world health organization

CHAPTER 1

Introduction

1.1. Staphylococcus aureus *epidemiology*

The genus *Staphylococcus*, belongs to the *Staphylococcaceae* family and *Bacillales* order. Among the microorganisms belonging to this family, *Staphylococcus aureus* is the most representative and studied, because of its potential pathogenicity against human and animals. The microorganism primary habitat is natural environment (soil, water and air) but it can also represent part of normal flora of human and animals, where it can be isolated from skin, mucous membranes and intestine. The ability of the pathogen to cause disease is closely related to the host susceptibility, such as immune system response, predisposing conditions or accidental factors (trauma, injury). Previous studies demonstrated no fundamental differences existing between colonizing and infecting *S. aureus* population (van Belkum et al., 2009).

S. aureus carriers could be classified into three different classes: those who always carry a strain (20%), those who intermittently carry different strains (60%) and the remaining hosts who never carry *S. aureus* strains (Von Eiff et al., 2001; Weems and Beck, 2002).

The hosts, especially asymptomatic host, play an important role in the spread of *S. aureus* strains into the environment. Moreover, this pathogen shows a wide adaptability to several environmental and host factors, thus permitting colonization of "susceptible environments" such as hospitals. Prevalence of *S. aureus* strains is around 11-32% in general population and about 25% in hospital personnel (Wenzel and Perl, 1995).

Presence of *S. aureus* in hospitals is a major concern for public health because of its ability to develop or to acquire antibiotic resistance, especially to methicillin.

In 1940, when penicillin was first used to treat infections in human medicine, all the *S. aureus* strains were susceptible. After few years approximately 50% of the strains showed resistance to penicillin because of the production of β-lactamase enzymes, which catalyze hydrolysis of β-lactam ring (Yuan et al., 2011), codified by *blaZ* gene. In 1959 has been introduced in human medicine the antibiotic methicillin, to treat infections caused by *S. aureus* resistant to penicillins (Enright et al., 2002). After only one year a study conducted by Jevons (1961) reported the appearance of *S. aureus* strains that showed resistance to celbenin (methicillin) isolated from

routine material in United Kingdom hospitals, afterwards referred to as methicillin resistant *Staphylococcus Aureus* (MRSA). In a few decades the percentage of MRSA strains alarmingly raised. A more recent study carried out in England by the National Health Service showed that the rate of MRSA involving bloodstream infection or colonization of other body sites increased from 5% in 1990s to over 40% in 2000s decades (Johnson et al., 2005).

MRSA can be included in two different groups, health care-associated (HA-MRSA) and community-associated (CA-MRSA) strains (Schwalm III et al., 2011). These two groups, although have similar microbiological characteristics; differ for risk factors, genetic structure, virulence determinants and antibiotic resistance. CA-MRSA carry type IV or V staphylococcal cassette chromosome *mec* (SCC*mec*) element, are usually producer of Panton Valentine Leukocidin (PVL) and are not multidrug resistant. HA-MRSA carry type I, II or III SCC*mec*, do not produce PVL and show multidrug resistance (Wang et al., 2010).

In recent years have been reported cases of MRSA strains linked to livestock classified as Livestock-associated MRSA (LA-MRSA). These strains, which are not HA or CA-MRSA related, belong mainly to a specific lineage 398 (ST398/CC398), although other minor sequence type (ST1, ST5, ST8, ST9, ST30, ST97) have been described (Cavaco et al., 2011). The emergence of LA-MRSA could be related to a selection and differentiation among the population of methicillin susceptible *Staphylococcus aureus* (MSSA) [Hasman et al., 2010]. Pigs and pig farms are recognized as one of the most important source of LA-MRSA in farmers, veterinarians and their families. The ST398 is the most represented and widely disseminated in countries with intensive pig farming (Cuny et al., 2010). A study carried out in Netherlands showed that 40% of the investigated farms and 80% of the animals were positive for MRSA-ST398 (de Neeling et al., 2007). Prevalence of LA-MRSA in dairy cattle is generally low and mainly associated with clinical and subclinical mastitis (Bengtsson et al., 2009). After the first report of MRSA involved in cases of cattle mastitis, this pathogen has been occasionally reported in dairy cattle farms (Cuny et al., 2010). Prevalence of MRSA in poultry flocks is low as well, although the rate of this resistant pathogen increases on mixed poultry-pig farms. In this case, the source of MRSA contamination for animals and the persistence in environment is

accountable to the farmer (Pletinckx et al., 2011). MRSA strains are detected in horses and companion animals too. The prevalence in horses ranges between 0 and 4.7% in horse farms and between 10.9 and 16% during hospitalization (van Duijkeren et al., 2010). Regarding companion animals different studies showed an increase of MRSA infection, mainly associated with post-operative infection and open wounds (Rich and Roberts, 2004, 2006).

Since their appearance LA-MRSA has been considered as an occupational hazard for people that work in contact with animals. Animals are a potential reservoir of LA-MRSA, while people can serve as carriers of these resistant strains into community and, occasionally, into hospitals (EFSA, 2009). All the animals can represent a potential source of MRSA strains, with differences in prevalence, as described above.

1.2. *Staphylococcus aureus* in small ruminants dairy chain

Raw milk is an ideal growth medium for several pathogen bacteria having an impact on both public health and economic aspects. Among these microorganisms, *S. aureus* is one of the pathogens causing one of the most health concern because of its ability to give disease in human, after consumption of contaminated milk and dairy products. Moreover the presence of *S. aureus* in small ruminant dairy farms can cause important economic losses, due its ability to cause diseases, especially against mammary gland.

S. aureus can occur in all the steps of the small ruminants dairy food chain.The most common transmission pathway of *S. aureus* into the flock is the transferring from an infected mammary gland to an uninfected gland, especially during milking time through teat clothes, milkers' hand and milking equipment (Petersson-Wolfe et al., 2010). *S. aureus* is one of the major causes of intramammary infection (IMI) in cows, sheep and goats, leading to heavy economic losses (Seegers et al., 2003). Many differences exist among dairy ruminants, thus requiring a specific approach to the mastitis problem in small ruminants and not by generalizing results obtained on mastitis in cows. Several studies have demonstrated that *Staphylococcus* spp. and *S. aureus* are the most frequently causal microorganisms of IMI in dairy sheep and goats, though other

pathogens, such as Coagulase Negative Staphylococci (CoNS), *Streptococcus* spp., *Corynebacteria*, *Enterobacteriacae*, *Pseudomonas* and *Mycoplasma* spp. can be involved (Contreras et al., 2007).

It is extremely difficult to control mastitis caused by *S. aureus* strains. Understanding the epidemiology of the microorganism (transmission pathway and risk factors) and the prevention of new intramammary infections are crucial steps to control *S. aureus* into dairy herds. Different works demonstrated that teat sanitization with germicidal agents (iodine, chlorhexidine, quaternary ammonium, etc.) especially after milking (post-dipping) plays an important role to control and prevent contagious mastitis. Post-dipping is very effective in preventing new IMI (Contreras et al., 2007), while have not effects in restoring udder health of animals with subclinical mastitis (Klinglmair, 2005). Unfortunately post-dipping practice is not frequently used in small ruminant dairy farms (Bergonier et al., 2003).

S. aureus can cause different IMI patterns, such as clinical peracute, acute and chronic mastitis, although is also responsible for subclinical mastitis, especially in cows (Akineden et al., 2001). Subclinical mastitis is the most fearful forms of udder disease because they don't cause macroscopic alterations in milk and udder. Animals with subclinical types of IMI are a reservoir of *S. aureus* into dairy environment and represent the most common cause of raw milk contamination.

Mastitis in dairy sheep and goats is a cause of major concern in milk and dairy products sector for economical and public health reasons. IMI can cause economic losses as consequence of decrease in milk production, undervaluation of milk with high Somatic Cell Count (SCC), sheep, goats and lambs mortality, treatment costs. In dairy sheep and goats the great part of subclinical mastitis are accountable to CoNS, with prevalence ranging from 25 to 93%, while *S. aureus* prevalence ranges from 3 to 43% (Vautor et al., 2005; Fotou et al., 2011). Into dairy farms, the presence of animals affected by *S. aureus* IMI can result into a public health problem. In small ruminants *S. aureus* is responsible of both clinical, mainly gangrenous mastitis, and subclinical mastitis. *S. aureus* produces a wide range of virulence factors involved in the pathogenesis of mastitis, which also play a role in foodborne disease. The ability of the

pathogen to cause food poisoning is mainly related to the SEs production. Although *S. aureus* can cause both clinical and subclinical mastitis, a study conducted by De Santis et al. (2005) have demonstrated that isolates from sheep with subclinical forms of IMI are less enterotoxigenic than the isolates from clinical cases. Therefore, prevention and control of IMI in small ruminant dairy farms is important not only for epidemiologic purposes, but also to prevent or reduce the risk of food poisoning.

Along small ruminant milk and dairy products chain, *S. aureus* growth can occur mainly in raw milk as a consequence of temperature abuse during storage, or in the first cheese-making steps, especially in raw milk cheeses, when the pathogen multiplication is not inhibited by the activity of lactic acid bacteria (Charlier et al., 2009). Technologies used to produce raw sheep's and goat's milk cheeses and the absence of heat treatments, such as thermization or pasteurization, do not give insurance to control *S. aureus* multiplication (FSANZ, 2009).

Different studies carried out on prevalence of the pathogen during cheese-making phases, showed an increase in *S. aureus* count up to six hours after molding, when the lowest pH of the cheese has been reached (Pisano et al., 2007; Jakobsen et al., 2011).

To date little information is available on *S. aureus* prevalence in raw sheep and goat milk, while more data can be find in literature on the prevalence in cow's milk. However, studies carried out on prevalence of Coagulase Positive Staphylococci (CPS) ranged between 11% and 29% in raw ewe's milk, and between 32% and 91% in raw goat's milk (Muehlherr et al., 2003; Scherrer et al., 2004; Jackobsen et al., 2011).

On the other hand more data are available in literature on the prevalence of *S. aureus* in small ruminant raw milk cheeses, probably due to the great impact of these products on public health. Cheeses, especially made from raw milk, are accountable for about 5% of the total outbreaks due to staphylococcal foodborne intoxication in Europe (European Commission, 2003). Prevalence of *S. aureus* in small ruminants raw milk cheeses ranged between 60% and 100%, six hours after molding (De Buyser et al., 2001; Tekinşen and Özdemir, 2006; Pisano et al., 2007; Scarano et al., 2007; Jackobsen et al., 2011). After dry or brine salting raw milk cheeses are ripened for a long time (three months or more). Ripening is a crucial step during raw milk

cheese production to limit *S. aureus* growth. During this phase, *S. aureus* mean count progressively decreases, due to the modifications of chemical parameters of the cheese, such as pH, water activity, redox potential and the presence of inhibitory substances other than lactic acid (Vernozy-Rozand et al., 1998).

1.3. *Staphylococcus aureus* virulence factors

S. aureus is an ubiquitous pathogen microorganism which can causes illness in human and animals. It commonly lives as commensal colonizing the skin and nasal mucosa of humans. *S. aureus* disease can be associated to skin and wound mild infections or with severe illness, such as septicemia, pneumonia, endocarditis, osteomyelitis, septic arthritis and toxic shock syndrome (Arvidson and Tegmark, 2001).

S. aureus is also involved in animal illness, especially during intramammary infections, which determine a significant loss in dairy industry (Sears & McCarthy, 2003). *S. aureus* can cause human illness either by direct infection (human to human or animal to human) or by assumption of contaminated foods with Staphylococcal Enterotoxins (SEs). Staphylococcal food poisoning results after ingestion of contaminated foods with a sufficient amount of preformed SEs. Symptoms are nausea, vomiting, abdominal cramps and sometimes diarrhea. This illness is usually self-limiting and resolves after 24-48 hours. Sometimes, especially for susceptible patients, such as young and immune compromised people, can lead to hospitalization (Argudín et al., 2010). Contamination of foods with *S. aureus* at levels $\geq 10^5$ cfu/g is compatible with production and excretion of SEs and their presence should be investigated on incriminated foods (Commission Regulation (EC) No 2073/2005).

S. aureus carries into genome several genes directly involved in the virulence of the strains or in the control and regulation of virulence determinants expression. Regulation in the expression of *S. aureus* virulence determinants is under control of the accessory gene regulator (agr) system. Agr locus, originally discovered in *Tn551*, consists of two transcriptional units, P2 and P3. Unit P2 carries four genes, *agrA*, *agrB*, *agrC* and *agrD*. *agrA* and *agrC* are the typical two

components signal transduction system, while *agrB* and *agrD* encoded an autoinducing peptide (figure 1), involved in cell to cell communication in Gram positive bacteria (Sturme et al., 2002). Later, other five additional loci (*sarA, sarH1, sae, rot, and 1E3*) have been identified as involved in the global regulation of *S. aureus* virulence factors (Arvidson and Tegmark, 2001).

To date approximately forty different extracellular and cell surface proteins, involved in *S. aureus* pathogenicity, have been identified (Arvidson and Tegmark, 2001). They can be classified as staphylococcal adherence factors and staphylococcal exoproteins.

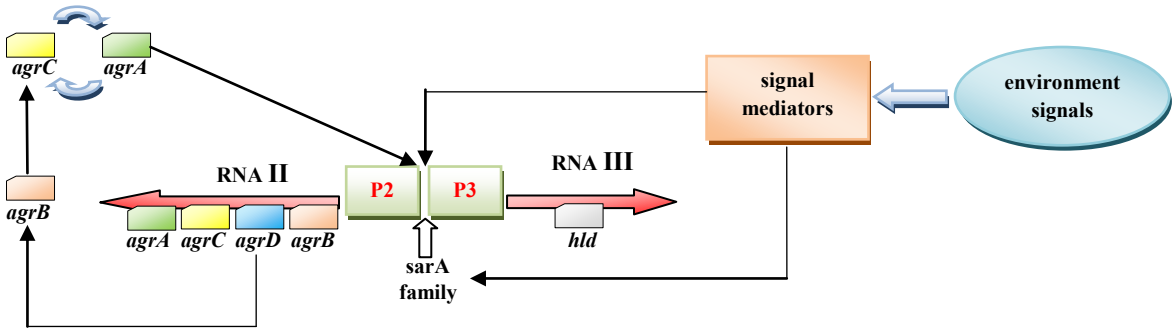

Figure 1. Schematic representation of accessory gene regulator system (Agr*BDCA*) in *S. aureus*.

1.3.1. *S. aureus* adherence factors (adhesines)

Adhesines are specific proteins involved in the adherence of *S. aureus* strains to the host cell surface and are essential in the first phases of cell colonization. They belong to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM). *S. aureus* adhesisnes can be classified on the basis of their adherence target (figure 2):

staphylococcal protein A (spA)

It binds the Fc fragment of the immunoglobulins, essential to avoid phagocytosis. spA protein is encoded by the *spa* gene, mapped on the chromosome, and it is expressed during the exponential phase of bacterial growth, while is down- regulated during the post exponential phase (Gao and Stewart, 2004).

fibronectin-binding proteins A and B (fnBPA and fBPB)

fBPA and fBPB recognize fibronectin, fibrinogen and elastin of the cell host and promote internalization in epithelial and endothelial cells by a fibronectin bridge between *S. aureus* and integrin. These proteins are encoded by *fbnA* and *fbnB* chromosomal genes (Burke et al., 2010).

collagen-binding protein

Collagen is the main component of the Extracellular Matrix (ECM). *S. aureus*, as other bacteria, can express a collagen-binding protein, referreed to as CNA, which plays a key role in the adherence to the external structure of the host cell. *S. aureus* strains carrying CNA protein show an increase in virulence when compared with CNA negative strains (Visai et al., 2000).

clumping factor A and B (ClfA and ClfB)

It is another fibrinogen-binding protein which allows the adherence of *S. aureus* strains to the host cell and its subsequent invasion. Fibrinogen molecule contains two different sites which can interact with the active region of the clumping factor. Expression of ClfA and B proteins, encoded by the chromosomal *clfA* and *clfB* genes, causes fibrinogen immobilization and induce platelet aggregation (Palmqvist et al., 2004).

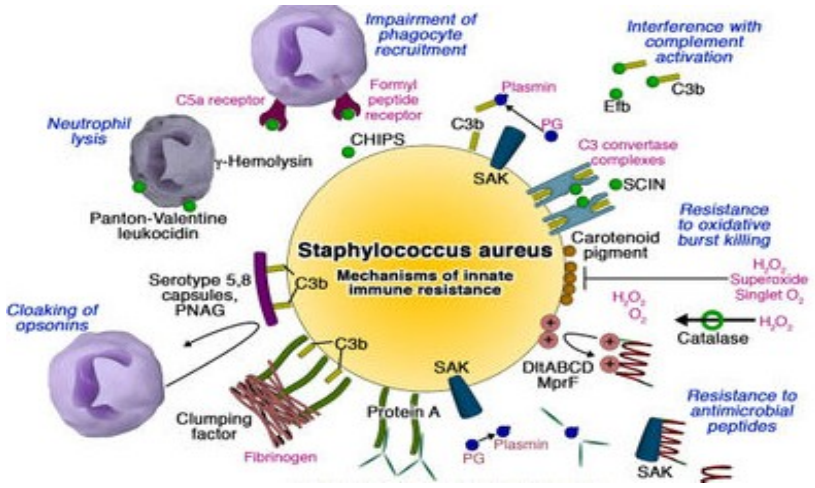

Figure 2. Schematic representation of adherence factors and mechanisms of immune resistance of *S. aureus* strains.

1.3.2. *S. aureus* exoproteins

S. aureus strains are able to produce several exoproteins, such as exotoxins and enzymes. The main function of these proteins is to convert host tissues in nutrients for bacterial growth. Some of the exoproteins produced by *S. aureus* are cytolytic toxins and have a direct impact on the virulence of the strains, allowing to overcome host defenses (Figure 2).

α, β, δ and γ-hemolysins

α-hemolysin (Hla), encoded by *hla* gene, is active against the red blood cells of a wide range of mammalian, especially against rabbit erythrocytes. It is secreted by the majority of *S. aureus* strains involved in human clinical cases. β-hemolysin (Hlb), encoded by *hlb* gene, is a sphingomyelinase with marked activity against sheep but not for rabbit erythrocytes. It is mainly associated with *S. aureus* isolated from small ruminants. δ-hemolysin (hlD), is encoded by *hld* gene and it is not a host specific hemolysin. About 97% of the *S. aureus* clinical isolates are producers of hlD (Burnside et al., 2010). γ-hemolysin (Hlg) as hlA is a pore-forming hemolytic toxin which can causes membrane damage of red blood cells. *hlg* gene, coding for three different Hlg proteins (HlgA, HlgB and HlgC) that are secreted in two functional pairs: HlgA+HlgB and HlgC+HlgB (Nilsson et al., 1999). *hla*, *hlb*, *hld* and *hlg* genes are located on the *S. aureus* chromosome.

leukocidins

They are exotoxins with high affinity towards leukocytes and are usually involved in severe cases of illness caused by *S. aureus*. Panton-Valentine leukocidin (PVL) is the most important toxin involved in necrotizing diseases and it is mainly associated with *S. aureus* strains isolated from humans (Francis et al., 2005). PVL is a two component pore-forming protein, especially active against neutrophils. It is encoded by the *lukPV* operon coding for the two components LukF-PV and LukS-PV. Strains carrying *pvl* gene have a rise in the virulence rate and are usually associated to CA-MRSA type IV or V (Löffler et al., 2010; Wang et al., 2010).

Other leukocidins are expressed by *S. aureus* strains, such as leukocidin M (LukM) and leukocidin E/D (LukE/D). As PVL they are pore-forming proteins which are active especially against neutrophils, but are mainly related to strains of animal origin. Leukocidin M and E/D are encoded by *lukM* and *lukE/D* genes, located on the chromosome (Barrio et al., 2006).

exfoliative toxin A and B (ETA, ETB)

Some of *S. aureus* strains are able to produce exfoliative toxins. They are mainly associated with *S. aureus* isolated from human clinical cases, although some study have demonstrated production of ETA in *S. aureus* strains isolated from mastitic cow's milk (Hayakawa et al., 2001). ETA and ETB can cause epidermolytic disease in human, from localized and not serious blisters to severe staphylococcal scalded-skin syndrome (SSSS), especially in children (Farrel, 1999). The gene encoding ETA (*eta*) is located on *S. aureus* chromosome, while ETB (*etb*) is carried on plasmid (Růžičková et al, 2003).

toxic shock syndrome toxin-1 (TSST-1)

It is a potent exotoxin produced by *S. aureus* strains isolated from human and animals too. TSST-1, together with staphylococcal exterotoxins, belongs to the large family of pyrogenic toxins which can cause fever, endotoxic shock, sometimes leading patients to death (Takeuchi et al., 1998). Moreover the toxin belongs to the staphylococcal superantigens (SAgs) family because of its ability to stimulate non-specific T cell proliferation. TSST-1 is encoded by *tst* gene, located in MGEs or in the pathogenicity island SaPI-1 together with *sec* and *sel* genes, coding for the correspondent staphylococcal enterotoxins (Mele and Madrenas, 2010).

staphylococcal enterotoxins (SEs)

As TSST-1, staphylococcal enterotoxins (SEs) belong to the pyrogenic toxins with superantigens activity (PTAgs) family. Currently are known about twenty different SEs which are functionally and genetically related. They are heat-stable toxins classified in five major

antigenic variants (SEA-SEE) and other minor variants, SEs-like, with structure and activity similar to the "traditional" SEs (Lina et al., 2004).

SEs are important determinants in food poisoning and *S. aureus* one of the most common causes of bacterial foodborne outbreaks in many countries (European Commission, 2003). Staphylococcal foodborne intoxication occurs after ingestion of contaminated foods with SEs and it is characterized by several symptoms, such as fever, vomiting and abdominal cramps. Genes coding for SEs are usually carried on MGEs, such as plasmids, transposons or pathogencity islands (Pinchuk et al., 2010).

1.4. Antibiotic resistance

Antibiotics are substances used to kill microorganisms or to stop them from growing. The term antibiotic has been introduced by S. A. Waksman in 1942. Antibiotics are natural drugs produced by several fungi or bacteria. In the first use of the term "antibiotic" was distinguisced by the term "chemotherapeutic drug" reffering to man-made drugs. This difference was abolished with the combination of natural antibiotics and synthetic products. The massive use of antibiotics in human and animals practices is constantly increasing. Antimicrobial agents are considered as a "miracle drugs" in the treatment of infection diseases (WHO, 2011). Antibiotics are commonly used in human and veterinary practices to fight infectious diseases caused by bacteria and others microbes. In addition, the administration of antimicrobial agents is routinely used in companion animals, plant agriculture and industrial applications (Aleem et al., 2003).

Worldwide the great part of antimicrobials are not directly administrated to human patients to treat diseases, but given to animals for food production purposes. In this case antibiotics are used to treat diseases in sick animals, to avoid the arise of illness. Antimicrobial agents were also used as growth promoters, in order to increase muscles in a short time using the same amount of animal feed. Antibiotics growth promoters (AGPs) were mainly used in pig and poultry breeding that in a few years have showed an increase in their production. The use of antibiotics in livestock production for non-medical purpose makes up at least 60% of the total antimicrobial production in the USA (Kenneth Todar, http://www.textbookofbacteriology.net/). Easy access, low costs and effectiveness have leaded to misuse antibiotics. Indiscriminate and irresponsible use of these substances allowed the development of mechanism of resistance to antimicrobial agents in target microorganisms. Antibiotic resistance occurs when bacteria change their molecular structure in order to reduce or eliminate the effects of the antibiotics.

Antibiotic resistance is a public health problem which caused an increase in morbidity and mortality of infectious diseases with serious socioeconomic costs (Alvan et al., 2011). A technical report of European Centre of Disease Prevention and Control (ECDC) showed that every year in Europe approximately 25,000 patients die because of infections caused by resistance microorganisms not treatable with suitable antibiotics (ECDC/EMEA, 2009).

Nowadays is estimated that about 70% of pathogen microorganisms which cause infection during hospitalization are resistant to one or more antibiotics used to treat several diseases. For this reason, since 2001 the European Commission started a program strategy to combat and control the problem of antibiotic resistance in human, animals, plants and environment. The program included the progressive phasing out of antibiotics for non-medical purpose in animals and outlined measures that Member States should take to prevent antibiotic resistance in human medicine. In 2003 has been published the Regulation (EC) No 1831 on additives for use in animal nutrition and finally, in January 2006 the European Commission banned antibiotics as growth promoters in animals feed.

1.5. Antimicrobial agents:

1.5.1. Beta-lactam antibiotics

This group of antibiotics, known as β-lactams include penicillins, cephalosporins, carbapenems and monobactams. They have a common β-lactam ring and a R-group which can give to the molecule different antibacterial properties. β-lactam structure derived from the appearance of two covalent bonds between cysteine and valine amino-acids.

Mechanism of action: inhibition of steps in cell wall synthesis (Alanis et al., 2005).

a) Penicillins: the group can be divided into 4 subgroups.

- *Natural penicillins*

Penicillin G and V, procain-penicillin and benzatinpenicillin. They have a broad spectrum against Gram positive and negative cocci, positive (rods?), spirochetes and most of anaerobes. These antibiotics are not effective against bacteria β-lactamase producers.

- *Anti-staphylococcal penicillin*

Methicillin**,** nafcillin, oxacillin, cloxacillin and dicloxacillin. These antibiotics are very effective against staphylococci β-lactamase producers (i.e. *S. aureus*) but they have no effects against other β-lactamases produced by Gram negative bacteria.

- *Amino-penicillins*

Ampicillin and amoxicillin sometimes in combination with clavulanic acid. The drugs have a spectrum similar to natural penicillin with extension against common gram-negative bacteria like *Escherichia coli*, *Salmonella* spp., *Shigella* spp. Moreover they are more effective than natural penicillin against Enterococci and *Listeria* spp. These penicillins are frequently used in combination with β-lactamase inhibitors such as bactam and clavulanic acid.

- *Penicillins effective against Pseudomonas spp*. which are effective against *Pseudomonas* spp. and other Gram negative naturally resistant to antibiotics.

Karbenicillin, ticarcillin, azlocillin etc.

b) Cephalosporins: they are semi-synthetic drugs derived from cephalosporin C. Their structure is very similar to the penicillins with a β-lactam ring fused with a 6-membered dihydrothiazine ring. The cephalosporins group is formed by 4 sub groups, arranged into generations, according to their spectrum of antimicrobial activity.

- *1st generation*

Cefalotin, cefazolin, cefalexin, cefadroxil, cefaclor. These drugs are effective against Gram positive cocci, mainly streptococci and staphylococci and some Gram negative bacteria, such as *Eschirichia coli* and *Proteus* spp.

- *2nd generation*

Cefamandole, cefuroxime, cefoxitin. This generation of cephalosporins has got a similar activity of the 1st generation with a spectrum extended to other Gram negative microorganisms (*Haemophilus* spp., *Moraxella* spp. and *Eschirichia coli* resistant to 1st generation cephalosporins).

- *3rd generation*

Cefoperazone, ceftriaxon, ceftiofur. This group of cephalosporins with an extended spectrum and more potency of activity against Gram negative bacteria, are more stable to hydrolysis by β-lactamases produced by these microorganisms. However the 3rd generation cephalosporins show a weak activity against staphylococci and other Gram positive microbes.

- *4th generation*

cefepime, cefpirome, cefquinome. The 4th generation cephalosporins summarized the spectrum of activity of the 1st, 2nd and 3rd generation though they are less effective than 1st generation against *Staphylococcus* spp. and then 3rd generation against *Pseudomonas* spp.

c) Carbapenems and monobactams**:** these drugs are structurally related to cephalosporins. They have a wide spectrum against Gram positive and negative bacteria and are resistant to β-lactamases. Carbapenems are not effective against Methicillin Resistant *Staphylococcus aureus* (MRSA).

1.5.2. Glycopeptides

They are bactericidal drugs. Glycopeptides are effective only against Gram positive bacteria. Because of their complex structure (large molecules) they cannot penetrate into the peri-plasmatic space of Gram negative bacteria. Among glycopeptides vancomycin and teicoplanin play an important role to fight infections caused by microbes resistant to others antibiotics.

Vancomycin and teicoplanin are the most suitable antibiotics to treat infections caused by MRSA, although since 1997 have been reported cases of infections caused by *S. aureus* resistant to methicillin with reduced susceptibility to vancomycin (Hiramatsu, 2001). Moreover teicoplanin is well tolerated by the organism and it can be used to treat patients with hypersensitivity to vancomycin (Sivagnanam and Deleu, 2003).

Mechanism of action: inhibition of steps in cell wall synthesis by the inactivation of the Pencillin Binding Protein (PBP) transpeptidase (Hiramatsu, 2001).

1.5.3. Aminoglycosides

This class of antibiotics have a very rapid bactericidal effect and work in synergism with β-lactams and glycopeptides. They are effective against Gram positive and negative bacteria,

Micobacteria and protozoa but they not show effects against anaerobes, spirochetae and obligatory intracellular pathogens.

Streptomycin is the oldest drug belonging to aminoglycosides group and was normally used to treat tuberculosis and brucellosis. The use of this antibioticis limited because of its toxicity, especially for the vestibular and renal systems (Durante-Mangoni et al., 2009).

Other drugs belonging to this antibiotic group are gentamicin, tobramycin, netilmicin and amikacin with similar activity to streptomycin but with reduced toxicity.

Mechanism of action: inhibition of proteins synthesis by binding to the aminoacyl site of 16S ribosomal RNA (rRNA) [Durante-Mangoni et al., 2009].

1.5.4. Quinolones

These antibiotics have a bactericidal effect but are less effective than β-lactams and aminoglycosides. Quinolones are a group of synthetic drugs derived from nalidix acid. The spectrum of activity is mainly against Gram negative bacteria, although the last generation of quinolones are effective to Gram positive, intracellular pathogens and some anaerobes as well. The quinolones can be divided into two major subgroups, quinolones without fluorine substituent on their ring (oldest quinolones such as nalidix, pipemidic and oxolinic acids) and with fluorine substituent (ciprofloxacin, ofloxacin and pefloxacin).

Mechanism of action: inhibition of DNA gyrase subunits (*gyrA* and *gyrB*) and topoisomerase IV which are involved in the replication and transcription of DNA (Dougherty et al., 2001).

1.5.5. Macrolides

This group of antibiotics is primarily bacteriostatic but some of them have a bactericidal effect, especially if used in high dosage. They show a broad spectrum against Gram positive and negative bacteria, anaerobes, spirochetes, and obligatory intracellular pathogens. Macrolides are characterized by the presence of a macrocyclic lactone ring bound to one or more sugars. The most important drug was the erythromycin but its use was limited because of the side-effects.

After erythromycin other macrolides have been introduced in medicine practices, such as roxitromycin, claritromycin, spiramycin and josamycin. They have similar effects of erythromycin but the side-effects are less important.

Mechanism of action: inhibition of proteins synthesis by reversible bond with the 50S ribosomal subunit. It inhibit the transpeptidation - translocation process, causing the cell death (Retsema and Fu, 2001).

1.5.6. Nitromidazoles

They are antibacterial and antiprotozoa drugs characterized by a common imidazole ring. Metronidazole is the most used nitromidazole to treat several diseases, especially for diseases caused by Gram negative bacteria, followed by tinidazole and ornidazole. Use of nitromidazoles in food-processing animals is not allowed in Europe (Zeleny et al., 2006).

Mechanism of action: direct interaction with DNA (disruption of DNA molecules, inhibition of nucleic acid synthesis) [Bendesky et al., 2002].

1.5.7. Tetracyclines

This group of antibiotics shows bacteriostatic and bactericidal effects. The spectrum of activity is broad and they are effective against Gram positive and negative bacteria and anaerobes too. Unfortunately, after indiscriminate use in veterinary and human practices, many pathogens have developed resistance. They are characterized by a common four hydrocarbon ring. Tetracyclines are also known as "derivatives of polycyclic naphthacene carboxamide". The first tetracyclines introduced in medicine routine were chlortetracycline, tetracycline and oxytetracycline. These drugs are natural products derived from *Streptomyces* spp. More recent tetracyclines as methacycline, doxycicline and minocycline are a semi-synthetic derivates.

Mechanism of action: inhibition of bacterial protein synthesis by preventing the association between tRNA and ribosomes (Chopra and Roberts, 2001).

1.5.8. Lincosamides

They have bacteriostatic and bactericidal effects, depending on the concentration. Lincosamides show a spectrum of activity similar to macrolides, although their molecular structure is quite different. In the past the diseases treatment with lincosamides such as clindamycin, lincomycin and pirlimycin was limited because of the rapid development of resistance and their side-effects. Recently the use of lincosamides has been revisited especially to treat infections caused by multidrug resistant pathogens, such as MRSA (Morar et al., 2009).

Mechanism of action: inhibition of proteins synthesis via binding to the 23S rRNA of the ribosomal subunit 50S (Morar et al., 2009).

1.5.9. Nitrofurans

Nitrofurans are bactericidal drugs with good effects against Enterobatteriacae and excellent effect against Enterococci. Belonging to this class of antimicrobial agents there are synthetic drugs such as furazolidone, furaltadone, nitrofurantoin and nitrofurazone. All these antibiotics contain a characteristic 5-nitrofuran ring in their structure. Because of their potential carcinogenic effects for humans, nitrofurans have been banned by the European Union (EU) from use in livestock production since 1995 (Vass et al., 2008). However, their use is still registered for clinical purposes in both human and animals (Vasheghani et al., 2008).

Mechanism of action: inhibition of proteins synthesis (DNA, RNA and cell wall proteins). The overall effect is the death of the microbial cell (http://www.drugbank.ca/drugs/DB00698).

1.5.10. Sulfonamides

Sulfonamides are a group of antibiotics analogues to the para-aminobenzoic acid (PABA). They have been used for many years to treat several diseases caused by pathogen microorganisms due to their bacteriostatic effects. The discovery of "new" drugs as penicillin, its derivates and other modern antibiotics, decreased the use of sulfonamides (Tommasino et al., 2011). Sulfonamides are classified in three different groups, on the basis of their rate of excretion: short-acting

sulfonamides including sulfapyridine, sulfadiazine, and sulfadimidine; medium-acting sulfonamides including Sulfamethoxazole; long-acting sulfonamides represented by sulfadimethoxine and sulfamethoxypyridazine.

Mechanism of action: inhibition of proteins synthesis by antagonism of PABA, a precursor of the folic acid, that is essential for bacterial growth (Tommasino et al., 2011).

1.5.11. Chloramphenicol

It is an antibacterial drug with a broad spectrum of activity against Gram positive and negative bacteria, anaerobes and obligatory intracellular pathogens. Due to its toxicity, especially for bone marrow, chloramphenicol has been banned in many countries. In Europe and USA its administration is prohibited in food-producing animals (Monari et al., 2008).

Mechanism of action: inhibition of proteins synthesis by binding the L16 protein of the 50S ribosomal unit (http://www.drugbank.ca/drugs/DB00446).

1.6. Mechanisms of antibiotic resistance in bacteria population

At least 17 different classes of antibiotics with bacteriostatic and bactericidal effects have been produced to date. The indiscriminate use and administration of drugs in human and veterinary medicine have determined antimicrobial resistance. Sometimes microorganisms show resistance to a single drug, but the rate of multiple resistance to different antibiotics is constantly increasing (Alanis, 2005). The treatment of diseases caused by microorganisms showing multiple resistance is extremely difficult and expensive. Moreover, these pathogens are usually associated with a rise in morbidity and mortality. In order to show antibiotic resistance is required the combination of two elements: an antibiotic effective against a heterogeneous colony of bacteria and at least one microorganism carrying a genetic determinant coding for drug resistance (Levy and Marshall, 2004).

Antibiotic resistance is given by changing at genetic level among microorganisms target. Modifications can be acquired by mutation and selection or by acquisition of new genetic elements from other bacteria. These modifications influencing one or more biological mechanisms that determine antibiotic resistance (Tenover, 2006).

In most of the cases drug resistance is associated to DNA segments that are capable to "jump" into a genome (intracellular mobility) or from a cell to another (intercellular mobility) typical of the bacteria cells. In prokaryotes the intercellular movement of genetic elements occurs by means of transformation, conjugation and transduction.

Transformation occurs between closely related microorganisms. The transfer process of the cellular DNA is mediated by proteins codified at chromosomal level (Frost et al., 2005). In contrast with transformation, conjugation needs independent genetic elements, such as plasmids and transposons (*Tns*), coding for the proteins for DNA transfer. These proteins mediate the DNA movement from a "donor" cell to a recipient cell. Transduction is another mechanism of DNA transfer, in which are involved independently replicating bacterial viruses, known as bacteriofages or fages. These viruses are able to package segment of host DNA in their capsid

and transfer it in the cellular chromosome of a new bacteria cell after infection (Frost et al., 2005).

Several mechanisms are involved in confering resistance to antibiotics in bacteria population and they can be classified into three functional categories: drug inactivation, exclusion of the antibiotic from the target (efflux pump and other mechanisms) and target modification.

Drug inactivation:

acquisition and activation of genes coding for enzymes that destroy the antibiotic molecule such as β-lactamases that cleave the amide bond in the β-lactam ring. To date are known 4 different classes of β-lactamases (A, B, C, D) and they are the major cause of bacteria resistance to β-lactam antibiotics (Tomanicek et al., 2011).

Exclusion of the antibiotic from the target:

acquisition and activation of an efflux pump which extrude the antibiotic from cells, before the drug reaches the bacteria target-site. To activate the efflux pump microorganisms need an overexpression of the genes encoding and regulating the activity of the pump. Efflux pumps are specific protein transporters, such as MefE and MefA for macrolides and tetK, tetL and tetZ for tetracyclines (Roberts et al., 1999; Tauch et al., 2000; Ramos et al., 2005). The activation of an efflux pump is one of the most important mechanisms that confer multiple resistance to bacteria. Acquisition of mutations limits accessibility of drugs molecules into bacteria cells, by a down-regulation of the porin genes. Normally the antibiotic active sites interact with porin channels, cross the cell wall in Gram positive or the outer membrane in Gram negative bacteria and reach the target site. Alteration of porin channel reduces the influx of the drugs into cells (Vinson et al., 2010).

Target modification:

acquisition and activation of new genes coding for an altered bacteria cell wall. In this case, bacteria show a modification in the binding site of the antibiotic, such as a modified Penicillin Binding Protein (PBP) referred to as PBP2a that confer methicillin resistance to *S. aureus* strains (García-Alvarez et al., 2011).

1.7. *Staphylococcus aureus* and antibiotic resistance

Among the microorganisms showing resistance to antibiotics *S. aureus* represents a major public health concern. The hazard of this pathogen is due to its intrinsic virulence, the ability to cause a large array of diseases, the adaptability to different environment conditions and multiple resistance to antibiotics (Lowy et al., 2003). In contrast to other microorganisms, such as some species of Enterococci which show an intrinsic resistance to several antibiotics due to their natural metabolism (Klare et al., 2003), *S. aureus* can be defined as a microorganism with acquired antimicrobial resistance. The mechanisms involved in the acquisition of *S. aureus* antibiotic resistance are the same described for other bacteria, although transformation and transduction are rare events (Al-Masaudi et al., 1991).

1.8. Molecular mechanisms of antibiotic resistance in *S. aureus* strains:

1.8.1. β-lactam resistance

S. aureus acquire resistance to β-lactams by two major mechanisms, production of penicillinases and production of a modified PBP. The penicillinase BlaZ, inactivate β-lactams antibiotics by hydrolysis of the β-lactam ring. On the basis of differences in β-lactam ring hydrolysis, penicillinases can be classified in four different classes: A, B, C and D. *blaZ* genes, coding for penicillinase A, C and D are usually located on plasmids, while *blaZ* encoding penicillinase B is located on the chromosome. Expression of *blaZ* structural gene is controlled by *blaI* repression gene and by *blaR1* (figure 3), a signal transducer-sensor protein, which are clustered together (Olsen et al., 2006).

Figure 3. *Staphylococcus aureus* N315, plasmid pN315. Genomic context of *blaZ* and related regulation genes (*blaI*, *blaR1*).

Another mechanism involved in β-lactams resistance is the production of an altered PBP, known as PBP2' or PBP2a, that shows a decreased affinity for β-lactams. Although PBP2a appears similar to the PBP2 found in Methicillin Susceptible *Staphylococcus aureus* (MSSA), they are not closely related (Ayliffe, 1997). Resistance to methicillin, antibiotic used to treat infections caused by *S. aureus* strains resistant to penicillinase, is codified by the structural gene *mecA*, located into the Staphylococcal cassette chromosomes (SCC*mec*). In the same manner of *blaZ*, *mecA* gene expression is regulated by a two-component system (TCS), *mecI* repression gene and *mecR1* (figure 4), a signal transducer-sensor protein (McCallum et al., 2010).

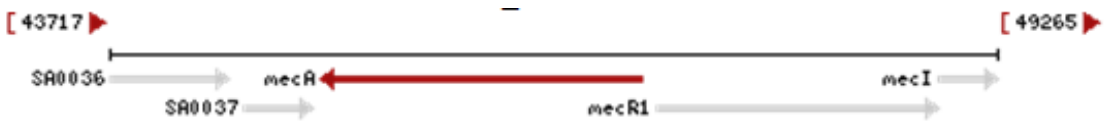

Figure 4. *Staphylococcus aureus* N315, chromosome. Genomic context of *mecA* gene and related regulation genes (*mecI, mecR1*).

1.8.2. glycopeptide resistance

Vancomycin resistance in *S. aureus* strains is a global issue. Vancomycin has been used for many years being the most suitable drug to treat MRSA infections. In 1996 was reported a first case of Vancomycin Resistance *Staphylococcus aureus* (VRSA) isolated from a Japanese patient. The recovery of VRSA is constantly increasing and the effectiveness of glycopeptides to fight MRSA infections is declining (Hiramatsu, 2001).Vancomycin resistance has been described for the first time in Enterococci. In 1992 Noble et al., have demonstrated that genes coding for vancomycin resistance could be transferred from Vancomycin Resistance Enterococci (VRE) to MRSA by conjugation.

Resistance to vancomycin is associated to *van* genes which codify for different resistance phenotypes, carrying the same name of the correspondent genes (Cetinkaya et al., 2000):

1) *vanA*, gene encoding high-level resistance to both vancomycin and teicoplanin;

2) *vanB*, gene encoding resistance only to vancomycin;

3) *vanC1*, *vanC2*, *vanC3*, genes encoding a low-level resistance to vancomycin;

4) *vanD*, a novel gene, found in *Enterococcus faecium*, coding for vancomycin resistance;

5) *vanE*, a novel gene, found in *Enterococcus fecalis*, coding for low-level resistance to vancomycin;

Genes coding for vancomycin resistance are carried on genetic mobile elements such as plasmids and transposons. VanA and VanB are the most prevalent phenotypes in nature. VanA is the phenotype usually associated to VRSA (Périchon and Courvalin, 2009).

In the same manner of methicillin resistance, *S. aureus* strains acquire vancomycin resistance by a mutation in the PBP structure. Expression of the *van* genes is controlled by a TCS: *vanS* (sensor) and *vanR* (response regulator) regulating the transcription of *vanHAX* gene cluster (figure 5) [Cetinkaya et al., 2000].

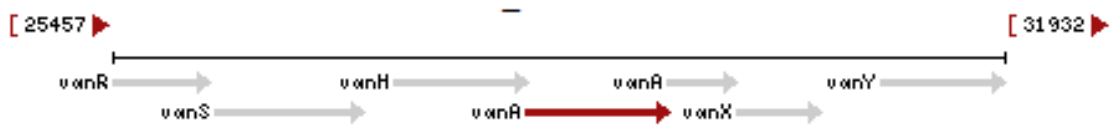

Figure 5. *Enterococcus faecalis*, plasmid pWZ1668. Genomic context of *vanHAX* gene cluster associated to VRSA.

1.8.3. aminoglycoside resistance

S. aureus strains show resistance to aminoglycosides too. Epidemiological analysis showed that resistance to aminoglycosides is generally associated to methicillin resistance (Udou, 2004). In fact, MRSA strains often carry a plasmid with *aacA/aphD* genes which codify for resistance to gentamicin, trobramycin, kanamycin and when overexpressed, amycacin (Strommenger et al., 2003). The product of the expression of *aacA/aphD* genes (figure 6) is a bifunctional aminoglycoside-modifying enzyme (AAC6'/APH2'') that inactivate the aminoglycoside drugs (Schmitz et al., 1999).

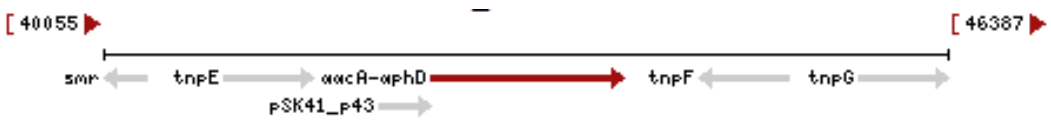

Figure 6. *Staphylococcus aureus*, plasmid pSK41. Genomic context of *aacA*/*aphD* genes.

1.8.4. macrolide and lincosamide resistance

Three different mechanisms are involved in the acquisition of resistance to macrolides and lincosamides in *S. aureus* strains (Lina et al., 1999):

1) modification of the target site by mutation or methylation of 23S rRNA subunit, encoded by one or more *erm* genes, mainly *ermA*, *ermB* and *ermC* genes located on plasmids or the chromosome (*ermC*);

2) activation of an efflux pump which belongs to the ABC transporter family, encoded by *mrsA* and *mrsB* genes; this resistance mechanism is more prevalent in Coagulase Negative Staphylococci (CoNS) than in *S. aureus*;

3) enzymatic inactivation of the antibiotic via activation of phosphotransferase C, encoded by *mphC* gene for macrolides resistance. Moreover, inactivation of lincosamides drugs is mediated by the activation of a lincosamide nucleotidyl transferase, encoded by *lnuA* gene.

In *S. aureus* strains the most represented mechanism for macrolides resistance is the methylation of the 23S rRNA, encoded by *ermA* gene (figure 7).

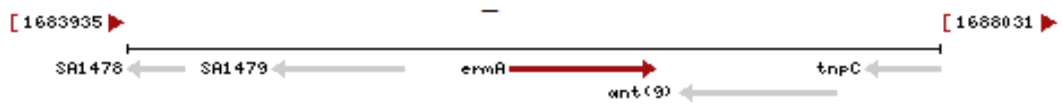

Figure 7. *Staphylococcus aureus* N315, chromosome. Genomic context of *ermA* gene.

1.8.5. quinolone resistance

Two mechanisms of resistance to quinolones are involved in *S. aureus* strains:

1) mutations of gyrase gene. GrlA of topoisomarase IV and gyrA subunits (figure 8) are the most common sites of mutation that confer quinolones resistance in *S. aureus* strains (Lowy, 2003);

Figure 8. *Staphylococcus aureus* MRSA252, chromosome. Genomic context of *gyrA* and *gyrB* genes.

2) activation of an efflux pump, belonging to the Nor multidrug resistance pumps. Expression of staphylococcal efflux pump is controlled by the transcriptional regulators *mgrA* (figure 9) and *norG* genes and by the ArlRS-TCS. An overexpression of the efflux pump can result in low-level quinolones resistance (Ng et al., 1994).

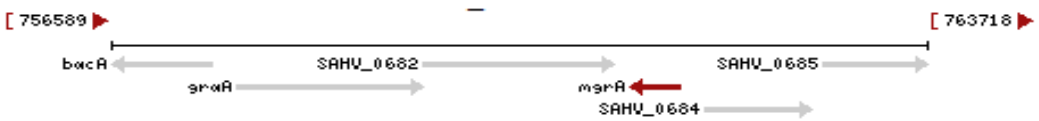

Figure 9. *Staphylococcus aureus* Mu3, chromosome. Genomic context of *mgrA* gene belonging to MarR family transcriptional regulator.

1.8.6. tetracycline resistance

The main mechanisms involved in *S. aureus* resistance to tetracyclines are the ribosomal protection by elongation of tetM or tetO proteins and activation of the efflux pump. Ribosomal protection is coded by *tetM* (figure 10) and *tetO* genes carried on chromosome (Connell et al., 2003), while efflux pump is coded by *tetK* (figure 11) and *tetL* genes, located on plasmids (Levy et al., 1999). Recently a new efflux pump, Tet38, has been identified. The Tet38 contributes to tetracycline resistance in *S. aureus* strains. The activation of this efflux pump is controlled by *mgrA* regulator gene as seen for quinolones resistance. MgrA protein is an indirect repressor of the pump Tet38, therefore inactivation of MgrA leads to the activation of the efflux pump (McCallum et al., 2010).

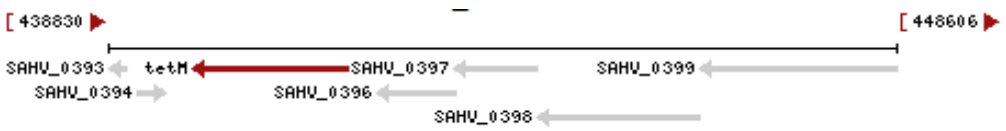

Figure 10. *Staphylococcus aureus* Mu3, chromosome. Genomic context of *tetM* gene coding for ribosomal protection.

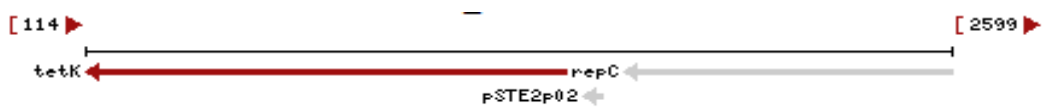

Figure 11. *Staphylococcus lentus*, plasmid pSTE2p01. Genomic context of *tetK* gene coding for efflux pump.

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CHAPTER 2

Thesis project

Thesis project

In recent years food safety issues and microbiological risk assessment of foodborne pathogens have become a central topic. Milk and dairy products may have an important impact on human health. Concern exists on the ability of several pathogen bacteria to cause illness by means of toxins production and excretion in foods. Foodborne disease is defined by the World Health Organization (WHO) as "diseases of infectious or toxic nature caused by, or thought to be caused by the consumption of food or water". Worldwide, production and consumption of milk and related products is constantly increasing. In 2009 were produced approximately 584 million tons of cow's milk, 9 million tons and 16 million tons of sheep's and goat's milk, respectively. Worldwide the cheese-making sector accounted for about 20 million tons of cheese produced in 2009 . European Union contribute for about 40% of the total cheese production, 10% of which was from raw milk. The main producer in EU is Germany (2 million tons), followed by France (1.8 million tons) and Italy (1.2 million tons) [FAOSTAT, 2011].

Staphylococcus aureus is one of the most common pathogens involved in food poisoning and milk and dairy products are accountable for about 5% of the total outbreaks due to staphylococcal foodborne intoxication in Europe (European Commission, 2003).

While cow's milk is mainly consumed as fluid milk, sheep's and goat's milk is generally used for cheese-making production, especially around Mediterrean basin. Most of the cheese products obtained from small ruminants are made with raw milk and are often linked to the tradition of the production area. Artisan cheese production is usually made by hand, sometimes with little mechanization, in small batches, with a great attention of the cheese marker to respect tradition during cheese-making phases.

Production and consumption of raw milk cheeses has increased during the last years. A possible explanation could be a rise in consumer's request of traditional dairy products with distinctive characteristics, such as stronger flavour and odour than cheeses made with pasteurized milk (FSANZ, 2009).

Unfortunately, the absence of heat treatment of the milk and the low standardization of the technologies, such as use of starter cultures, do not give insurance to control *S. aureus* growth in traditional raw milk cheeses, mainly in the first cheese-making steps (Jakobsen et al., 2011).

S. aureus can cause food poisoning after production and excretion of SEs in foods, when its count increases over 10_5-10_6 cfu/g (Regulation (EC) No 2073/2005). In the first cheese-making phases of raw milk cheese production only pH reduction and particularly competition of lactic acid flora could limit *S. aureus* multiplication. Ripening time and temperature are an important steps for progressive decreasing of *S. aureus* contamination level, though they do not reduce risks of foodborne disease related to the presence of the pre-formed SEs, produced during the microorganism exponential phase (Scarano et al., 2007). Even with the advent of pasteurization in the 20th century, raw milk cheeses continue to be associated with foodborne infections, with a rate of incidence of about 70% of the total outbreaks accountable to cheese consumption (FSNAZ, 2009).

Table 1. Microbiological hazards associated with raw milk cheeses.

Microorganism	contaminant of raw milk	severity of illness	implication of cheeses in foodborne illness*
Brucella spp.	yes	serious	++
Campilobacter spp.	yes	serious	+
Escherichia coli	yes	serious	++
Listeria monocytogenes	yes	severe	++
Salmonella spp.	yes	serious	++
Staphylococcus aureus	yes	mild	++

* + = rare; ++ = commonly associated with human illness

Application of Hazard Analysis and Critical Control Points (HACCP), Good Manufacturing and Good Hygiene Practices (GMP, GHP) can contribute to increase the food safety level in dairy chain production (NSW Food Authority, 2009).

In order to keep under control *S. aureus* contamination in milk and cheeses, especially made with raw milk, it is essential to understand the epidemiology of the microorganism, pathways of transmission, adaptability to different environmental conditions, role of both human and animals as reservoir of the pathogen.

Moreover, to ensure human health, worldwide should be implemented an effective surveillance systems, to monitor trends of foodborne intoxications and outbreaks related to the consumption of milk and cheeses contaminated with staphylococcal SEs. Again, surveillance systems should give an accurate outline on the food category (milk, cheese, raw milk cheese) involved in staphylococcal food poisoning to develop an adequate risk assessment which requires detailed information on manufacturing process, physiochemical characteristics and challenge data of these products (Lindqvist et al., 2002; FSNAZ, 2009).

Unfortunately, both surveillance systems and risk assessment have showed difficulty in evaluating safety of raw milk and related products due to the lack or the extreme variability of available data. In the same manner except for France and USA, no data are available on the rate of hospitalization caused by staphylococcal foodborne intoxication (European Commission, 2003).

The aim of the present Ph.D. thesis was to trace and characterize *S. aureus* strains collected along the small ruminant dairy chain.

The first objective was to study antibiotic resistance in *S. aureus* strains and CoNS isolated from goats with subclinical mastitis. An adequate assessment of *S. aureus* resistant pattern is essential to prevent the development and the selection, at farm level, of resistant strains, which could reach to consumers with milk and dairy products (**Chapter 3**).

Moving forward in small ruminat dairy chain, the second study was aimed to characterize *S. aureus* virulence pattern in goat's bulk tank milk. Acquisition of epidemiological data, the knowledge of virulence factors arrays and antibiotic resistance characteristics are important elements to develop strategies to control and limit the environmental spread of the pathogen (**Chapter 4 and 5**).

The third step of the current study was to evaluate the prevalence of *S. aureus* in sheep's cheeses, especially made with raw milk, during the first few hours of cheese-making process, when *S. aureus* count is supposed to be highest and compatible with enterotoxins production (**Chapter 6**).

In **Chapter 7** a selection of *S. aureus* strains, isolated from raw milk sheep's cheese, has been tested for resistance to antibiotics commonly used in medicine and veterinary practices. It is important to evaluate the prevalence of resistant strains in dairy chain. *S. aureus* antibiotic resistance is a major public health problem, particularly methicillin-resistant *S. aureus*. Although the spread of resistant strains through foods to human population is sporadic, presence in farms and cheese-making plants of persistent resistant strains is important for epidemiological studies. Indeed farm environment can be considered as a potential reservoir of *S. aureus* resistant to antibiotics.

In **Chapter 8** are presented the results obtained in an extensive study on 100 *S. aureus* strains isolated from raw milk sheep's cheese. Results on virulence determinats and antibiotic resistance are important factors to trace a complete profile of *S. aureus* strains and to evaluate the risk associated with the presence of the pathogen in sheep's cheese. Moreover the study of the genetic variability carried out by Analysis of Molecular Variance (AMOVA) allowed to trace the most likely origin (human or animal) of the cheese contamination by *S. aureus*.

CHAPTER 3

Antibiotic Resistance in *Staphylococcus aureus* and Coagulase Negative Staphylococci isolated from goats with subclinical mastitis.

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Antibiotic Resistance in *Staphylococcus aureus* and Coagulase Negative Staphylococci isolated from goats with subclinical mastitis.

3.1. Introduction

In the last years consumption of goat milk as fluid milk and related products, mainly cheeses and yogurt, is constantly increasing. Worldwide goat milk production increased from 12 million tons in 2000 to approximately 16 million tons in 2009 (FAOSTAT, 2011). It is probably due to the intrinsic characteristics of this milk, having an impact on human nutrition. Indeed, goat milk could be used to treat people with cow milk intolerance or allergies and gastro-intestinal disorders, which mainly afflicted people of industrialized countries, where cow milk is largely consumed (Haenlein, 2004).

Regarding economic aspects, goat milk and dairy products are taking a great importance, mainly for two reasons: it is a good source of livelihood for people in the developing world, where goats' breeding is widespread. Moreover goat milk and derivates represent a niche products which are essentially demanded by a consumer's category which appreciate the characteristic flavor due to typical fatty acid, such as capric, caproic and caprilic acids (Haenlein, 2004; Soryal et al., 2005).

Raw goat's milk can be considered as a potential source of antibiotic resistant pathogens for human and animals. Raw milk contamination can occurs directly from animals, especially those affecting by clinical and subclinical mastitis, or by farm environment and equipments.

In dairy goats with Subclinical Mastitis (SCM), Coagulase Negative Staphylococci (CoNS) make up 44.7% to 95.9% of the isolated pathogens from milk samples, and *S. aureus*, which is usually considered to have the greater pathogenicity, accounts from 4.1% to 18.0% of SCM agents (Contreras et al., 2003).

The intramammary administration of antibiotics used on farm level is constantly increasing and it could be explained with the effectiveness of the antimicrobial agents to treat SCM in dry small ruminants (Mavrogianni et al., 2011). Unfortunately, the widespread use and misuse of these antibiotics could lead to the selection and to the emergence of antibiotic resistant bacterial strains.

Antibiotic resistance pattern for staphylococci isolated from SCM refers mainly to cows, and little is known about dairy goats. Studies on antimicrobials susceptibility of these pathogens have been mainly conducted using the agar disc diffusion method. The broth microdilution and the agar dilution methods instead allow the evaluation of the minimum inhibitory concentrations (MIC). The evaluation of the antimicrobial susceptibility of *Staphylococcus* spp. isolated from goats with SCM is of great interest for clinical purposes in order to decide which antibiotics should be administered, as well as, for monitoring the spread of multiple resistant strains on farms.

The aim of the present study was to provide new additional information about antibiotic resistance in *S. aureus* stains and CoNS isolated from goats with SCM. The data obtained could be used to support the sparse literature on the problem of resistance to antibiotics in extensive rearing goats system.

For this purpose, MICs and in vitro susceptibilities to ten antibiotics used in medical and veterinary practices were determined against 25 *S. aureus* and 75 CoNS strains. On these strains, the frequencies of single and multiple antibiotic resistance were also evaluated.

3.2. Material and methods

3.2.1. Dairy Farms

The milk samples were collected from eight goats flocks located in Sardinia (Italy) where mainly Sarda and Sarda-Maltese breeds were reared. The rearing system was extensive and the animals were hand-milked. The goats, fed on bushes and grass, occasionally were supplemented with concentrates. The mean flock size was 187.5 ± 25.2 (mean ± SD) with a range from 160 to 234. Goats were not treated with systemic or intramammary antimicrobial agents during lactation previous to the enrollment in this study nor during the dry period.

3.2.2. Sampling

A clinical examination of half-udders was conducted in order to exclude animals with signs of clinical mastitis (Donovan et al., 1992). A total of 3,000 half-udder milk samples were collected in a single sampling time in each of the eight flocks during the early lactation period (from

January to April). The first few streams of foremilk were discarded, and duplicate half-udder milk samples were aseptically collected into sterile tubes after cleaning and disinfection of each teat end. One sample (10 mL) was used for bacteriological analysis and the other one (50 mL) was added with bronopol (2-bromo-2-nitropropane-1,3-diol) and analyzed for Somatic Cell Count (SCC). The milk samples were stored at +4 °C, and bacteriological and SCC examinations were carried out within 6 and 72 hours after sampling.

3.2.3. Bacteriological analysis and isolation procedures

Each half-udders milk sample was mixed by inversion and 10 µL were then inoculated onto 5.0% Sheep Blood Agar (SBA) plates. The SBA plates were then incubated aerobically at +37 °C and examined after 48 and 72 hours. A significant bacterial count was considered when a growth of ≥500 identical cfu/mL was detected. Bacterial strains were isolated on Brain Heart Infusion agar (BHI, Oxoid, Basingstoke, UK) and identified using routinary microbiological procedures such as colony morphology, microscopic characteristics and Gram staining, hemolysis pattern on SBA, catalase and oxidase reactions. The strains were identified using API ID32 STAPH system (bioMérieux, Lyon, France) and the strips were read by mini API instrument and associated software V 1.5.2 (bioMérieux, Lyon, France). Strains were then frozen at -80 °C in BHI broth with glycerol (15.0% v/v).

3.2.4. Bacterial characterization

Among the isolated microorganisms 100 *Staphylococcus* spp. strains were selected, 25 *S. aureus* (all isolates) and 75 CoNS (randomly). Each strain was analyzed for: haemolysis pattern on SBA at 5.0%, lecithinase activity on Baird Parker Medium (BPM, Oxoid, Basingstoke, UK), supplemented with Egg Yolk Tellurite Emulsion (EYTE) at 5.0% (Oxoid, Basingstoke, UK); thermostable DNase (TDNase, Biolife, MI, Italy) tested on toluidine blue-DNA agar plates (Lachica et al., 1971), free coagulase (Coagulase plasma-EDTA, bioMérieux, Lyon, France) and bound coagulase production (Staphylase test, bioMérieux, Lyon, France), following the manufacturer's instructions.

3.2.5. Somatic cell count (SCC)

The SCC was determined by the fluoro-opto-electronic cell counting method according to the FIL-IDF No. 148 (1995) method C [FIL-IDF, 1995], using a Fossomatic 5000 (Foss Electric, Hillerød, Denmark).

3.2.6. Definition of subclinical mastitis

For the purpose of this work, a mammary gland was considered as affected by subclinical mastitis when having no clinical signs or abnormal milk, in which ≥500 cfu/mL were isolated and a somatic cell count was ≥300,000 cells/mL (Ribeiro et al., 1998).

3.2.7. Antimicrobial agents and minimal inhibitory concentration (MIC)

On each strain the MICs of ten antibiotics used in human and veterinary medicines were determined. The antibiotics tested were ampicillin (AM), cephalothin (KF), cefoperazone (CFP), ceftriaxone (CRO), cloxacillin (OB), kanamycin (K), novobiocin (NV), ofloxacin (OFX), oxytetracycline (OT), and vancomycin (VA). The MICs were determined by the broth microdilution method (CLSI, 2006a; NCCLS, 2002a) using cation-adjusted Mueller-Hinton broth (CAMHB, Oxoid, Basingstoke, UK). Each antimicrobial agent, in powder form (Sigma-Aldrich-Fluka, MI, Italy), was weighed and dissolved in an appropriate solvent [CLSI, 2006a, NCCLS, 2002a], thus obtaining a stock solution (2,560 μg/mL). Stock solutions were stored at -80 °C until used. From each stock solution, 12 serial twofold working dilutions in deionized water (only for AMP, the diluents were phosphate buffer, pH 6.0, 0.1 mol/L) was prepared according to CLSI standard protocols, and the antimicrobial agent final concentrations in each microplate ranged between 0.06 and 128 μg/mL. Each strain stored at -80 °C until testing were subcultured twice on BHI agar before inoculum preparation.

3.2.8. Detection of mecA gene

Genomic DNA used as target for polymerase chain reaction (PCR) assay was extracted using the following procedure: strains grown in BHI broth at +37 °C overnight were centrifuged (10 minutes at 3,000 x *g*) and resuspended in 500 μL of Tris-EDTA buffer saline (Tris base 10mM+

EDTA 1mM). The suspension was added with 10 µL of a 1.5 mg/mL lysostaphin solution (Sigma-Aldirich, MI, Italy) and incubated for 1 hour at +37 °C. Then, 5 µL of a 20 mg/mL proteinase K solution (Eurobio, Sarreguemines, France) were added and the incubation was continued at +50 °C for 60 minutes. An equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1) was added and mixed by inversion. After centrifuging (15 minutes at 10,000 x g), the upper layer was collected and 500 µL of chloroform isoamyl alcohol (24:1) solution were added. The mixture was centrifuged again (15 minutes at 10,000 x g) and the upper aqueous phase was transferred into a new tube. A volume of 800-1,000 µL of refrigerated absolute ethanol was added and gently mixed until DNA precipitation. DNA was resuspended in 100 µL of sterile deionized water. The DNA concentration was estimated spectrophotometrically. The *mecA* gene coding for methicillin resistance was detected by PCR as previously described (Murakami et al., 1991). The primers used for the detection of the *mecA* gene were 5'-AAAATCGATGGTAAAGGTTGGC-3' (forward) and 5'AGTTCTGCAGTACCGGATTTGC-3' (reverse). *S. aureus* HT 2004 0874 reference strain was used as positive control (Vandenesh et al., 2003).

3.3. Results

3.3.1. Isolates

Bacteriological cultures were positive in 469 (15.6%) out of 3,000 half-udder milk samples. The intramammary infection rates in the eight flocks were, respectively, of 16.7% (range 15.0%-18.6%) and 14.6% (range 12.6%-17.4%) for the left and right half-udders. Bacterial strains isolated from milk samples were 415 CoNS (88.5%), 25 *S. aureus* (5.3%), 4 *Micrococcus* spp. (0.9%), while 21 (4.5%) were identified as belonging to other species (*Bacillus* spp., *Enterococcus* spp. and Gram-Negative Bacilli), and 4 (0.9%) could not be identified by API system. The CoNS strains were identified as follows: 187 *S. caprae* (45.1%), 64 *S. warneri* (15.4%), 41 *S. simulans* (9.9%), 31 *S. chromogenes* (7.5%), 16 *S. epidermidis* (3.9%), 9 *S. xylosus* (2.2%), 8 *S. haemolyticus* (1.9%), 7 *S. capitis* (1.7%), 6 *S. cohnii* (1.4%), 6 *S. lugdunensis* (1.4%), 5 *S. equorum* (1.2%), 5 *S. hominis* (1.2%), and 30 *Staphylococcus* spp.

(7.2%). The mean SCC of bacteriological positive samples was 6.3 Log_{10} cells/mL, while in the negative ones it was 5.7 Log_{10} cells/mL. The mean SCC was greater in milk samples positive for *S. aureus* (6.8 Log_{10} cells/mL) when compared to those positive for CoNS (6.4 Log_{10} cells/mL).

3.3.2. Selected isolates

A total of 100 isolates were selected for antimicrobial agent susceptibility testing as follows: *S. aureus* (25), *S. caprae* (25), *S. warneri* (16), *S. simulans* (15), *S. chromogenes* (7), *S. epidermidis* (6), *S. equorum* (2), *S. cohnii* (1), *S. haemolyticus* (1), *S. lugdunensis* (1), and *S. xylosus* (1). The *S. aureus* strains were isolated from five out of eight flocks ranging between 2 and 8 for each one. Fifteen (60.0%) of the *S. aureus* strains showed hemolytic activity and among these, 5 (20.0%) produced α-hemolysin, 8 (32.0%) β-hemolysin and 2 (8.0%) were α and β-hemolytic. Thirteen (17.3%) of the CoNS strains showed a weak hemolytic activity on SBA. Free coagulase was produced by all the *S. aureus* strains tested, while three of these (12.0%) were negative to the bound coagulase test. All the CoNS strains were found to be negative for the free coagulase and only one *S. lugdunensis* strain (1.3%) was clumping factor positive. TDNase was produced by all the *S. aureus* strains and by 17 (22.7%) of the CoNS strains. Lecithinase was produced by 12 *S. aureus* (48.0%) and 27 CoNS strains (36.0%).

3.3.3. Antimicrobials susceptibility

The MICs of antibiotics and the susceptibility of *S. aureus* and CoNS strains isolated from goats with SCM are shown Tables 1-2. Fourteen (56.0%) of 25 *S. aureus* and thirty-one (41.3%) out of 75 CoNS strains were resistant to one or more antimicrobials. The susceptibility of *S. aureus* was 92.0% or greater for seven out of ten antibiotics tested but was lower for kanamycin (60.0%), oxytetracycline (84.0%), and ampicillin (88.0%). The susceptibility of CoNS was between 94.0%-100.0% for eight antimicrobials, but was somewhat lower for ampicillin (64.0%) and kanamycin (78.7%). *Staphylococcus* spp. isolates showed a poor susceptibility to AMP. The MIC_{90} (1-4 μg/mL) of this antibiotic was higher than the breakpoint value for susceptibility. Among CoNS resistant to AM, the most prevalent species were *S. caprae*

(37.0%) and *S. chromogenes* (22.2%), while only 12.0% of the *S. aureus* strains were resistant. On the other hand, almost all staphylococci (98.0%) were susceptible to OB and only 2 out of 75 (2.7%) of the CoNS were resistant. Cephalosporins showed high activity against *Staphylococcus* spp. isolates. The percentages of susceptible staphylococci were 98.0%, 99.0% and 96.0% for KF, CFP, and CRO, respectively. The *mecA* gene was not found in any of the tested isolates. For K a low susceptibility of the isolates (74.0%) was recorded. The MIC_{90} of K against the CoNS and *S. aureus* strains (32-≥128 μg/mL) was higher than the reference breakpoint for antimicrobial susceptibility. A different susceptibility to OT was observed in CoNS isolates (94.7%) when compared to that of *S. aureus* (84.0%). The MIC_{90} of OFX for both *S. aureus* and CoNS was comparable with the reference breakpoint for antimicrobial susceptibility (≤1 μg/mL). All the tested staphylococci were susceptible to VA (100.0%), and 98.0% of these to NV. The NV resistant strains belonged to the *S. chonii* (*n* = 1) and *S. xylosus* (*n* = 1) species. The isolates which were resistant to two or more antimicrobial agents are shown in Table 3. It is remarkable that one *S. epidermidis* strain was resistant to six different antibiotics (AM, CRO, K, OB, OFX, and OT).

3.4. Discussion

In the present study, the average prevalence of SCM was 15.6%, which is within the range (6.5%-67.0%) reported in previous studies carried out on dairy goat farms (Contreras et al., 2003). Staphylococci, which made up 88.5% of the isolated microorganisms in this study, are the most common pathogens associated with SCM in dairy goats (Poutrel et al., 1996). AM was less effective than the other β-lactam antimicrobial agents. The susceptibility of the isolates against AM was within the range reported by other authors (Ebrahimi et al., 2007), even if Moroni et al. (Moroni et al., 2005a), have found a markedly greater prevalence of AM resistant *S. aureus* (67.9%). These findings are consistent with AM sensitivity to the penicillinases, frequently produced by *Staphyloccus* spp. and particularly by CoNS strains. OB, a penicilinase-resistant penicillin (PRP), was very effective in vitro. Two CoNS resistant to OB were simultaneously resistant to other β-lactamic antimicrobials, such as AM (n=1) and AM-CRO (n=1). The Cephalosporins showed a strong activity with regard to staphylococci. The MIC_{90}

values of CFP ranged between 0.25 and 8 μg/mL, that were comparable (1.87–3.75 μg/mL) to those found by Moroni et al (Moroni et al., 2005b). Nevertheless, a low susceptibility to CFP was previously found in some CoNS strains isolated from goats with SCM, particularly with regard to *S. chromogenes*, *S. warneri*, *S. simulans*, and *S. kloosii* (Moroni et al., 2004). Other authors found that the susceptibility to KF of CoNS isolated in goats with SCM was between 86.0% and 100.0% (da Silva et al., 2004), that is, comparable with the results (98.7%) obtained in this study. The *mecA* gene was not found in any of the strains tested, in agreement with the results of a previous study carried out on *S. aureus* strains isolated from sheep with SCM (De Santis et al., 2005). The results of the present study confirm that methicillin-resistant staphylococci prevalence is still low in ruminants as observed in previous research (Alves et al., 2009). The finding of some *mecA*-negative isolates which were phenotipically resistant to β-lactam antimicrobial agents could be related to a less common type of resistance due to either overproduction of β-lactamase or the presence of altered Penicillin Binding Protein (PBP) not related to 2a or 2*'* (Georgopapadakou, 1993). The susceptibility to OT was lower in *S. aureus* (84.0%) than in CoNS (94.7%). In previous studies, a number of authors have observed a marked variability in the susceptibility of both these microorganisms to tetracycline, as it ranged between 10.0% and 100.0% (Bochev and Russenova, 2005; Bedidi-Madani et al., 1992; da Silva et al., 2004). The susceptibility of CoNS against NV was 97.3% and this peculiarity is of interest in the taxonomy for bacterial typing since it is also well related with pathogenic activity (Deinhofer and Pernthaner, 1995). All staphylococci were sensitive to VA, thus confirming the results of other authors (Moroni et al., 2004; De Santis et al., 2000). The VA breakpoint value was recently reduced from ≤4 μg/mL to ≤2 μg/mL in testing the susceptibility of bacterial strains isolated from humans (CLSI, 2006b). Some staphylococci isolated from milk samples taken from goats with SCM showed MIC values of 4 μg/mL. When these strains are transferred from animals to humans, they could increase the spreading of vancomycin-intermediate *S. aureus* (VISA) strains. Indeed, comparing the MIC values of VA that we found with the breakpoint actually used for human origin strains, a relevant percentage of *S. aureus* (16.0%) and CoNS (13.3%) would be classified as intermediate.

3.5. Conclusion

This study confirms that staphylococci are the most common pathogens associated with SCM in dairy goats. As expected, the bacteriological positive milk samples had a SCC greater than the negative ones. Over 40.0% of the tested staphylococci were resistant to at least one antimicrobial agent. Single resistance against β-lactamics or aminoglicosides is the most common trait observed. Multiple antibiotic resistance was found in few of the tested Staphylococci strains, mainly in CoNS. Although the methicillin-resistant staphylococci represent the most important pathogens responsible of humans severe hospital-acquired infections, the absence of *mecA* gene and the low prevalence of single and multiple antibiotic resistance suggest that SCM in goats does not play a significant role in the spreading of multi-resistant staphylococci and it does not represent a great public health concern.

3.6. Tables

3.6.1. Table 1. MIC (μg/mL) of antimicrobials against *S. aureus* and CoNS strains isolated from goats subclinical mastitis.

3.6.2. Table 2. Antimicrobial susceptibility of *S. aureus* and CoNS strains isolated from goats subclinical mastitis.

3.6.3. Table 3. Staphylococcus spp. with multiple resistance to antibiotics (μg/mL).

Table 1. MIC (μg/mL) of antimicrobials against *S. aureus* and CoNS strains isolated from goats subclinical mastitis

	Staphylococcus aureus				CoNS			
Antimicrobial agents	MIC_{50}	MIC_{90}	mode	range	MIC_{50}	MIC_{90}	mode	range
Ampicillin[a]	0.12	1.0	≤0.06	≤0.06-16.0	≤0.25-≥0.5	22	≤0.06	≤0.06-16.0
Cefoperazone[b]	2.0	8.0	1.0	1.0-8.0	≤16.0-≥64.0	25	2.0	≤0.06-32.0
Ceftriaxone[b]	4.0	8.0	4.0	1.0-16.0	≤8.0-≥64.0	23	2.0	≤0.06-≥128.0
Cephalothin[a]	0.25	0.25	0.25	≤0.06-≥128.0	≤8.0-≥64.0	24	0.25	≤0.06-16.0
Cloxacillin[a]	0.25	0.5	0.25	1.0-0.12	≤2.0-≥4.0	25	0.5	≤0.06-≥128.0
Kanamycin[a]	16.0	≥128.0	32.0	1.0-≥128.0	≤16.0-≥64.0	15	16.0	≤0.06-≥128.0
Novobiocin[c]	0.12	0.5	≤0.06	≤0.06-0.5	≤4.0	25	≤0.06	≤0.06-32.0
Ofloxacin[b]	0.5	1.0	0.5	0.25-2.0	≤1.0-≥4.0	23	0.5	≤0.06-32.0
Oxytetracycline[a]	0.25	64.0	0.25	0.12-≥128.0	≤4.0-≥16.0	21	0.25	≤0.06-≥128.0
Vancomycin[a]	2.0	4.0	2.0	0.5-4.0	≤4.0-≥32.0	25	2.0	≤0.06-4.0

[a] = Breakpoints NCCLS, 2002; [b] = CLSI, 2006; (Thornsberry et al., 1997)

Table 2. Antimicrobial susceptibility of *S. aureus* and CoNS strains isolated from goats subclinical mastitis.

		Staphylococcus aureus			CoNS		
Antimicrobial agents	MIC breakpoints	susceptible %	intermediate %	resistant %	susceptible %	intermediate %	resistant %
Ampicillin[a]	≤0.25-≥0.5	88.0	-	12.0	64.0	-	36.0
Cefoperazone[b]	≤16.0-≥64.0	100.0	-	-	98.7	1.3	-
Ceftriaxone[b]	≤8.0-≥64.0	92.0	8.0	-	97.3	1.3	1.3
Cephalothin[a]	≤8.0-≥64.0	96.0	-	4.0	98.7	1.3	-
Cloxacillin[a]	≤2.0-≥4.0	100.0	-	-	97.3	-	2.7
Kanamycin[a]	≤16.0-≥64.0	60.0	12.0	28.0	78.7	14.7	6.7
Novobiocin[c]	≤4.0	100.0	-	-	97.3	-	2.7
Ofloxacin[b]	≤1.0-≥4.0	92.0	8.0	-	94.7	1.3	4.0
Oxytetracycline[a]	≤4.0-≥16.0	84.0	-	16.0	94.7	-	5.3
Vancomycin[a]	≤4.0-≥32.0	100.0	-	-	100.0	-	-

[a]= Breakpoints NCCLS, 2002; [b]= breakpoints CLSI, 2006; [c]= breakpoint (Thornsberry et al., 1997).

Table 3. *Staphylococcus* spp. with multiple resistance to antibiotics (μg/mL).

	n (%)	AM[a]	CRO[b]	K[a]	OB[a]	OFX[b]	OT[a]
S. aureus	1 (4)	-	-	≥128	-	-	≥128.0
S. caprae	3 (12)	0.25-2-8	-	-	-	4	≥128.0
S. epidermidis	2 (33)	8.0	≥128.0	≥128.0-64.0	≥128.0	8.0-32.0	≥128.0
S. simulans	1 (7)	0.5	-	-	16.0	-	-
S. warneri	1 (6)	1.0	-	-	-	-	≥128.0

AM=Ampicillin; CFP=Cefoperazone; CRO=Ceftriaxone; KF= Cephalothin; K=Kanamycin; NV=Novobiocina.; OB=Cloxacillin; OFX=Ofloxacin; OT=Oxitetracycline; VA=Vancomicin.
[a] = Breakpoints NCCLS, 2002; [b] = CLSI, 2006.

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CHAPTER 4

The pattern of enterotoxins and virulence factors genes in *Staphylococcus aureus* strains isolated from goat's bulk tank milk.

The pattern of enterotoxins and virulence factors genes in *Staphylococcus aureus* strains isolated from goat's bulk tank milk.

4.1. Introduction

Raw goat milk represents a good substrate for the growth of several pathogen bacteria, which could cause disease in human and animals. Among these microorganisms *Staphylococcus aureus* is a major public health concern, because of its ability to grow in bulk tank milk at levels compatible with enterotoxins (SEs) production. *S. aureus* is also able to produce a large array of secreted and cell-surface-associated virulence factors, such as staphylococcal protein A (spA), fibronectin binding protein, clumping factor A-B, leukocidins and haemolysins which are involved in the adherence, invasion and multiplication in host cells.

The most important source of contamination of bulk tank milk by *S. aureus* are animals with clinical and subclinical mastitis, even if contamination can occurs by milkers' hand and equipment (Sherrer et al., 2004).

Virulence pattern of *S. aureus* stains isolated from goat's bulk tank milk can allow to trace back the most likely origin of the contamination (strains of human or animal origin) and it is essential to characterize spread and genetic modification of *S. aureus* population in goat dairy farms.

The aim of the present study was to determine the virulence pattern of *S. aureus* strain isolated from goat's bulk tank milk and to assess whether the contamination is of human or animal origin, by the detection of the genes coding for the virulence factors, the *in vitro* production of the SEs (SEA-SEE). Results obtained in this study can implement the knowledge on *S. aureus* strains isolated during small ruminants dairy chain. Indeed, studies carried out up to date on the characterization *S. aureus* virulence factors focused mainly on strains of human origin (Alves et al., 2009), while there is very little data regarding *S. aureus* isolated from goat's milk.

4.2. Material and methods

4.2.1. Sampling

In 26 machine milked goat flocks located in Sardinia (Italy), during the period between February and May 2009, 3 different sterile bulk tank milk samples (100 mL) were collected for the detection of Coagulase Positive Staphylococci (CPS). After collection, the samples were transported refrigerated (+4 °C) to the laboratory and analyzed within 6 hours.

4.2.2. Bacteriological procedure and identification of Coagulase Positive Staphylococci

Detection and enumeration of CPS were carried out according to ISO 6888-1/2 (1999a, 1999b). From each positive sample, a single CPS colony was picked, isolated on Brain Heart Infusion (BHI) agar (Oxoid, Basingstoke, UK) and identified by standard microbiological procedures. On each isolate were determined thermolabile and thermostable DNase production (BioRad, California, USA), free (bioMérieux, Lyon, France) and bound (Oxoid, Basingstoke, UK) coagulase production. The strains were also identified by biochemical metabolic tests system API ID32 Staph (bioMérieux, Lyon, France). Stock cultures were stored at -80 °C in BHI broth with glycerol (15% v/v).

4.2.3. DNA extraction

Total genomic DNA was obtained by fhenol-chloroform-isoamilic alchol (25:24:1), lysostaphin (Sigma-Aldrich, MI, Italy) and proteinase K (Invitrogen, UK) according to the protocol proposed by De Buyser et al. (1989). DNA concentration was estimated spectrophotometrically (Shimadzu, Duisburg, Germany).

4.2.4. Oligonucleotide primers

The sequences of the oligonucleotide primers (Invitrogen Corporation, Carlsbad, CA, USA) used for the amplification of the *gyr* (gyrase), *agr* (accessory gene regulator, I–IV), *sea*, *seb*,

sec, *sed*, *see*, *seh*, *sek*, *sel*, *sem*, *seo*, *sep* (encoding for SEs and SEls), *hlb* (ß- haemolysin), *hlg* and *hlgv* (γ-and γ-variant-haemolysin), *lukE/D* (leukocidins LukE/D), *lukM* (leukocidin LukM), *lukF-PV* and *lukS-PV* (Panton-Valentine Leukocidins, PVL), *tst* (TSST-1), *eta*, *etb* and *etd* (exfoliative toxin genes), *edinA/B/C* (epidermal cell differentiation inhibitor genes) and *mecA* (coding for methicillin resistance) genes, have been described by Jarraud et al. (2002). The reference strains used as positive control for the different virulence factors are described by Jarraud et al. (2002).

4.2.5. Multiplex PCR condition and electrophoresis of the PCR products

Different multiplex polymerase chain reactions (PCRs) were prepared, in order to determine the *agr* alleles and the virulence factors genes of *S. aureus* strains isolated from goat's milk. Fifty µL of the PCR mixture conteined: DNA (5 µL), 10X Taq Buffer (Eurobio, Lyon, France), 50 mM $MgCl_2$ (Eurobio, Lyon, France), 1.25 mM of each dNTPs (Invitrogen Corporation, Carlsbad, CA, USA), 5 pmol/µL of primers (Invitrogen Corporation, Carlsbad, CA, USA), 5U/µL Taq polymerase (Eurobio, Lyon, France). DNA amplification was performed using a Thermal Cycler Gene Amp PCR 9700 (Applied Biosystems, Carlsbad, CA, USA) under the followings conditions: initial denaturation at +94 °C for 5 min followed by 25 cycles of denaturation at +94 °C for 30 s, annealing at +55 °C for 30 s, extension at +72 °C for 30 s, followed by a final extension at +72 °C for 7 min.

Amplified products were resolved by linear electrophoresis, visualized using a UV transilluminator (Gel DOC XR - BioRad Laboratories, Hercules, CA, USA) and analyzed by correspondent software (Biorad Laboratories, Hercules, CA, USA).

4.2.6. Statistical analysis

The associations of the genes coding for the tested virulence factors were assessed using the Jaccard similarity coefficient (S3). A complete association between genes is represented by a value of 1, while a total lack of association is represented by a value of 0. The Fisher's exact test was used to investigate if the association found among different genes was statistically

significant with the significance value (P) set at 0.05. All analyses have been carried out using the Statgraphics Centurion XVI software. The strains were included in different pathogenicity profiles according to a binary numerical sequence formed by the presence (=1) or the absence (=0) of the investigated genes.

4.2.7. Pulsed Field Gel Electrophoresis

Pulsed Field Gel Electrophoresis for the characterization of the strain lineage was carried out according to the harmonization protocol suggest by Murchan et al. (2003). Total genomic DNA was digested using *smaI* restriction enzyme (Invitrogen Corporation, Carlsbad, CA, USA). Plugs were loaded in 0.8% agarose gel and the run was performed in Tris Borate EDTA 0.5X. The genetic pattern of *S. aureus* isolates was investigated using a Chef Mapper Drive II (Biorad Laboratories, Hercules, CA, USA). The DNA banding patterns were analyzed with GelCompar II software version 6.0 (Applied Math, Kortrijk, Belgium). The Pearson's correlation coefficient was calculated with 0.5% of optimization setting, and the Unweighted Pair Grouping by Mathematical Averaging was used for cluster analysis. A cut-off of 80% similarity was used to obtain PFGE patterns.

4.2.8. Staphylococcal Enterotoxins detection

S. aureus enterotoxins production was determined on BHI broth cultures after 18-24 hours of incubation. After centrifugation of the broth cultures, the supernatant was filtered (0.22 µm) on nitrocellulose membranes and the filtrates were tested for the presence of the enterotoxins A, B, C, D and E by an ELISA immunoenzymatic kit (Ridascreen Set, R-Biopharm, Darmstadt, Germany) in accordance with the manufacturer instructions. The Optical Density has been measured at 450 nm (TECAN, Grödig, Austria). The interpretation of the results was obtained with Ridasoft Win software (R-Biopharm, Darmstadt, Germany).

4.2.9. Detection of MRSA

The minimal inhibitory concentration (MICs) of oxacillin (Sigma-Aldrich, MI, Italy), was determined with the broth microdiluition method in accordance with the guidelines and the interpretative criteria recommended by the Clinical Laboratory Standards Institute (CLSI, 2006a, 2006b). Reference strains, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were used as quality control.

4.3. Results

4.3.1 Enumeration, isolation and identification of Coagulase Positive Staphylococci

The results of the bacteriological analysis showed the presence of CPS in 60 (76.9%) of the 78 milk samples collected from 20 different farms. Coagulase positive staphylococci count was 2.9±1.1 (mean ± SD) log cfu/mL (range 1.0-4.9 cfu/mL). The 60 CPS strains (one for each positive milk sample) isolated from selective medium were Gram+ cocci, catalase+ and able to produce thermolabile and thermostable Dnase. All the CPS isolated were identified as *S. aureus* according to API ID 32 Staph identifying system and the presence of the specific *gyrA* gene.

4.3.2. SEs and TSST-1 genes associations

The results in Table 1 show that 29 (48.3%) of *S. aureus* strains carried at least one gene for SEs and SEls. 26 out 29 strains carried one or more genes coding for the SEA-SEE while 3 strains were exclusively carriers of SEls genes. The combined presence of *sea*, *seh* and *sek* genes was found in 3 strains (5.0%). The *sec* and *sel* genes were found in 23 (38.3%) strains in 21 of which they were present in association ($P < 0.01$). The *seo* and *sep* genes were associated in 1 strain (1.7%), while the *sep* gene alone was found in 5 strains (8.3%). The *sed* gene was detected in only 1 strain (1.7%), and it was not associated to any other gene that codifies for the SEs or the SEls. *seb*, *see* and *sem* genes were never detected. The *tst* gene was present in 26 (43.3%) strains, in 21 of which was co-detected to the *sec* and *sel* genes. In 2 strains, *tst* was associated exclusively to the *sec* gene and in 3 strains to *sea* gene ($P < 0.05$). The associations among genes

that codify for the SEs-SEls and the other determined virulence factors, are shown in Table 2. The associations among genes that codify for the enterotoxins and the *agr* groups detected are shown in Table 3.

4.3.3. S. aureus virulence factors and agr alleles

All strains carried at least one of the genes that codify for the hemolysins (Table 1). The genes detected with the higher frequency were *hlb* and *hlgv*, found in 55 (91.7%) and 59 (98.3%) strains, respectively. The *hlg* gene was found only in one strain (1.7%). The genes that codify for the *lukE/D* leukocidins were found in 51 strains (85.0%) and in 25 of these (41.7%) it was in association with the *lukM* gene. The *lukE/D* and the *sel* genes, present at the same time in 23 (45.1%) strains were significantly associated ($P < 0.05$). The *edinA/B/C* genes were found in 29 (48.3%) strains. None of the tested strains carried the *eta*, *etb*, *etd*, *lukF-PV*, *lukS-PV* and *mecA* genes.

As shown in Table 1, selected strains were divided into different groups, on the basis of the *agr* profile. Twenty-four strains (40.0%), belonged to *agrI* group and were isolated from 13 farms. Thirty-two strains (53.3%) isolated from 17 farms, belonged to *agrIII* 168 group. Only 3 strains (5.0%) and one strain (1.7%) showed *agr* profile *II* and *IV* respectively.

4.3.4. Association among virulence factors and pathogenicity profile

The association between the presence of the genes encoding the virulence factors and the *agr* groups were as follow: *lukM* gene with the *agrI* group ($P < 0.01$), *seo* gene with *agrII* group ($P < 0.01$), *sec*, *tst*, *lukE/D*, *lukM* and *edinA/B/C* genes with *agrIII* group ($P < 0.05$, $P < 0.01$, $P = 0.05$, $P < 0.01$, $P < 0.001$, respectively). The presence of the *tst+* gene was associated with the presence of *sec*, *sel*, *lukM* and *edinA/B/C* genes ($P < 0.01$) found in 29 strains (48.3%) isolated from 16 (80.0%) of the farms enrolled in the study. According to their pathogenicity profiles (P) the strains were classified in 18 groups, of which the more frequent (P01) included 15 strains (25.0%) belonging to the *agrI* group (Table 1). All the P01 strains carried the following genes: *lukE/D*, *hlb* and *hlgv*. The P15 (12 strains), P05, P12 and P16 with 4 strains each all belonged to

the *agrIII* group. The gene profiles are described in Table 1. All the other patogenicity profile included at the most 3 strains.

4.3.5. PFGE clusters

Among the *S. aureus* isolates strains, one was not typeable by PFGE. Cluster analysis of the 59 isolates (figure 1), using a similarity rate ≥80%, showed that the strains belonged to 12 different clusters (CL01-CL12). Cluster 01 was the most represented with 29 strains (48.3%) isolated from 16 farms, followed by the clusters 11 (18.3%) and 9 (11.6%). These three clusters accounted up to 78.3% of the isolates. The remaining 9 clusters consisted of a number of strains varying from 1 to 3.

4.3.6. Staphylococcal Enterotoxins detection and oxacillin susceptibility

The ELISA method showed the *S. aureus* strains as SED (n = 1), SEA (n = 3) and SEC (n = 23) producers. The agreement of the results obtained by ELISA technique with the molecular techniques used to detect the corresponding genes was of 100%. The SEB and SEE enterotoxins were never detected.

All the tested strains showed to be oxacillin-sensitive, with values of MIC_{50}, MIC_{90}, mode equal to 0.25 μg/mL and range of the MICs ranging from 0.12 to 0.25 μg/mL.

4.4. Discussion

High prevalence of *S. aureus* strains belonging to the *agrIII* (53.3%) and *agrI* (38.3%) groups observed in the current study, is characteristic of isolates from small ruminants (De Santis et al., 2005; Vautor et al., 2009). Overall 29 strains of *S. aureus* carried at least one gene which codifies staphylococcal enterotoxins. This prevalence is lower as compared to what has been observed in previous studies carried out on strains of *S. aureus* isolated from milk of small ruminants (Scherrer et al., 2004; Katsuda et al., 2005). In the current study only three strains (5%) were carriers of the sea gene, which is not frequently observed in goat's milk and it is predominantly related to strains of human origin involved in cases of food toxinfection (Wei

and Chiou, 2002; Ikeda et al., 2005). The presence of the *sea*, *seh* and *sek* genes was significantly associated (P<0.001), as reported in previous studies (Kérouanton et al., 2007; Fischer et al., 2009). *sec* gene, typically associated with *S. aureus* strains isolated from goat's skin, udder and teat skin, mucosa of the nostrils, raw milk, mammary gland secretions of goats with mastitis and goat's raw milk cheese, was found in 38.3% of the isolates. The *tst* gene was found in 26 strains, constantly associated with the *sec* or *sea* genes, and in 21 strains associated also with *sel*. The association of the three *sec*, *sel* and *tst* genes, located on the same pathogenicity island (Becker et al., 2003; De Santis et al., 2005), was observed in 35% of the strains of presumable animal origin. This is in agreement with findings of rare co-occurrence of the *sec* and *tst* genes in human isolates from blood and nasal mucosa (Becker et al., 2003). The frequency of strains carrying both *sec* and *tst* genes found in the current work (38.3%), is lower as compared to previous studies carried out on *S. aureus* isolated from raw tank goat milk (Jørgensen et al., 2005) or isolated from goat mastitis. Leukocidins genes are frequently observed in *S. aureus* isolated from mastitis of dairy ruminants (Rainard et al., 2003). In the current study the *lukM* gene was found in 41.6% of the isolates and its presence has not been reported in strains of human origin (Monecke et al., 2007). The high frequency of *lukE/lukD* genes observed in the present study is in agreement with previous findings of *S. aureus* strains of human and bovine origin (Von Eiff et al., 2004; Yamada et al., 2005). The *PVL* gene is not associated with *S. aureus* strains of dairy ruminants (Aires-de-Sousa et al., 2007) which is confirmed in the current study, where no strain carried *PVL* gene. Indeed, the presence of the *PVL* gene is mainly associated with human strains (Monecke et al., 2007). The hlb gene was found in 85% of the strains, and this outcome is comparable with data reported dairy sheep with subclinical mastitis (De Santis et al., 2005), supporting the animal origin of these strains. None of the tested strains carried the exfoliatin genes. They are not normally present in the isolates from bovine mastitis (Salasia et al., 2004) and from sheep with subclinical mastitis (De Santis et al., 2005). The presence of *et* genes have been observed in *S. aureus* strains belonging to the *agr* group *IV* of typical human origin (Jarraud et al., 2000). The finding of no *et* genes and only one strain belonging to the *agr* group *IV* of our study confirms the probable animal origin

of the S. *aureus* strains as observed in a previous study (Vautor et al., 2007). The available data in literature related to the presence of the *edinA/B/C* genes in strains of *S. aureus* isolated from ruminant milk are limited and they exclusively concern isolates from sheep subclinical mastitis (De Santis et al., 2005), where the prevalence is higher (71.8%) as compared to the current work (48.3%). Most of the strains belonged to 3 clusters and 18 different pathogenicity profiles were detected. The profiles P01 and P15 had a wide distribution among farms and showed a pathogenicity profile typical of strains of animal origin. The current study found a limited number of strains with a genetic profile accountable to human biotypes and all the microorganisms were *mecA* negative and susceptible to oxacillin, patterns frequently observed in strains of animal origin.

4.5. Conclusion

The combination of bio-molecular and immune-enzymatic techniques, with the antibiotic resistance profile, allows to define a virulence pattern and homology traits among *S. aureus* strains isolated from goat's bulk tank milk. Data can be used to study the diffusion and to trace at farm level *S. aureus* strains and to identify the source of contamination (animal or human) of raw milk. The results of this study suggest that a little number of *S. aureus* isolated from goat's bulk tank milk is likely to be of human origin. The *S. aureus* gene virulence patterns contribute in a significant measure to strains characterization and in the definition of their relation with the host.

4.6. Tables

4.6.1. Table 1. Gene profile of the *agr* genes and virulence factors of *S. aureus* isolated from raw goat's milk.

4.6.2. Table 2. Association among genes codifying for enterotoxins and other virulence factors in *S. aureus* isolated from dairy goat's milk cheese through Jaccard coefficient (S3): Sij=a/(a+b+c) and significance test (Fisher's exact test).

4.6.3. Table 3. Association among genes that codify for the enterotoxins and other *agr* groups found in *S. aureus* isolated from dairy goat's milk cheese through Jaccard coefficient (S3): Sij=a/(a+b+c) and significance test (Fisher's exact test).

4.7. Figures

4.7.1. Figure 1. Dendrogram of the 59 *S. aureus* isolated from bulk tank goat milk. The five columns on the right represent *S. aureus* isolate code, *agr* group, virulence factors genes and virulence profile.

Table 1. Genetic pattern of *S. aureus* isolated from raw goat's milk on the basis of *agr* and virulence factors genes.

agrI	*agrII*	*agrIII*	*agrIV*	*sea*	*sec*	*sed*	*seh*	*sek*	*sel*	*seo*	*sep*	*tst*	*lukE/D*	*lukM*	*hlb*	*hlg*	*hlgv*	*edinA/B/C*
15	-	-	-	-	-	-	-	-	-	-	-	-	15	-	15	-	15	-
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-
2	-	-	-	-	-	-	-	-	2	-	-	-	2	-	2	-	2	-
2	-	-	-	-	2	-	-	-	1	-	-	2	2	1	2	-	2	1
4	-	-	-	-	4	-	-	-	4	-	-	4	4	4	4	-	4	-
-	1	-	-	-	-	-	-	-	-	-	-	-	1	-		-	1	-
-	1	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	1	-
-	1	-	-	-	-	-	-	-	-	1	1	-	1	-		-	1	-
-	-	1	-	-	-	-	-	-	-	-	-	-	1	-	1	-	1	-
-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	3	-	3	3
-	-	3	-	-	-	-	-	-	-	-	-	-	3	3	3	-	3	3
-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	4	-	4	4
-	-	1	-	-	-	1	-	-	-	-	-	-	1	1	1	-	1	1
-	-	1	-	-	1	-	-	-	-	-	-	1	-	-	1	-	1	1
-	-	12	-	-	12	-	-	-	12	-	-	12	12	12	12	-	12	12
-	-	4	-	-	4	-	-	-	4	-	4	4	4	4	4	-	4	4
-	-	3	-	3	-	-	3	3	-	-	-	3	3	-		-	3	-
-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	1	-	1	-

Table 2. Association among genes coding for enterotoxins and other virulence factors in *S. aureus* isolated from dairy goat's milk cheese through Jaccard coefficient (S3): Sij=a/(a+b+c) and significance test (Fisher's exact test).

	sea	*sec*	*seh*	*sek*	*sel*	*seo*	*sep*	*tst*	*LukDE*	*LukM*	*hlb*	*hlg*	*hlgv*	*edinABC*
sea	1.0	0	1.0**	1.0**	0	0	0	0.115	0.058	0	0	0	0.05	0
sec		1.0	0	0	0.875**	0	0.166	0.884**	0.423	0.777**	0.418	0	0.389	0.529**
seh			1.0	1.0**	0	0	0	0.115	0.058	0	0	0	0.05	0
sek				1.0	0	0	0	0.115	0.058	0	0	0	0.05	0
sel					1.0	0	0.166	0.75**	0.450*	0.777**	0.418	0	0.389	0.485*
seo						1.0	0.2	0	0.019	0	0	0	0.016	0
sep							1.0	0.148	0.098	0.153	0.071	0	0.084	0.133
tst								1.0	0.5	0.724**	0.396	0	0.440	0.486*
LukDE									1.0	0.490*	0.766	0	0.864	0.355*
LukM										1.0	0.454	0	0.423	0.636**
hlb											1.0	0.018	0.9	0.527*
hlg												1.0	0	0
hlgv													1.0	0.491
edinABC														1.0

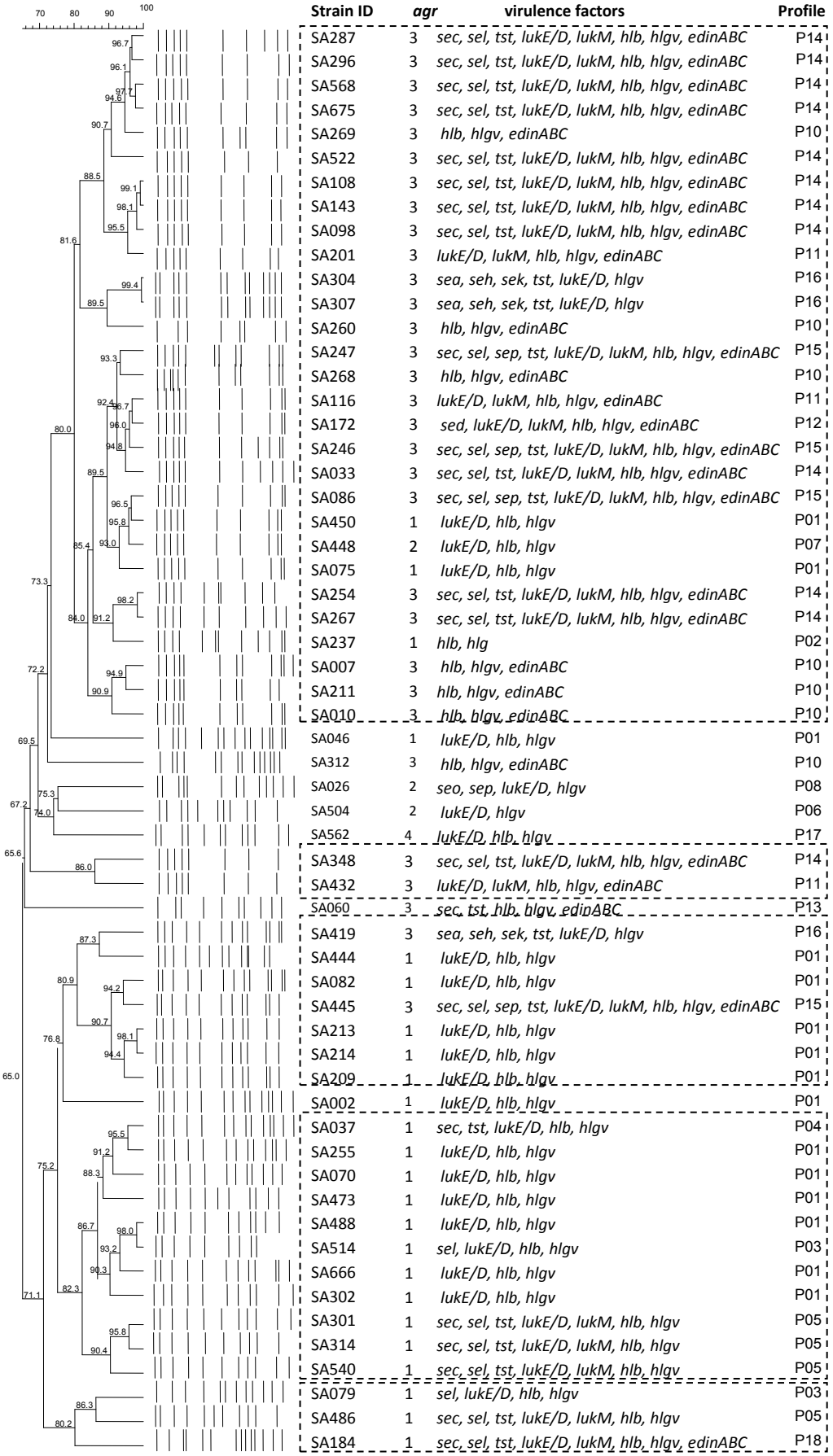
*<0.05 **<0.001

Table 3 - Association among genes coding for the enterotoxins and other *agr* groups in *S. aureus* isolated from dairy goat's milk cheese through Jaccard coefficient (S3): Sij=a/(a+b+c) and significance test (Fisher's exact test).

	sea	*sec*	*seh*	*sek*	*sel*	*seo*	*sep*	*tst*
agrI (n =24)	0	0.146	0	0	0.175	0	0	0.136*
agrII (n = 3)	0	0	0	0	0	0.333	0	0
agrIII (n=32)	0.093	0.447*	0.093	0.410	0	0.121	0.526*	0
agrIV (n= 1)	0	0	0	0	0	0	0	0

*<0.05
**<0.001

Figure 1. Dendrogram of the 59 *S. aureus* isolated from goat bulk tank milk. The four columns on the right represent *S. aureus* isolate code, *agr* group, virulence factors genes and virulence profile.



Strain ID	*agr*	virulence factors	Profile
SA287	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA296	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA568	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA675	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA269	3	*hlb, hlgv, edinABC*	P10
SA522	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA108	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA143	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA098	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA201	3	*lukE/D, lukM, hlb, hlgv, edinABC*	P11
SA304	3	*sea, seh, sek, tst, lukE/D, hlgv*	P16
SA307	3	*sea, seh, sek, tst, lukE/D, hlgv*	P16
SA260	3	*hlb, hlgv, edinABC*	P10
SA247	3	*sec, sel, sep, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P15
SA268	3	*hlb, hlgv, edinABC*	P10
SA116	3	*lukE/D, lukM, hlb, hlgv, edinABC*	P11
SA172	3	*sed, lukE/D, lukM, hlb, hlgv, edinABC*	P12
SA246	3	*sec, sel, sep, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P15
SA033	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA086	3	*sec, sel, sep, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P15
SA450	1	*lukE/D, hlb, hlgv*	P01
SA448	2	*lukE/D, hlb, hlgv*	P07
SA075	1	*lukE/D, hlb, hlgv*	P01
SA254	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA267	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA237	1	*hlb, hlg*	P02
SA007	3	*hlb, hlgv, edinABC*	P10
SA211	3	*hlb, hlgv, edinABC*	P10
SA010	3	*hlb, hlgv, edinABC*	P10
SA046	1	*lukE/D, hlb, hlgv*	P01
SA312	3	*hlb, hlgv, edinABC*	P10
SA026	2	*seo, sep, lukE/D, hlgv*	P08
SA504	2	*lukE/D, hlgv*	P06
SA562	4	*lukE/D, hlb, hlgv*	P17
SA348	3	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P14
SA432	3	*lukE/D, lukM, hlb, hlgv, edinABC*	P11
SA060	3	*sec, tst, hlb, hlgv, edinABC*	P13
SA419	3	*sea, seh, sek, tst, lukE/D, hlgv*	P16
SA444	1	*lukE/D, hlb, hlgv*	P01
SA082	1	*lukE/D, hlb, hlgv*	P01
SA445	3	*sec, sel, sep, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P15
SA213	1	*lukE/D, hlb, hlgv*	P01
SA214	1	*lukE/D, hlb, hlgv*	P01
SA209	1	*lukE/D, hlb, hlgv*	P01
SA002	1	*lukE/D, hlb, hlgv*	P01
SA037	1	*sec, tst, lukE/D, hlb, hlgv*	P04
SA255	1	*lukE/D, hlb, hlgv*	P01
SA070	1	*lukE/D, hlb, hlgv*	P01
SA473	1	*lukE/D, hlb, hlgv*	P01
SA488	1	*lukE/D, hlb, hlgv*	P01
SA514	1	*sel, lukE/D, hlb, hlgv*	P03
SA666	1	*lukE/D, hlb, hlgv*	P01
SA302	1	*lukE/D, hlb, hlgv*	P01
SA301	1	*sec, sel, tst, lukE/D, lukM, hlb, hlgv*	P05
SA314	1	*sec, sel, tst, lukE/D, lukM, hlb, hlgv*	P05
SA540	1	*sec, sel, tst, lukE/D, lukM, hlb, hlgv*	P05
SA079	1	*sel, lukE/D, hlb, hlgv*	P03
SA486	1	*sec, sel, tst, lukE/D, lukM, hlb, hlgv*	P05
SA184	1	*sec, sel, tst, lukE/D, lukM, hlb, hlgv, edinABC*	P18

4.8. References

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CHAPTER 5

Antibiotic resistance in *Staphylococcus aureus* strains isolated from bulk tank goat's milk in Sardinia.

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Antibiotic resistance in *Staphylococcus aureus* strains isolated from bulk tank goat's milk in Sardinia.

5.1. Introduction

Staphylococcus aureus can show single or multiple resistance to antibiotics commonly used in medicine and veterinary practices. The presence of *S. aureus* strains resistant to antibiotics in small ruminant dairy farms is cause of concern for public health. The contamination/infection can origin from direct contact of workers with infected animals or can be the consequence of the ingestion of contaminated food. Small ruminants can serve as a reservoir of resistant strains and are involved in the spread of the pathogens in the environment. Although transmission of *S. aureus* resistant strains through foods is sporadic (EFSA, 2008), the arise of resistant strains, especially methicillin resistant, livestock associated, is becoming a public health emergency (Cavaco et al., 2011). These strains, referred to as Livestock-Associated MRSA (LA-MRSA), are not linked to HA or CA-MRSA and their occurence could be related to selection and differentiation among the population of methicillin susceptible *Staphylococcus aureus* (MSSA) [Hasman et al., 2010].

Since *S. aureus* can be isolated with high frequency in goat milk and related products (Jakobsen et al., 2011), dairy chain could represent the means for the introduction of resistant strains into human population.

The aim of the present research was to carry out an extensive study on the presence of *S. aureus* resistant strains in bulk tank milk using genotypic and phenotypic methods. Results obtained could increase knowledge on *S. aureus* strains resistant to antibiotics in bulk tank goat's milk.

5.2. Material and methods

5.2.1. Sampling and isolation of Coagulase Positive Staphylococci

In 26 goat flocks located in Sardinia (Italy), 3 different sterile bulk tank milk samples (100 mL) were collected (one month apart from each other o during the lactation) for the detection of

Coagulase Positive Staphylococci (CPS). After collection, the samples were transported refrigerated (+4 °C) to the laboratory and analyzed within 6 hours.

Detection and enumeration of CPS were carried out according to ISO 6888-1/2 (1999a, 1999b) standard methods. From each positive sample, a single CPS colony was picked and subcoltured on Brain Heart Infusion (BHI) agar (Oxoid, Basingstoke, UK) and identified by standard microbiological procedures. On the total of the isolates, 40 strains (2 strains for each flock positive for the presence of CPS) were selected and tested for thermolabile and thermostable DNase production (BioRad, California, USA), free (bioMérieux, Lyon, France) and bound (Oxoid, Basingstoke, UK) coagulase production. The strains were also identified by biochemical metabolic tests (API ID32 Staph; bioMérieux, Lyon, France). The presence of the specific *gyrA* gene was also assessed by PCR in order to confirm biochemical identification. Stock cultures were stored at -80 °C in BHI broth with glycerol (15% v/v).

5.2.2. Susceptibility to antimicrobial agents

On each strain the MICs of 11 antibiotics used in human and veterinary medicines were determined. The antibiotics tested were ampicillin (AM), penicillin (P), amoxicillin (AMX), oxacillin (OX), tetracycline (TE), cephalotin (CF), erythromycin (E), vancomycin (VA), cloxacillin (OB), cefoperazone (CFP) and ceftriaxone (CRO).

The MICs were determined by the broth microdilution method (CLSI, 2006a, 2006b) using cation-adjusted Mueller-Hinton broth (CAMHB, Oxoid, Basingstoke, UK). Each antimicrobial agent, in powder form (Sigma-Aldrich-Fluka, MI, Italy), was weighted and dissolved in an appropriate solvent, thus obtaining a stock solution (2,560 μg/mL). Stock solutions were stored at -80 °C until used. From each stock solution, 12 serial twofold working dilutions in deionized water (only for AM, the diluents were phosphate buffer, pH 6.0, 0.1 mol/L) was prepared according to CLSI standard protocols, and the antimicrobial agent final concentrations in each microplate ranged between 0.06 and 128 μg/mL. Each strain stored at -80 °C until testing were subcultured twice on BHI agar before inoculum preparation.

5.2.3. DNA extraction

Briefly, after overnight incubation at +37 °C, bacterial cells were suspended in 5 mL of BHI broth and centrifuged at 3,000 x g for 10 minutes. Then pellet was resuspended in Tris EDTA buffer. Total genomic DNA was obtained by fhenol-chloroform-isoamilic alchol (25:24:1), lysostaphin (Sigma-Aldrich, MI, Italy) and proteinase K (Invitrogen, UK) according to the protocol proposed by De Buyser et al. (1989). DNA concentration was estimated spectrophotometrically (Shimadzu, Duisburg, Germany).

5.2.3. Detection of genes coding for antibiotic resistant

Primers used to amplify antibiotic resistance genes are reported in table 1. Multiplex polymerase chain reactions were prepared, in order to determine the presence of 10 different genes (*mecA*, *ermA*, *vanA* and *vanB*, *blaZ*, *tetK*, *L*, *M*, *S*, *W*) coding for antibiotic resistance of *S. aureus* strains isolated from goat's bulk tank milk.

Twenty µL of the PCR mixture conteined: DNA (1 µL), 5X Taq Buffer (Promega Corporation, MA, USA), 50 mM $MgCl_2$ (Promega Corporation, MA, USA), 0.2 mM of each dNTPs (Invitrogen Corporation, CA, USA), 5 µM of primers forward and reverse (Invitrogen Corporation, CA, USA), 5U/µL Taq hot start polymerase (Promega Corporation, MA, USA).

DNA amplification was performed using a Thermal Cycler Gene Amp PCR 9700 (Applied Biosystems, Carlsbad, CA, USA) under the followings conditions: initial denaturation at +94 °C for 5 min followed by 30 cycles of denaturation at +94 °C for 1min, annealing at +55 °C for 1 min, extension at +72 °C for 1 min, followed by a final extension at +72 °C for 7 min.

PCR products were submitted to electrophoresis and visualized using a UV transilluminator (Gel DOC XR - BioRad Laboratories, Hercules, CA, USA). Gel images were analyzed using the corresponding software Quantity One (BioRad Laboratories, Hercules, CA, USA).

5.3. Results

All the selected strains were identified as *S. aureus* on the basis of the phenotypic characteristics and the presence of the *gyrA* gene, specific for *S. aureus*. For each tested antibiotic MIC_{50}, MIC_{90}, mode and range were determined (table 2). All the strains were susceptible to 10 out of 11 tested antibiotics. For all the 11 antibiotics, MIC_{90} were lower than corresponding breakpoint values. Only two *S. aureus* strains, isolated from the same farm, showed resistance to TE, with a MIC value of 128 μg/mL. Resistance to TE was confirmed by the presence of *tetK* gene, coding for an efflux pump. None of the tested strains showed resistance to β-lactam antibiotics (P, AM, AMX, OB, OX, CF, CFP, CRO), although 2 strains carried *blaZ* gene. Resistance to E and VA, antibiotics commonly used to treat MRSA.

Moreover all the *S. aureus* strains selected in the present study, were susceptible to OB, antibiotic used to test methicillin resistance.

5.4. Discussion and Conclusion

Results obtained in the present investigation indicated that *S. aureus* strains, isolated from bulk tank goat milk have limited *spectrum* of resistance to antibiotics commonly used in human medicine and veterinary practices. These data are supported by other studies carried out on goat's and cow's milk (Moroni et al., 2005; Hata et al., 2008) where only few strains showed resistance to antibiotics. Moreover, in the current research none of the strains was simultaneously resistant to different antibiotics, while is a common finding in strains isolated from pigs, poultry or human infection (Wang et al., 2010; Waters et al., 2011).

Single or multiple resistance to antibiotics in *S. aureus* strains linked to small ruminants is more frequent when animals are affected by mastitis, while is a rare finding in strains isolated from milk (Tueber, 1999).

5.5. Tables

5.5.1. Table 1. Primers used to amplify different genes coding for antibiotic resistance in *S. aureus* strains isolated from bulk tank goat milk.

5.5.2. Table 2. MIC_{50}, MIC_{90}, mode and range of antimicrobials against *S. aureus* strains isolated from bulk tank goats milk.

Table 1. Primers used to amplify different genes coding for antibiotic resistance in *S. aureus* strains isolated from bulk tank goat milk.

gene target	resistance to	sequence (5'-3')	length (bp)	reference
mecA	Oxacillin	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	533	Vandenesch et al. (2003)
ermA	Erythromycin	TCTAAAAAGCATGTAAAAGAA CTTCGATAGTTTATTAATATTAGT	645	Vancraeynest et al. (2004)
blaZ	β-lattamic	TCAAACAGTTCACATGCC TTCATTACACTCTGGCG	792	Rosato et al. (2003)
vanA	Vancomycin	CATGACGTATCGGTAAAATC ACCGGGCAGRGTATTGAC	832	Patel et al. (1997)
vanB		CATGATGTGTCGGTAAAATC ACCGGGCAGRGTATTGAC	832	
tetL	Tetracycline	ATAAATTGTTTCGGGTCGGTAAT AACCAGCCAACTAATGACAATGAT	696	Trzcinsky et al. (2000)
tetM		ACAGAAAGCTTATTATATAAC TGGCGTGTCTATGATGTTCAC	740	Aminov et al. (2001)
tetS		GAAAGCTTACTATACAGTAGC AGGAGTATCTACAATATTTAC	169	
tetW		GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	

Table 2. MIC50, MIC90, mode and range of antimicrobials against *S. aureus* strains isolated from bulk tank goats milk.

Antibiotics	MIC_{50}	MIC_{90}	Mode	Range
AM[1]	0,12	0,25	0,12	0,06-0,25
P[2]	0,6	0,12	0,06	0,06-0,12
AMX[3]	0,25	0,5	0,25	0,12-1,0
OX[4]	0,25	0,25	0,25	0,12-0,5
TE[5]	4,0	4,0	4,0	1,0-128,0
CF[6]	0,25	0,25	0,25	0,06-0,5
E[7]	0,12	0,25	0,06	0,06-0,5
VA[8]	1,0	2,0	1,0	0,5-2,0
OB[9]	0,25	0,25	0,25	0,12-1,0
CFP[10]	4,0	8,0	4,0	2,0-8,0
CRO[11]	4,0	8,0	4,0	4,0-8,0

[1]=Ampicillin, [2]=Penicillin, [3]=Amoxicillin, [4]=Oxacillin, [5]=Tetracycline, [6]=Cephalotin, [7]= Erythromycin, [8]=Vancomycin
[9]=Cloxacillin, [10]=Cefoperazone, [11]=Ceftriaxone

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CHAPTER 6

Prevalence of *Staphylococcus aureus* strains in raw milk sheep's cheese and enterotoxigenic profile.

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Prevalence of *Staphylococcus aureus* strains in raw milk sheep cheese and enterotoxigenic profile.

6.1. Introduction

Presence of *Staphylococcus aureus* in milk and dairy products could represent a concern for public health because of the ability to produce and excretes enterotoxins (SEs) in food, when its count exceed 10^5 cfu/g (Commission Regulation (EC) No 2073/2005).

Dairy products, especially raw milk cheeses are accountable for 5% of the total outbreaks caused by staphylococcal SEs (European Commission, 2003). *S. aureus* growth can occur primarily in raw milk as a consequence of temperature abuse during storage or in the first cheese-making steps, especially in raw milk cheeses (Charlier et al., 2009). Moreover, productions of raw milk cheeses do not include a heat milk treatment and it do not give insurance to control *S. aureus* multiplication during cheese-making (FSANZ, 2009). Different studies carried out on the prevalence of *S. aureus* in small ruminants dairy chain, have showed that the pathogen count present in raw milk increases untill six hours after molding, when pH of the cheese is approximately 5.3-5.4 (Pisano et al., 2007; Jakobsen et al., 2011). Although raw milk cheese production does not include use of high temperatures to reduce *S. aureus* contamination, its count progressively decrease during ripening time. Even low *S. aureus* contamination at the end of the ripening phase does not give insurance of the healthiness of the cheese, because of the presence of preforming SEs (European Commission, 2003).

SEs are thermostable exotoxins belonging to the PTAgs family, which can cause food poisoning. Symptoms of staphylococcal foodborne intoxication are nausea, vomiting, abdominal cramping and sometimes diarrhea. Generally the illness is self-limiting and symptoms resolve after 24-48 hours. Sometimes this toxinfection can lead to hospitalization, especially in susceptible patients, such as young and older people, pregnant and immune compromised (Argudin et al., 2011).

Usually staphylococcal foodborne intoxication after consumption of raw milk cheeses is accountable to SEA, followed by SED in cow milk cheeses and SEC in sheep and goat milk cheese (Morandi et al., 2007; Argudin et al., 2011).

Different studies showed that *S. aureus* prevalence in raw milk sheep's cheese ranged between 60% and 100% six hours after molding and then decreased during ripening. Although *S. aureus* prevalence was high, only in few cases isolates produced one or more SEs (Lamprell et al., 2004; Tekinşen and Özdemir, 2006; Ertas et al., 2010).

The aim of the present study was to determine prevalence of *S. aureus* strains in raw milk sheep's cheeses produced in artisan cheese-making plants in Sardinia. Cheese samples with S. aureus prevalence $\geq 10^5$ cfu/g were tested for the SEs production (SEA-SED) using a Reverse Passive Latex Agglutination (RPLA) kit. Moreover, for each isolate has been defined a virulence pattern by the detection of the genes encoding SEs (*sea, seb, sec, sed, see*, *seh*, *sek*, *sel*, *sem*, *seo* and *sep*). Results obtained could be used to enhance knowledge among the prevalence of *S. aureus* in raw milk sheep's cheese and to evaluate risk to human health associated to the presence of the pathogen in artisan dairy products.

6.2. Material and methods

6.2.1. Sampling

Sixteen raw milk sheep cheese samples, collected from eight artisan cheese-making plants (A-H), were analyzed for the detection and enumeration of CPS. Samples were representative of two different production batches and were collected six hours after molding, when its count is supposed to be highest (Commission Regulation (EC) No 2073/2005). On the total of the dairies, 5 out of 8 used an auto-produced whey starter culture during cheese-making. For each sample, pH and temperature (pH-meter pH340i/SET, WTW, Germany) were detected during six hours before collection.

6.2.2. Detection and identification of Coagulase Positive Staphylococci

Twenty-five grams of each cheese sample were added to 225 mL of Buffered Peptone Water (BPW, Oxoid, Basingstoke, UK) and homogenized for 3 min using a stomacher 400 circular (International P.B.I., Milan, Italy). 0.1 mL of homogenate were plated out onto Baird-Parker + Rabbit Plasma Fibrinogen (BP+RPF, bioMérieux, Lyon, France) and incubated for 24 hours at +37 °C ± 2 °C according to ISO 6888-1/2 (1999a, 1999b).

From each positive plate, 2 typical CPS colonies were picked up and tested for: Gram staining, catalase and oxidase tests, free (bioMérieux, Lyon, France) and bound (Oxoid, Basingstoke, UK) coagulase, TDNase (BioRad, California, USA). Haemolytic activity of the strains was also assessed on blood agar plates. Presumptive identification was confirmed by biochemical metabolic tests (API ID32 Staph; bioMérieux, Lyon, France). Presence of the specific *gyrA* gene was also carried out by PCR, in order to confirm biochemical identification. After identification strains were stored at -80 °C in Brian Heart Infusion broth (BHI, Oxoid, Basingstoke, UK) with glycerol (15% v/v).

6.2.3. Staphylococcal enterotoxins detection

Cheese samples which showed a SCP count $\geq 10^5$ cfu/g were tested for the presence of enterotoxins A, B, C and D using a SET-RPLA kit (Oxoid, Basingstoke, UK), according to the manufacturer's instruction. Sensitivity level for each tested SE was ~0,5 ng/mL.

6.2.4. Detection of the genes coding for staphylococcal enterotoxins

After DNA extraction according to the protocol proposed by De Buyser et al. (1989), *sea, seb, sec, sed, see*, *seh*, *sek*, *sel*, *sem*, *seo* and *sep* genes were detected by PCR.

Fifty µL of the PCR mixture conteined: DNA (5 µL), 10X Taq Buffer (Eurobio, Lyon, France), 50 mM $MgCl_2$ (Eurobio, Lyon, France), 1.25 mM of each dNTPs (Invitrogen Corporation, California, USA), 5 pmol/µL of primers forward and reverse (Invitrogen Corporation, California, USA), 5U/µL Taq polymerase (Eurobio, Lyon, France). DNA amplification was performed using a Thermal Cycler Gene Amp PCR 9700 (Applied Biosystems, California,

USA) under the followings conditions: initial denaturation at +94 °C for 5 min followed by 25 cycles of denaturation at +94 °C for 30 s, annealing at +55 °C for 30 s, extension at +72 °C for 30 s, followed by a final extension at +72 °C for 7 min.

Amplified products were resolved by linear electrophoresis, visualized using a UV transilluminator (Gel DOC XR - BioRad Laboratories, California, USA) and analyzed by correspondent software (BioRad Laboratories, California, USA). Electrophoretic band pattern was analyzed and then used to obtain different virulence profiles. The presence of a given band/gene was coded as "1", while the absence was coded as "0".

6.2.5. Statistical analysis

Student's t test was performer to compare pH values of cheese obtained with the use of starter and without any starter culture. General linear model was used to investigate the effect of cheese making pant and production batch on CPS count. Analysis was performed using Statgraphics Centurion XVI software.

6.3. Results

All the analyzed cheese samples were positive for CPS contamination with a count of 4,78±1,05 $\log_{10}$ cfu/g ($\bar{x}$ ±SD) and ranged between 3.9 and 6.4 $\log_{10}$ cfu/g. CPS count and pH value for each dairy are reported in table 1. pH values of the cheese samples from time 0 (curd after cutting) to time 6 (six hours after molding) were 6.61±0.13 and 5.65±0.57 ($\bar{x}$ ±SD) respectively. pH detected at time 6 was lower in cheese samples collected from dairy plants which used the whey starter culture (P <0.01). Differences in pH values among dairies which used or not whey starter culture are reported in table 2.

On the CPS count, general linear model (GLM) did not show differences related to the dairy of origin (P >0.05) and the production batch (P >0.05). Although 50% of the cheese samples showed a CPS contamination >10^5 cfu/g, enterotoxins A-D were never detected.

Selected CPS were identified as *S. aureus* based on the phenotypic characteristics and the presence of *gyrA* gene. All the strains were TDNase, free and bound coagulase producers and

belonged to 4 different haemolytic groups: α-haemolysis (n. 7), β-haemolysis (n. 10), α/β-haemolysis (n. 8), while the remaining strains (n. 7) did not show haemolytic activity.

Linear combination of the binary codes for the presence or absence of the investigated genes allowed to obtain 3 different virulence profiles, P1 (*sec*+*sel*), P2 (*sem*+*seo*) and P3 (*sea*+*sem*+*seo*) [table 3].

6.4. Discussion and conclusion

S. aureus is one of the main microorganisms involved in foodborne disease associated with consumption of dairy products, especially raw milk cheeses (Jarraud et al., 2002). In the present study all the cheese samples were contaminated with *S. aureus* (prevalence = 100%). Similar prevalence (60%-100%) was found in previous studies carried out on raw milk sheep cheese (and Özdemir, 2006; Argudín et al., 2010).

S. aureus growth can occurs primarily in raw milk as a consequence of temperature abuse during storage or in the first cheese-making steps, especially in raw milk cheeses, when the pathogen multiplication is not inhibited by the activity of lactic acid bacteria (Charlier et al., 2009). The role of the pH to keep under control *S. aureus* growth is still debated. Different studies showed that did not exist a close relationship between utilization of a starter culture and CPS count during the first 24 hours of the cheese-making (Gomez-Lucia et al., 1987; Charlier et al., 2009). In the present research, dairy plants which used an auto-produced whey starter culture showed the highest CPS count, although cheese acidification at six hours after molding was proper. Natural lactic flora of the milk or starter culture with low acidifying power is effective to control *S. aureus* multiplication as well.

Technologies used to produce traditional raw sheep's milk cheeses and the absence of heat milk treatment do not give insurance to control *S. aureus* multiplication (FSANZ, 2009). Therefore, for this category product is essential understanding the role of the pH and the inhibitory potential of Lactic Acid Bacteria (LAB) on *S. aureus* multiplication. Unfortunately, these factors compose a complex network and it is difficult evaluate the effectiveness of one factor over another on the inhibition of *S. aureus* growth.

6.5. Tables

6.5.1. Table 1. Average values of pH_0 (curd after cutting) , pH_6 (6 hours after molding) and CPS count (log_{10} cfu/g) in raw milk sheep cheese samples, collected from 8 artisan dairies.

6.5.2. Table 2. Differences in pH values (pH_0 and pH_6) among dairies which used or not whey starter culture and SCP count (log_{10} cfu/g).

6.5.3. Table 3. Genes coding for SEs in 32 *S. aureus* strains isolated from 8 artisan dairies.

Table 1. Average values of pH_0 (curd after cutting), pH_6 (6 hours after molding) and CPS count (log_{10} cfu/g) in raw milk sheep cheese samples, collected from 8 artisan dairies.

Dairy	A[1]	B[1]	C	D	E[1]	F[1]	G[1]	H
pH_0	6,87	6,54	6,65	6,67	6,65	6,42	6,50	6,60
pH_6	5,27	5,24	6,12	6,46	5,13	5,40	5,20	6,39
CPS[a]	5,4	5,5	4,8	3,4	5,5	5,0	6,4	3,9

[a]= Coagulase Positive Staphylococci; [1]= daries which used an auto-produced whey starter culture.

Table 2. Differences in pH values (pH_0 and pH_6) among dairies which used or not whey starter culture and SCP count (log_{10} cfu/g).

	samples	whey starter culture utilization	samples	whey starter culture utilization	p-value
	n.	yes	n.	no	
pH_0	10	6,60±0,17	6	6,64±0,04	ns
pH_6	10	5,25±0,10	6	6,32±0,18	<0,01
SCP[a]	10	5,21±0,95	6	4,05±0,81	<0,05

[a]= Coagulase Positive Staphylococci

Table 3. Genes coding for SEs in 32 *S. aureus* strains isolated from 8 artisan dairies.

dairy	SEs										
	sea	*seb*	*sec*	*sed*	*see*	*seh*	*sek*	*sel*	*sem*	*seo*	*sep*
A	-	-	4/4	-	-	-	-	4/4	-	-	-
B	-	-	2/4	-	-	-	-	2/4	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-
D	-	-	2/4	-	-	-	-	2/4	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	2/4	2/4	-
H	4/4	-	-	-	-	-	-	-	4/4	4/4	-

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CHAPTER 7

Antibiotic resistance assessment in *S. aureus* strains isolated from raw sheep's milk cheese

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Antibiotic resistance assessment in *S. aureus* strains isolated from raw sheep's milk cheese

7.1. Introduction

S. aureus is involved in food poisoning outbreaks and in local or systemic human diseases. Antibiotic resistance in *S. aureus* isolated from food of animal origin has been reported. The European Food Safety Authority (EFSA) identifies animals as a possible reservoir of *SA* antimicrobial-resistant (AMR) strains.

Transmission through foods of AMR strains involved in human outbreaks was reported in sporadic cases (EFSA, 2008). Several mechanisms are involved in the antibiotic resistance of *S. aurues*. The β-lactamase enzyme encoded by plasmids inactivates penicillins. Methicillin and other semi-synthetic penicillins were considered effective against β-lactamase producer strains. However, the emergence of Methicillin-resistant *SA* (MRSA) strains with multiple resistance, has been reported. MRSA strains harbour the *mecA* gene, which encodes for a low-affinity penicillin-binding protein (PBP2a). Nosocomial infections caused by MRSA sometimes can lead patients to death (Moreillon and Que, 2004). Although MRSA are mainly related to human, in recent years have been reported cases of MRSA strains linked to livestock classified as Livestock-associated MRSA (LA-MRSA). The emergence of LA-MRSA is not clear yet but it could be related to a selection and differentiation among the population of methicillin susceptible *Staphylococcus aureus* (MSSA) [Hasman et al., 2010]

Macrolide resistance in *S. aureus* can be caused by a target modification of a 23S rRNA methylase, encoded by the plasmid *erm* genes. *S. aureus* can also show resistance to Vancomycin, which has been successfully used to treat MRSA strains. Vancomycin resistance is encoded by several genes, and it seems to be originated from mutation under antibiotic selective pressure.

Antibiotic resistance is frequently acquired by exchange of mobile DNA elements during food processing between pathogenic and non pathogenic microorganisms. In dairy sheep industry, *S.*

aureus AMR strains can be recovered from raw milk, from secretion of animal with mastitis and from dairy products.

The aim of the present study was to assess a protocol to characterize *S. aureus* isolates and their resistance to antibiotics using phenotypic and molecular techniques. In *S. aureus* strains isolated from raw sheep's milk cheese, in samples collected at early stage of cheese-making process, Minimum Inhibitory Concentration (MICs) and susceptibility against antibiotics used in human and veterinary practice have been determined. The presence of the genes *ermA*, *blaZ*, *mecA* and *vanA-B* was also investigated. On the isolated microorganisms, a population study was carried out by Pulsed Field Gel Electrophoresis (PFGE).

7.2. Material and methods

S. aureus strains were recovered from cheese made from raw sheep's milk, collected 6 hours after molding, phase generally associated with the highest level of *S. aureus* (Commission Regulation (EC) No 2073/2005). Twelve cheese wheels were collected from 6 household plants (A-F) located in Sardinia (Italy). Each sample was representative of a different production batch, two for each plant. Samples were analyzed according to ISO 6888-1/2 (1999a, 1999b).

Thirty-six strains were isolated from typical colonies and tested for: Gram staining, catalase and oxidase tests, free (bioMérieux, Lyon, France) and bound (Oxoid, Basingstoke, UK) coagulase, TDNase (BioRad, California, USA). Presumptive identification was confirmed by biochemical metabolic tests (API ID32 Staph; bioMérieux, Lyon, France). Presence of the specific *gyrA* gene was also evaluated by PCR, in order to confirm biochemical identification. After identification strains were stored at -80 °C in Brian Heart Infusion broth (BHI, Oxoid, Basingstoke, UK) with glycerol (15% v/v).

On each strain the MIC was tested by the broth microdilution method (CLSI, 2006a, 2006b) for the following antibiotics: Ampicillin (AM), Cefalotin (CF), Cefoperazone (CFP), Cloxacillin (OB), Eritromicin (E), Gentamicin (GM), Kanamicin (K), Novobiocin (NV), Oflaxicin (OFX), Oxacillin (OX), Penicillin (P), Rifampicin (RA), Streptomicin (S), Tetraciclin (TE), Tobramicin

(TM) and Vancomicin (VA). Twelve serial dilutions of each antibiotic were made in Cation-Adjusted Mueller Hinton Broth (CAMHB; Oxoid LTD Basingstoke, UK) added with NaCl (2%), obtaining final concentrations of antibiotic ranging from 0.06 to 128 μg mL^{-1} (0.006-12.8 μg mL^{-1} for RA). The MICs mode, range, MIC_{50} and MIC_{90} of antimicrobial agents were calculated and the antibiotic susceptibility was defined comparing the MICs with the reference breakpoints (CLSI, 2006b). After the DNA extraction according to De Buyser et al. (1989), multiplex PCR was carried out to detect *gyrA* encoding the DNA gyrase, *agr* (I–IV), accessory gene regulator (Jarraud et al., 2002), *blaZ* encoding a penicillinase, *ermA*, one of the genes encoding resistance to macrolide, *mecA* encoding PBP2a, and *vanA-B*, genes encoding for the VA resistance (Vandenesh et al., 2003; Rosato et al., 2003; Vancraeynest et al., 2004). The templates were loaded into agarose gel 1.5%, resolved by electrophoresis and visualized using a UV transilluminator (Gel DOC XR - BioRad Laboratories, California, USA). Banding pattern was analyzed using the Quantity One software (BioRad Laboratories, California, USA).

The population study was conducted by PFGE on *S. aureus* strains, according to the harmonization protocol proposed by Murchan et al. (2003) , after *Sma*I restriction (Invitrogen, CA, USA) of bacterial DNA. The correlation between the isolates was determined by Unweighted Pair Group Method using Arithmetic averages (UPGMA) using the Pearson's correlation coefficient with 1.0% optimization setting. PFGE clusters were obtained considering a cut off of 70%. All the PFGE analyses were performed using GelCompar II software version 6.0 (Applied Math, Sint-Martens-Latem, Belgium).

7.3. Results

CPS in cheese samples ranged from 3.9 to 5.8 Log_{10} cfu mL^{-1}. Thirty-six isolates were identified as *S. aureus* on the basis of biochemical and metabolic profile, and the presence of the *gyrA* gene. All the isolates produced TDNase, free and bound coagulase. The isolates were characterized by PFGE clusters (P1-P10), by the *agr* group and by antibiotic resistance genes (Table 1). None of the tested isolates showed resistance to CF, CFP, E, GM, K, NV, OFX, RA,

S and TM. Only two isolates showed intermediate levels of resistance to K (5.6%), and one isolate to OFX (2.8%).

Four *S. aureus* strains could not be typed by PFGE. All the non-typeable strains were isolated from the same dairy and showed resistance to TE.

The *S. aureus* isolates exhibited resistance to AM (36.1%), P (33.3%), TE (11.1%), OB (2.8%) and the MIC_{90} for these antibiotics was respectively 16.0, 32.0, 64.0 and 32.0 μg mL^{-1}. MIC_{90} of the isolates was below the sensitivity breakpoint for 13 out of the 16 tested antibiotics. With regards to AM and P resistance the results of broth microdilution method and molecular technique (*blaZ* gene detection) were in agreement, showing a concordance of 94.4%. The disagreements between the results obtained with the two methods were referred to a *S. aureus blaZ*+ isolate which showed resistance only to AM and one isolate *blaZ*- with resistance to AMP, P and OB.

7.4. Discussion

The *S. aureus* strains isolated during cheese-making process were resistant only to AM, P, OB and TE, which are among the most commonly used antibiotic in dairy sheep farms. In *S. aureus* isolated from sheep, the *agrIII* allele is the most frequently observed (De Santis et al. 2005; Vautor et al., 2007). Fifty percent of the *S. aureus* isolated from 4 different dairies belonged to *agrIII* group, while 27.8% belonged to *agrII* group and were isolated from 3 dairies (C, D and F). The strains belonging to *agrI* group (dairies C and F) and to *agrIV* group (dairy B) showed low prevalence. The dendrogram showed a specific distribution of isolates for each dairy. Only isolates included in P1 cluster were observed in two different dairies (A and B). Each of the isolates of P3, P4 and P10 clusters were recovered in only one dairy (D, E and F, respectively), but in both production batches.

Our results show a good correspondence between PFGE cluster and the antibiotic resistance profile of the isolates. In two dairies (A and D) all the isolates were susceptible to the tested antibiotics. Four strains belonging to *agrI* group (dairy C) were non-typeable by PFGE, and

exhibited resistance to TE. In agreement with previous studies on *S. aureus* isolated from raw sheep's milk (De Santis et al., 2005), the *mecA* gene was not carried in any of the tested strains. The recovery of MRSA strains from half udder milk samples is sporadic and usually they can refer to human contamination (De Santis et al., 2005). The agreement between the antibiotic resistance profile and the detection of the corresponding genes was 100% for E, OX and VA, and it was 94.4% for AM and P. The determination of the antibiotic resistance profile and the population study, allow to understand the source of contamination and to trace it back to each step of cheese-making process, and to determine spreading of strains with the same profile and their persistence within the cheese plant.

7.5. Tables

7.5.1. Table 1. Genotypic characterization and antibiotic susceptibility of 36 *S. aureus* strains isolated from raw sheep's milk cheese.

Table 1. Genotypic characterization and antibiotic susceptibility of 36 *S. aureus* strains isolated from raw sheep's milk cheese.

dairy	batch	*agr*	cluster	genes				Antibiotics						
				mecA	*blaZ*	*vanAB*	*ermA*	AM	P	OX	TE	E	VA	OB
A	L1	III	P1(4)[a]-P2(2)	-	-	-	-	-	-	-	-	-	-	-
	L2			-	-	-	-	-	-	-	-	-	-	-
B	L1	IV	P8(3)	-	-	-	-	-	-	-	-	-	-	-
	L2	III	P1(2)-P7(1)	-	+(1)	-	-	+(1)	+(1)	-	-	-	-	-
C	L1	I	nt*(3)	-	-	-	-	-	-	-	+(3)	-	-	-
	L2	I(1)-II(2)	nt(1)-P9(2)	-	-	-	-	-	-	-	+(1)	-	-	-
D	L1	II	P3(6)	-	-	-	-	-	-	-	-	-	-	-
	L2	III		-	-	-	-	-	-	-	-	-	-	-
E	L1	III	P4(6)	-	+(3)	-	-	+(3)	+(2)	-	-	-	-	-
	L2			-	+(3)	-	-	+(3)	+(3)	-	-	-	-	-
F	L1	I(1)-II(2)	P6(1)-P10(2)	-	+(2)	-	-	+(3)	+(3)	-	-	-	-	+(1)
	L2	II	P5(1)-P10(2)	-	+(3)	-	-	+(3)	+(3)	-	-	-	-	-

* nt= non-typeable; +=resistant; -= sensitive; [a]= number of the strains

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CHAPTER 8

Virulence factors and genetic variability of *Staphylococcus aureus* strains isolated from raw sheep's milk cheese.

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Virulence factors and genetic variability of *Staphylococcus aureus* strains isolated from raw sheep's milk cheese.

8.1. Introduction

Staphylococcus aureus is one of the most common causative agent of food poisoning associated with the consumption of raw milk cheese (De Buyser et al., 2001). Contamination of raw milk and dairy products with *S. aureus* arises at different stages of the food chain. Main sources of contamination of raw milk at farm level are animal skin, mucosal surfaces, infected glands, milking equipment, milkers' hands and environment (Bergonier et al., 2003). In cheese-making plants product contamination origins from food handlers, food-contact and non-contact surfaces. *S. aureus* growth could occur during storage of milk under temperature abuse or in the first cheese-making steps, if the microorganism is not inhibited by the activity of lactic acid bacteria (Charlier et al., 2009). The maximum level of *S. aureus* contamination in raw milk cheese has been observed in the first few hours after production (Jakobsen et al., 2011). Foodborne disease is associated with enterotoxins production and requires an increase of *S. aureus* counts above 10^5-10^6 cfu/g (Ertas et al., 2010). *S. aureus* is a common pathogen for human and animals, and its virulence depends on a large array of factors mainly extracellular proteins, such as enzymes and exotoxins, that contribute to cause disease (Haveri et al., 2007). *S. aureus* strains can harbor different virulence genes coding for Staphylococcal Enterotoxins (SEs), leukocidins, exfoliatins, haemolysins, Toxic Shock Syndrome Toxin 1 (TSST-1), accessory gene regulator alleles (*agr*) and antibiotic resistance. Among the factors contributing to the virulence of this pathogen, antibiotic resistance plays an important role. *S. aureus* strains can be characterized by single drug and multiple antibiotic resistance and they represent a major threat to public health, particularly methicillin-resistant *S. aureus* (Pereira et al., 2009). The presence or the absence of these genes is essential to determine the potential virulence of the strains. The pattern of virulence genes and the genetic polymorphism can be used to determine the biovar and the relationship with the origin of the isolates (Hennekinne et al., 2003). Several *S. aureus* biovars

have been described according to their host specificity. Differences in the virulence factor profiles allow to discriminate between *S. aureus* strains of animal and human origin (Bhatia & Zahoor, 2007; Larsen et al., 2000). The aim of this study was to characterize *S. aureus* isolated from raw sheep's milk cheese based on the presence of the genes coding for different virulence factors and to test the susceptibility to antimicrobial agents. The study was also intended to investigate genetic differences between *S. aureus* strains isolated from different dairy plant and between animal and human biovar strains.

8.2. Material and methods

8.2.1. Cheese sampling

From 10 artisan sheep cheese dairies located in Sardinia (Italy), 20 cheese wheels representative of two different production batches, were collected 6 hours after molding. The cheese-making process differed among dairies mainly for the rennet (calf or lamb rennet) and starter culture (selected, natural or none).

8.2.2. Identification of S. aureus

Samples were processed for the detection and enumeration of Coagulase Positive Staphylococci (CPS), according to ISO 6888-1/2 (1999a, 1999b). From each positive sample, 5 typical CPS colonies were tested using standard microbiological procedures such as Gram staining, catalase and oxidase reactions, free and bound coagulase and TDNase. Strains were also streaked on blood agar plates to test haemolytic activity. Identification of *S. aureus* was confirmed with biochemical test API ID 32 STAPH (bioMérieux, Lyon, France) and the presence of *S. aureus gyrA* gene (Vandenesch et al., 2003). After identification, strains were stored at -80 °C in Brain Heart Infusion Broth (Oxoid, Basingstoke, UK) with glycerol (15% v/v).

8.2.3. Detection of virulence genes by PCR

Total genomic DNA was extracted using a standard phenol-chlorophorm-isoamyl alcohol (25:24:1) procedure with the addition of 1.5 mg/mL lysostaphin (Sigma-Aldrich, St. Louis,

USA) and 20 mg/mL proteinase K (Invitrogen Ltd, Paisley, UK), as described by De Buyser et al. (1989). After extraction, DNA concentration was estimated spectrophotometrically (Shimadzu, Duisburg, Germany). Amplification of the genes coding for *gyrA*, *agr* (I-IV), SEs (*sea-see*), SEs-like (*seh, sek, sel, sem, seo, sep*) and other virulence factors genes such as leukocidins (*pvl, lukM, lukE/D,*), haemolysins (*hlb, hlg, hlgv*), exfoliatins (*eta, etb, etd),* epidermal cell differentiation inhibitor genes (*edinABC*), toxic shock syndrome toxin-1 (*tst*) was carried out using primers described by Jarraud et al. (2000; 2002). Primers used to amplify different genes coding for antibiotic resistance (*mecA*, *blaZ*, *vanAB*, *ermA*, *tetL*, *tetM*, *tetS*, *tetW*) are reported in table 1. 5 µL of the extracted DNA were added to PCR mixture containing 1X PCR buffer, 5 mM $MgCl_2$, 0.125 mM of each dNTP, 1.5 µM of forward and reverse primers (Invitrogen Ltd, Paisley, UK) and 2U/µL of taq polymerase (Promega Corp, Madison, USA). Amplification of the genes coding for virulence factors and antibiotic resistance was performed as described by Vandenesch et al. (2003), using Thermal Cycler Gene Amp PCR 9700 (Applied Biosystems, Carlsbad, USA). 5 µL of the PCR product were loaded into agarose gel (Invitrogen Ltd, Paisley, UK) and submitted to electrophoresis. Amplicons were visualized using an UV trans-illuminator (Gel Doc XR, Bio-Rad Laboratories, Hercules, USA) and gel images were analyzed with Quantity One software (Bio-Rad Laboratories, Hercules, USA).

8.2.4. Virulence pattern

The presence of a given gene was coded as "1", and its absence as "0". The linear combination of the binary response for each gene was used to obtain the pathogenicity profile of the isolates. The relationship between genes coding for SEs, other virulence factors and the host has been previously investigated (Bania et al., 2005; Dinges et al., 2000; Fitzgerald et al., 2001; Fueyo et al., 2001). It was used to classify the strains into four different biovars and to estimate the most likely origin of the contamination of cheese samples. The genetic profile was defined as "animal" biovar with two subgroups "enterotoxigenic" when positive for *sec* and/or *sel* genes and "non-enterotoxigenic " if positive for *lukE/D*, *lukM*, *hlb*, *hlgv* genes, "human" biovar when

carrying *sea*, *sem*, *seo*, *hlg* genes. “Non-host specific” biovar were the strains sharing *hlb*, *hlg*, *hlgv* genes.

8.2.5. Pulsed-Field Gel Electrophoresis (PFGE)

Population study was carried out according to the PFGE harmonization protocol proposed by Murchan et al., (2003). Genomic DNA was digested using *smaI* restriction enzyme (Invitrogen Ltd, Paisley UK). Electrophoretic run was performed in Tris Borate EDTA (TBE) 0.5X using CHEF Mapper XA System (Bio-Rad Laboratories, Hercules, USA). Plugs were loaded into 0.8% PFGE agarose gel (Bio-Rad Laboratories, Hercules, USA) with the addition of lysosthaphin (1.5 mg/mL). *S. aureus* strain NCTC 8325 was used as reference standard. After run, gels were stained with ethidium bromide and visualized with UV trans-illuminator. Gel images were analyzed with GelCompar II software version 6.0 (Applied Math, Sint-Martens-Latem, Belgium) using the Pearson’s correlation coefficient with 1.0% optimization setting and represented by Unweighted Pair Group Method using Arithmetic averages (UPGMA). PFGE clusters were obtained using a cut off of 70% of similarity. Gels were also analyzed by visual examination of the banding patterns to differentiate the strains between undistinguishable, closely related, possibility related or unrelated (Tenover et al., 1995). The presence of a band representative of a restriction site was coded as "1" while its absence as "0".

8.2.6. Susceptibility to antimicrobials

The minimum inhibitory concentration (MIC) of Ampicillin (AM), Cephalothin (CF), Cefoperazone (CFP), Cloxacillin (OB), Erythromycin (E), Oxacillin (OX), Penicillin (P), Tetracycline (TE) and Vancomycin (VA) antibiotics (Sigma-Aldrich, St. Louis, USA), was determined using the broth microdiluition method (CLSI, 2006a; 2006b). Reference strains *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 seved as quality control. For each antibiotic MIC_{50}, MIC_{90}, mode and range were computed.

8.2.7. Statistical analysis

Fisher's exact test was used to investigate pair-wise association between genes within the enterotoxins group. Association between antibiotic resistance genes and their expression was also investigated. Significance was defined as $P < 0.05$. Statistical analysis was conducted using Statgraphics Centurion XVI software. The binary codes obtained from PCR and PFGE analysis were used to perform Analysis of Molecular Variance, AMOVA (Excoffier et al., 1992) for partitioning the genetic variability of *S. aureus* strains into within and among dairy plant components. AMOVA analysis was also performed to estimate the genetic variation accountable to the defined biovar. Analysis was conducted using Arlequine software (Excoffier & Lischer, 2010).

8.3. Results

8.3.1. Identification of S. aureus

All 20 cheese samples were positive for CPS contamination. The 100 CPS strains originating from the cheese were identified as *S. aureus*. Counts ($\bar{x}$ ± SD) were 4.8 ± 1.0 $\log_{10}$ cfu/g and ranged between 2.3-6.4 $\log_{10}$ cfu/g.

8.3.2. Virulence patterns

Genes coding for SEs B, D, E, H, K, P, Panton Valentine Leukocidin and exofoliatins A, B and D were never detected. The strains carrying one or more genes for the production of SEs and other virulence factor are summarized in table 2. The gene encoding SEC was found in 23 strains and it was associated with *sel* and *tst* genes ($P < 0.001$). In 7 out of 10 strains *sea* gene was associated with *sem* and *seo* genes ($P < 0.001$). Linear combination of the binary codes for the presence or absence of the investigated genes allowed to obtain 28 different pathogenicity profiles.

8.3.3. PFGE patterns

PFGE analysis conducted on the strains revealed 15 different clusters (CLs). Six strains, isolated from 1 dairy were not typeable. The CL2 was the most represented pattern, with 22 strains isolated from 4 different dairies, followed by CL1 and CL4, with 13 and 11 strains, respectively. In 13 out of 15 cases, clusters included strains collected from 1 to 2 dairies. One dairy showed a greater variability, as compared to the other dairies, with strains distributed in 5 different CLs. Visual examination of PFGE banding pattern allowed to demonstrate that the strains were closely related, except one, which was possible related.

8.3.4. Antibiotic resistance

Relationship between genes encoding antibiotics resistance and their expression are shown in table 3. All the strains were susceptible to CF, CFP and OX. In 19 strains, isolated from 4 dairies, was observed resistance to AM and P, while 1 was resistant to AM, P and OB. The presence of the *blaZ* gene was statistically associated with the resistance to AM and P ($P < 0.001$). The presence of the *tetM* gene was detected in 21 strains, 6 of which showed in vitro resistance to TE. All the strains that showed resistance to TE were not typeable by PFGE. *TetL*, *tetS* and *tetW* genes were detected in 7 strains isolated from 3 different dairies, although these strains did not show resistance to TE. All the selected *S. aureus* strains did not carry *erm* and *vanAB* genes and were susceptible to E and VA. None of the strains showed the *mecA* gene coding for methicillin resistance.

8.3.5. Genetic structure of S. aureus strains

The distribution of genetic variation of *S. aureus* strains computed on PFGE and PCR analysis showed that great part of the variability was accountable to diversity among dairies (table 4). The "animal" biovar was the most represented with 57 strains included in the "non-enterotoxigenic" profile and 24 strains in the "enterotoxigenic" profile. The "human" biovar was attributable to 16 strains while 3 strains were classified as "non-host specific". Overall the

genetic distance between “animal” and “human” biovars was high with F_{ST} values ranging from 0.84768 (“animal enterotoxigenic” versus “human”) to 0.70952 (“animal non-enterotoxigenic” versus “human”). The greater genetic diversity was observed between “animal enterotoxigenic” and “non-host specific” strains (F_{ST} = 0.89514). Intermediate distance was observed between “animal non-enterotoxigenic” and “human” as compared to “non-host specific” (F_{ST} = 0.57275 and F_{ST} = 0.53280 respectively). Pair-wise comparison of genetic distance between the biotypes were all significant (P<0.001).

8.4. Discussion

The present study was aimed to characterize the virulence factors and genetic variability of *S. aureus* strains isolated from raw sheep’s milk cheese. The virulence profile of the strains was also evaluated in order to assess whether the contamination was of human or animal origin. All cheese samples were contaminated and in 50% of the cases *S. aureus* exceeded the EU microbiological criteria, posing a potential risk to human health. Previous studies, reported prevalence of *S. aureus* contamination >10^5 cfu/g in sheep’s raw milk cheese ranging from 6 to 25% (Ertas et al., 2010; Giammanco et al., 2011). Among the isolated strains the virulence pattern analysis showed a wide variability. Some of the virulence factors investigated can be considered important determinants for host-pathogen relationship providing information that allow to trace the most probable source of the contamination. The high prevalence of strains carrying *sec, sel, tst* genes, either alone or combined with *hlb* gene, observed in the current research, suggests the animal origin of the isolates (Fitzgerald et al., 2001; Ohkura et al., 2009). The *agr* regulator locus is another important factor to define correlation between *S. aureus* and its host. The majority of the strains (40%) carried the *agr* group *III* allele, frequently observed in *S. aureus* strains of ovine origin (Vautor et al., 2007). Population study conducted by PFGE showed that clusters were mainly constituted by strains isolated from the same dairy, thus indicating that great part of the genetic variability was among dairies. The importance of the dairy as component explaining the genetic polymorphism of *S. aureus* strains was also

confirmed by AMOVA analysis. The lower genetic polymorphism observed in each dairy could be related with the selection of resident strains well adapted to colonize farm environment (Smith et al., 2005). Sources of introduction of new strains into a farm are represented by personnel, moving of animals, trading of milk and other supplies. This picture is seldom in farmstead cheese productions, making it more difficult the occurrence of cross contamination of strains from one farm to another. Contamination of cheese with *S. aureus* is usually of animal origin although food handlers have been reported to serve as a potential source of the pathogenic bacteria (Callon et al., 2008). Overall 81% of the strains were reconducted to the "animal" biotype, 16% of the strains were linked to the "human" biotype while only 3% of the isolates were "non-host specific". Previous study carried out on *S. aureus* isolated from milk and dairy products reported the presence of strains not associated with a single host species (Kapur et al., 1995). In the present research the strains defined as "non-host specific" were genetically closer to "human" than to "animal" profile. The high prevalence of "animal biotype" strains recovered from raw milk sheep's cheeses was in agreement with the origin of the strains and demonstrated the feasibility of using the genetic profile to estimate the source of contamination. Investigation on antibiotic resistance showed a low prevalence of resistant strains to antibiotics used in veterinary practice. When antibiotic resistance was observed, it was limited to a single class of antibiotics. An overall strict association existed between the presence of genes encoding antibiotic resistance and *in vitro* susceptibility. Despite this association, one strain, *mecA*- and *blaZ*-, showed resistance to cloxacillin. This could be due by an alteration of a Penicillin Binding Protein (PBP), not PBP2a or PBP2' correlated. MRSA strains were not detected, as previously observed on isolates of ovine origin (De Santis et al., 2005). Since multiple resistance and methicillin resistance is typical of human strains, their absence supports our hypothesis that the great part of the isolates were of animal origin. Combination of genotypic and phenotypic methods used contributes to trace the host origin of the strains. The virulence pattern observed in most of the isolates, lead us to conclude that the contamination of

traditional and artisan sheep cheese with *S. aureus* is likely of animal origin and that the detection of human biotype is sporadic.

8.5. Tables

8.5.1. Table 1. Primers used to detect antibiotic resistance genes.

8.5.2. Table 2. Distribution of accessory regulator and virulence genes of *S. aureus* strains.

8.5.3. Table 3. Distribution of antibiotic resistance genes in *S. aureus* strains and their *in vitro* antimicrobial activity.

8.5.4. Table 4. AMOVA analysis on PFGE and PCR characterization of 100 *S. aureus* strains isolated from raw sheep's milk cheese.

8.6. Figures

8.6.1. Figure 1. Dendrogram of *S. aureus* strain isolated from raw sheep's milk cheese and their virulence profiles.

Table 1. Primers used to detect antibiotic resistance genes.

gene target	resistance	sequence (5'-3')	length (bp)	reference
mecA	Oxacillin	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	533	Vandenesch et al. (2003)
ermA	Erythromycin	TCTAAAAAGCATGTAAAAGAA CTTCGATAGTTTATTAATATTAGT	645	Vancraeynest et al. (2004)
blaZ	β-lattamic	TCAAACAGTTCACATGCC TTCATTACACTCTGGCG	792	Rosato et al. (2003)
vanA	Vancomycin	CATGACGTATCGGTAAAATC ACCGGGCAGRGTATTGAC	832	Patel et al. (1997)
vanB		CATGATGTGTCGGTAAAATC ACCGGGCAGRGTATTGAC	832	
tetL	Tetracycline	ATAAATTGTTTCGGGTCGGTAAT AACCAGCCAACTAATGACAATGAT	696	Trzcinsky et al. (2000)
tetM		ACAGAAAGCTTATTATATAAC TGGCGTGTCTATGATGTTCAC	740	Aminov et al. (2001)
tetS		GAAAGCTTACTATACAGTAGC AGGAGTATCTACAATATTTAC	169	
tetW		GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	

Table 2. Distribution of accessory regulator and virulence genes of *S. aureus* strains.

	acessory gene regulator allels				virulence factors genes											
dairy	*agrI*	*agrII*	*agrIII*	*agrIV*	*sea*	*sec*	*sel*	*sem*	*seo*	*tst*	*LukE/D*	*lukM*	*edinABC*	*hlb*	*hlg*	*hlgv*
A	-	-	10	-	-	10	10	-	-	10	10	10	8	10	-	10
B	1	-	4	5	1	3	3	-	-	3	-	-	4	9	1	1
C	5	5	-	-	-	-	-	-	-	-	5	5	-	9	3	8
D	4	-	6	-	-	10	10	-	-	10	10	10	10	10	-	10
E	-	10	-	-	-	-	-	-	-	-	10	-	-	-	-	10
F	1	-	9	-	-	-	-	-	-	-	10	10	8	8	-	8
G	5	-	-	5	-	-	-	5	5	-	5	-	-	5	5	5
H	1	-	9	-	9	-	2	7	7	6	3	-	-	2	8	3
I	-	10	-	-	-	-	-	-	-	-	9	-	-	-	-	10
L	-	9	1	-	-	-	-	-	-	-	10	1	1	3	-	10

Table 3. Distribution of antibiotic resistance genes in *S. aureus* strains and their *in vitro* antimicrobial activity.

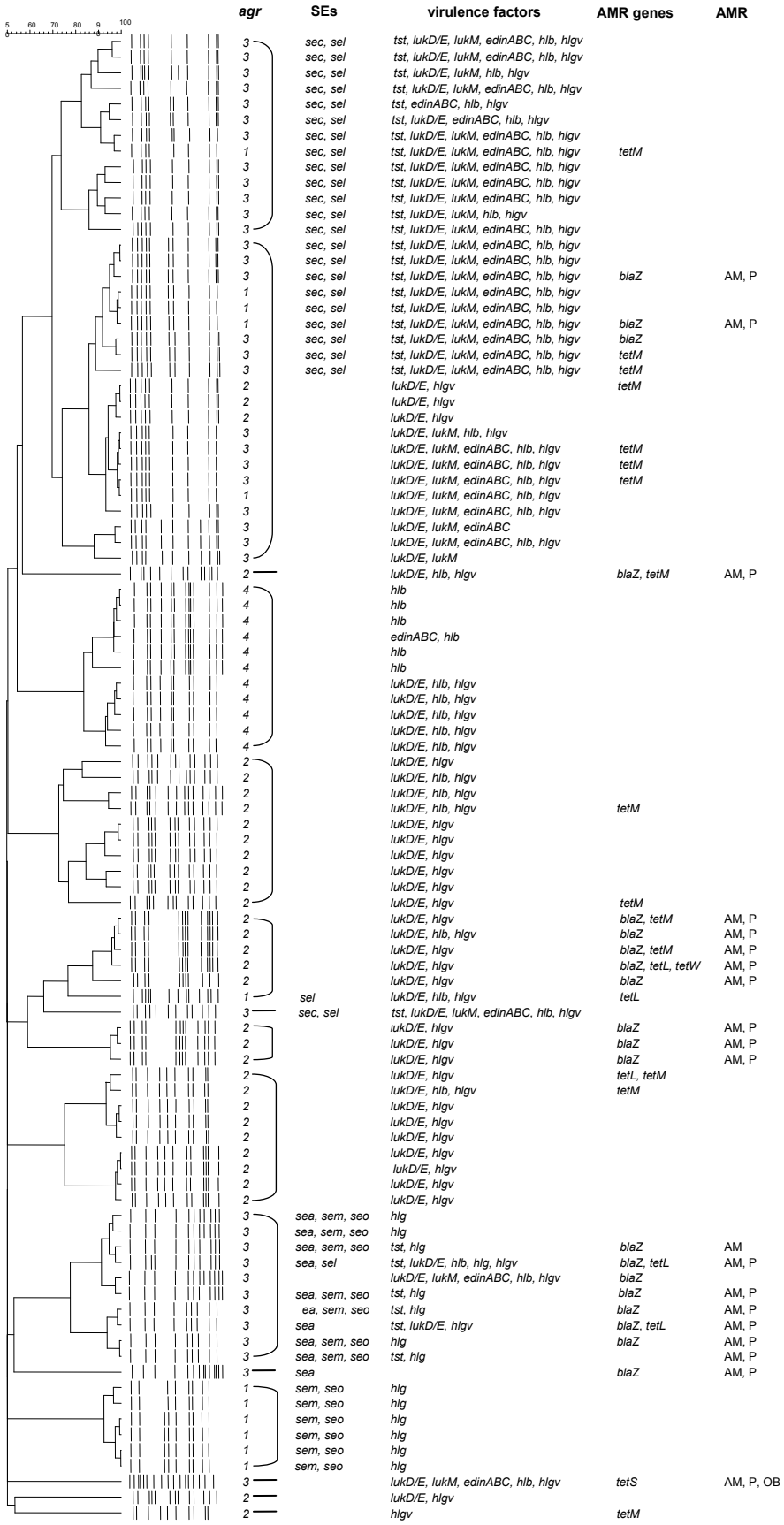
	antibiotic resistance genes								antibiotic resistance								
dairy	*mecA*	*blaZ*	*vanAB*	*ermA*	*tetL*	*tetM*	*tetS*	*tetW*	AM	P	OX	CF	E	VA	OB	CFP	TE
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	-	1	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-
C	-	-	-	-	-	7	-	-	-	-	-	-	-	-	-	-	6
D	-	1	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
H	-	8	-	-	3	-	-	-	9	8	-	-	-	-	-	-	-
I	-	1	-	-	1	3	-	-	1	1	-	-	-	-	-	-	-
L	-	8	-	-	1	3	1	1	9	9	-	-	-	-	1	-	-

AM, ampicillin; P, penicillin; OX, oxacillin; CF, cefoperazone; E, erythromycin; VA, vancomycin; OB, cloxacillin; CFP, cephalothin; TE, tetracycline.

Table 4. AMOVA analysis on PFGE and PCR characterization of 100 *S. aureus* strains isolated from raw sheep's milk cheese.

type of data	source of variation	degrees of freedom	sum of squares	variance components	percentage of variation	Fixation index (F_{ST})	p-value
PFGE	Among dairies	9	274.421	2.95043	50.7	0.50689	<0.001
	Within dairies	84	241.100	2.87024	49.3		
	Total	93	515.521	5.82066			
PCR	Among dairies	9	189.088	1.97992	66.1	0.66121	<0.001
	Within dairies	84	92.318	1.01449	33.9		
	Total	93	281.406	2.99441			

Figure 1. Dendrogram of 94 *S. aureus* strain isolated from raw sheep's milk cheese and their virulence and antibiotic resistance profiles.



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CHAPTER 9

General conclusion

General conclusion

The main objectives of the present thesis were to investigate the impact on human health of *Staphylococcus aureus* along small ruminant dairy supply chain. The rate of risk depends on the prevalence of raw milk and related products contamination, on the virulence determinants and on the antibiotic resistance pattern of *S. aureus* strains.

Raw milk can be contaminated with *S. aureus* shed in the milk during intramammary infection. Although the prevalence of staphylococcal subclinical infection in dairy goat's is relative high, *S. aureus* was accountable only for approximately 5% of the cases. These strains can have a direct effect on human health, related to enterotoxins production, or indirect, related to resistance to antimicrobial agents. The result presented in chapter 3 showed low prevalence of *S. aureus* resistant strains. In most of the cases was observed resistance against a single drug and in the very few cases of multiple drug resistance this was associated with coagulase negative staphylococci. Great concern exist on the possible presence of methicillin strain isolated from animals. However, no *in vitro* resistance nor presence of *mecA* gene was demonstrated on the isolated strains. During storage of raw milk *S. aureus* can growth and reach level potentially harmful due to enterotoxins production. The studies presented in chapter 4 and 5 were aimed to assess the pathogenicity profile of *S. aureus* recoverd from goat's bulk tank milk. The prevalence of *S. aureus* was around 80%, demonstrating that in bulk tank milk the risk of contamination was higher than the level observed in milk collected from individual animals. Although bulk tank milk was characterized by high prevalence, the presence of at least one genes encoding enterotoxins was observed in 40% of the isolates. Antibiotic resistance was mainly directed to a single drug, while multiple resistance was never detected. Likewise the strains isolated from half udder milk samples, the presence of *mecA* gene was never observed.

Chapter 6 was conducted to estimate the prevalence of *S. aureus* contamination in raw milk sheep's cheese. The detection of the pathogen was conducted at the time during the manufacturing process when the number of staphylococci is expected to be highest

(acidification). Although 50% of the cheese samples showed CPS counts > 10^5 cfu/g none of them was positive for the presence of preformed enterotoxins.

In chapter 7 and 8 *S. aureus* strains isolated from raw sheep's milk cheese were extensively characterized for the virulence and antibiotic resistance patterns. The profiles obtained were used to relate the pathogen with the host origin. Most of the strains were characterized by a pattern that could be linked with animal origin. The presence in cheese of strains belonging to an animal profile suggests that the contamination is not of human origin.