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## Invited Review

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**Abstract**

Staphylococci have been isolated from various sites of the body of healthy sheep, as well as from many infections of those animals, the main one being mastitis. The objective of this review is to appraise the importance and significance of staphylococci in causing mastitis in ewes. The review includes a brief classification and taxonomy of staphylococci and describes the procedures for their isolation and identification, as well as their virulence determinants and the mechanisms of resistance to antibacterial agents. Various staphylococcal species have been implicated in staphylococcal mastitis and the characteristics of isolates are discussed with regards to potential virulence factors. Staphylococcal mastitis is explicitly described, with reference to sources of infection, the course of the disease and the relevant control measures. Finally, the potential significance of staphylococci present in ewes' milk for public health is discussed briefly.

Staphylococcal mastitis in sheep was first studied towards the end of the 19<sup>th</sup> century by the French veterinarian and microbiologist Edmund Nocard, who described the experience of a practicing veterinarian of the time as follows: 'This disease is rightly considered to be the plague of the cheese-making flocks; it is not rare to see one tenth of the animals affected by the terrible evil; those, which recover, are invariably lost for milk-production. All the treatments have been tried equally unsuccessfully; this, what the shepherds have found the best to save the animal, is to cut open the udder in different directions from the start of the disease and to treat the wounds with any detergent lotion. The majority of veterinarians of the country consider this disease as a simple mastitis, caused by the milk engorgement and the knocks given on the mammary gland by the milkers; but the owners refuse to believe in these possible causes of the disease and the majority of them thinks that this mastitis is related to anthrax. This opinion should be rejected, because the animals never exhibit, during their life or after their death, the signs or lesions of anthrax; microscopic examination of the blood has never allowed me to see bacteria ...' (Nocard, 1887).

Nocard (1887) described staphylococcal mastitis from pathological and microbiological examinations. He isolated the causative microorganism from the milk of an affected ewe and described its morphological, cultural and biochemical characteristics. Also, he studied the effects of experimental intramammary inoculation of mammary secretion from an affected mammary gland, by means of which he successfully reproduced a pathological condition similar to the natural disease. Thereafter, Bridre (1907) undertook a systematic investigation of the disease in dairy flocks in France. He found that the incidence of the disease was approximately 5%, with a mortality of 20%. Further, he attempted to protect sheep by means of immunisation.

In general, the onset and outcome of staphylococcal infections are dependent on the combination of the virulence of the invading isolate and the defence abilities of the host. Protection of a host against staphylococci is to a large degree dependent upon (a) the integrity of the skin and mucous membranes, which form an important barrier to entrance of staphylococci into the body, and (b) the number and functionality of leucocytes, which are important for phagocytosing and destroying the invading bacteria (Murray *et al.*, 2008). Moreover, recent work has also presented a role for anti-staphylococcal antibodies, which seem to clear staphylococcal isolates more effectively (Vasileiou, 2019, Vasileiou *et al.*, 2019a).

Staphylococcal infections are usually preceded by colonisation and are characterised by intense tissue inflammation. They occur when the bacteria enter through skin or mucous membrane breakdowns (e.g. injuries, ulcerations, surgical incisions). The invading bacteria multiply locally and produce enzymes and toxins, leading in tissue destruction, influx of polymorphonuclear cells, severe tissue damage and abscess formation, which characterise the staphylococcal infections (Lowy, 1998). By means of this mechanism and in accord with the characteristics of staphylococci, local infections occur initially at the point of entry. Thereafter, more severe infections, consequently to bacterial dissemination in tissues or haematogenously can occur in other areas of the body of the host.

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**Table 1.** Clustering of staphylococcal species into 11 groups based on results of 16s rRNA sequencing

Group	Species
<i>S. aureus</i>	<i>S. anaerobius</i> (coagulase +ve), <i>S. argenteus</i> , <i>S. aureus</i> (coagulase +ve), <i>S. schweitzeri</i> , <i>S. simiae</i> – possible classification: <i>S. aureus</i> subsp. <i>anaerobius</i> and <i>S. aureus</i> subsp. <i>aureus</i> (both coagulase +ve)
<i>S. auricularis</i>	<i>S. auricularis</i>
<i>S. carnosus</i>	<i>S. carnosus</i> , <i>S. condimenti</i> , <i>S. massiliensis</i> , <i>S. piscifermentans</i>
<i>S. epidermidis</i>	<i>S. capitis</i> , <i>S. caprae</i> , <i>S. epidermidis</i> , <i>S. saccharolyticus</i>
<i>S. haemolyticus</i>	<i>S. devriesei</i> , <i>S. haemolyticus</i> , <i>S. hominis</i> (subspecies <i>hominis</i> novobiocin sensitive – subspecies <i>novobiosepticus</i> novobiocin resistant)
<i>S. hyicus</i> – <i>intermedius</i>	<i>S. agnetis</i> , <i>S. chromogenes</i> , <i>S. cornubiensis</i> , <i>S. felis</i> , <i>S. delphini</i> (coagulase +ve), <i>S. hyicus</i> (coagulase +ve), <i>S. intermedius</i> (coagulase +ve), <i>S. lutrae</i> (coagulase +ve), <i>S. microti</i> , <i>S. muscae</i> , <i>S. pseudintermedius</i> (coagulase +ve), <i>S. rostri</i> , <i>S. schleiferi</i> subsp. <i>coagulans</i> (coagulase +ve) and subsp. <i>schleiferi</i> (coagulase –ve)
<i>S. lugdunensis</i>	<i>S. lugdunensis</i>
<i>S. saprophyticus</i> (novobiocin-resistant species)	<i>S. arlettae</i> , <i>S. caeli</i> , <i>S. cohnii</i> , <i>S. edaphicus</i> , <i>S. equorum</i> , <i>S. gallinarum</i> , <i>S. kloosii</i> , <i>S. nepalensis</i> , <i>S. saprophyticus</i> , <i>S. succinus</i> , <i>S. xylosus</i>
<i>S. sciuri</i> (novobiocin-resistant, oxidase-positive species)	<i>S. fleurettii</i> , <i>S. lentus</i> , <i>S. sciuri</i> , <i>S. stepanovicii</i> , <i>S. vitulinus</i> (synonym to <i>S. pulvereri</i> )
<i>S. simulans</i>	<i>S. simulans</i>
<i>S. warneri</i>	<i>S. pasteurii</i> , <i>S. warneri</i>
Not clustered in any group	<i>S. argensis</i> , <i>S. petrasii</i> , <i>S. pettenkoferi</i>

Note. Reports regarding other species, e.g. *S. leei* (coagulase +ve), *S. lyticans*, *S. pseudolugdunensis*, have also been published, but their valid taxonomic status has not yet been confirmed. Source: Euzebay (1997).

Staphylococci have been isolated from various sites of the body of healthy sheep (online Supplementary Table S1). In sheep, the most important problem caused by staphylococci is mastitis, clinical or subclinical. Moreover, the bacteria have been implicated also in various other disorders (online Supplementary Table S1), the significance of which varies. Some of these occur frequently, e.g. impetigo or abscesses; some sporadically, e.g. vaginal infections or abortion; whilst others have been described only experimentally, e.g. osteomyelitis or rhinosinusitis.

In dairy sheep flocks, mammary infections have an obvious financial significance (Selvaggi *et al.*, 2017), due to the reduction in milk yield, the downgrading of milk quality and the rejection of milk consequently to antibiotic administration. Mammary infections are important also in meat production flocks, as reduced milk yield of ewes has been shown to lead to suboptimal growth of their lambs (Fthenakis and Jones, 1990a). Other costs associated with the disease include those for replacement animals and the relevant veterinary expenses. Mastitis is also a great welfare concern (European Food Safety Authority, 2014).

The objective of this review is to appraise the importance and significance of staphylococci in mastitis in ewes. Although staphylococci are important as causal agents for the disease, there is a lack of a systematic review of relevant knowledge.

## Methodology

The review includes primarily references published in journals cited in the Web of Knowledge database (<http://www.webofknowledge.com>); papers published in these journals have been refereed. Various search terms have been employed to identify relevant publications (e.g. 'sheep', 'goat\*', 'mastitis', 'somatic cell count\*', 'milk', 'staphylococcus', 'teat', 'carriage', 'carrier'). Subsequently, the full papers have been retrieved through the websites of the respective journals. Citations of other material are scarce; these

include a few references to book chapters and doctoral theses (i.e. material that has been reviewed) and one reference to edited conference material. References cited in the review appear at the end of the review, and additional supporting references are included in the online Supplementary File.

## The microorganism

### Classification – taxonomy

Taxonomically, the genus *Staphylococcus* (*S.*) is classified in the bacterial family Staphylococcaceae (order: Bacillales, class: Bacilli, phylum: Firmicutes, domain: Bacteria). Currently, the genus includes more than 50 species, with many having subspecies. Apart from speciation, various other taxonomic schemes have been proposed for staphylococci, which are based on phenotypic characteristics or molecular findings.

A well-known approach refers to the reaction of staphylococcal species to the coagulase test, which identifies whether a staphylococcal isolate produces the exoenzyme coagulase: staphylococcal species are classified as coagulase-positive or coagulase-negative, with a third class, coagulase-variable, having been established recently. Another approach refers to natural susceptibility of the species to novobiocin: staphylococcal species are classified as novobiocin-sensitive or novobiocin-resistant. Further, clustering into groups based on results of 16s rRNA sequencing has also been performed and 11 groups have been distinguished (Table 1).

### Isolation and identification

The procedure of isolation of staphylococci starts with culture of the clinical samples, which for mastitis are, usually, milk samples collected from ewes under examination or from the bulk milk tank. Less often, other types of samples may need to be processed,

e.g. swabs from mammary lesions or udder skin, or material from the teat duct or mammary tissue samples (Fragkou *et al.*, 2014).

Enrichment of samples is not necessary. They can be inoculated directly onto conventional (e.g. blood agar) or selective (e.g. Chapman's medium) media. The plates should be incubated at 35–37 °C for up to 48 h (Fragkou *et al.*, 2014, Vasileiou *et al.*, 2018b). If no apparent growth has occurred the media can be reincubated for a further 24 h. Due to the tolerance of staphylococci in increased salt concentrations (as high as 10%), mannitol salt agar is an appropriate selective medium (e.g. Chapman's medium) for growth and isolation. Chromogenic agar can be used for the isolation of *S. aureus* (Ariza-Miguel *et al.*, 2015). False negative results may always occur with culture technologies. A prolonged transportation time or inappropriate maintenance of samples can trigger bacterial survival mechanisms, e.g. slime production and biofilm formation. In such cases, the bacteria do not produce obvious colonies when transferred onto agar plates (Skrlin, 2016). Further, in long-standing infections, staphylococci may not grow on solid media (Ehrlich *et al.*, 2014).

Evaluation of the appearance of colonies is the first step in identification of staphylococci. Staphylococcal species usually form distinctive colonies on sheep blood agar, being smooth and butyrous, with a low convex profile and an entire edge (Winn and Koneman, 2006). Most *Staphylococcus aureus* isolates are pale light yellow colonies, due to staphyloxanthin, a carotenoid pigment with a notable role in pathogenicity of the bacteria (Lan *et al.*, 2010). Other species, most often, produce white-coloured colonies.

Microscopically, Gram-staining confirms a Gram-positive microorganism. Staphylococcal colonies are differentiated from other Gram-positive bacteria (e.g. streptococci, enterococci) by the catalase test, which detects cytochrome oxidase enzymes, staphylococci show a positive reaction in the test. This is followed by the coagulase test, which serves to distinguish coagulase-positive isolates (*S. anaerobius*, *S. aureus*, *S. delphini*, *S. hyicus*, *S. intermedius*, *S. lutrae*, *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans*).

Thereafter, identification to species level can be by commercial assays which use modified carbohydrate fermentation tests or adaptations of standard bacteriological identification biochemical tests (Donay *et al.*, 2004), or by molecular identification techniques, e.g. the Polymerase Chain Reaction (PCR) or Matrix-Assisted Laser Desorption/Ionisation-Time of Flight (MALDI-TOF). The latter analyses the protein profile of bacterial cells (Katsafadou *et al.*, 2015).

MALDI-TOF technology allows a greater number of bacterial isolates to be rapidly and accurately identified to species level (Cameron *et al.*, 2018, Hulland *et al.*, 2018). This is particularly relevant for coagulase-negative staphylococci, the identification of which to species level is often omitted and thus these organisms are cumulatively reported to the genus level only.

### Virulence factors

Pathogenicity of staphylococcal infections is based on the virulence factors of the bacteria that allow them to survive within the host and cause relevant damage. The bacteria carry a wide array of potential virulence factors (Table 2), being capable of (i) adhering to host tissues, (ii) avoiding, overcoming or invading the host immune system and (iii) releasing harmful toxins or enzymes (Sahuquillo Arce *et al.*, 2013). During infections, the bacteria produce the respective virulence determinants in a sequence tightly coordinated by relevant regulatory systems. The

expression of both virulence factors and regulatory mechanisms is controlled by specific virulence genes. Factors contributing to the pathogenicity of staphylococci can be classified as (i) bacterial cell surface components (adherence factors) and (ii) secreted variants.

Adherence factors include various proteins which mainly act during the early phase of infection. Their principal function is to facilitate attachment of the bacteria to the host cell surface, simultaneously leading to the cascade host immune response evasion (Foster and Höök, 1998). Various capsular polysaccharides, located on the bacterial cell wall, are involved in the inhibition of phagocytosis by neutrophils, while teichoic acids are implicated in bacterial adherence to the mucosal surface. Most of these are microbial surface components recognising adhesive matrix molecules including fibrinogen, fibronectin and collagens. Staphylococcal protein A (SpA), fibronectin-binding protein A and fibronectin-binding protein B (FnbpA and FnbpB), collagen-binding protein and clumping factor A and B proteins have also been determined to play a keynote role in the virulence of staphylococci (Palmqvist *et al.*, 2002, Heying *et al.*, 2007).

Slime production by staphylococcal isolates contributes to biofilm formation by these bacteria and plays an important role in the pathogenesis of staphylococcal infections and mastitis specifically (Schönborn *et al.*, 2017), particularly at the early stage of the infection, when the bacteria adhere to the mammary epithelial cells. Genes *icaA*, *icaB*, *icaC*, *icaD* are responsible for production of *icaADBC*-encoded polysaccharide intercellular adhesion and *ica*-independent chemically diverse slime (Cramton *et al.*, 1999, 2001). More specifically, the *icaA* gene product is a transmembrane protein which, for optimal action, requires presence of the *icaD* gene product. The *icaC* gene encodes a product contributing to chain formulation of N-acetyl-glucosamine oligomers produced by the *icaAD* combination (Gerke *et al.*, 1998); this product is also considered to be involved in translocation of the polysaccharide on cell surface (Gerke *et al.*, 1998). The *icaB*-encoded protein is considered responsible for the bacterial evasion process from the phagocytosis process (Cerca *et al.*, 2007). Moreover, the *clfa* gene encodes a surface protein, demonstrated as clumping factor A, which has a crucial role in bacterial initial attachment and evasion of host immune responses (Stutz *et al.*, 2011). The *bap* gene also encodes an important surface protein, termed 'biofilm associated protein'; beyond its contribution to initial bacterial attachment, it has also been considered that this protein is capable to induce a polysaccharide intercellular adhesion/poly-N-acetyl glucosamine-independent slime production process, especially on abiotic surfaces (Latasa *et al.*, 2006). Finally, the *eno* gene encodes a laminin-binding surface protein (Carneiro *et al.*, 2004).

Secreted virulence factors are mainly present during the late phase of infection and usually have a more distinct role in microbial pathogenicity (Otto, 2013). Based on their principal activity, secreted virulence determinants are further classified in four categories: (i) super-antigens, (ii) cytolytic toxins, (iii) exoenzymes and (iv) miscellaneous proteins. Tissue shock syndrome toxin-1 (TSST-1) and enterotoxins are the most prominent superantigens, usually causing clinical conditions of increased severity. Cytolytic toxins (i.e.  $\alpha$ -haemolysin,  $\beta$ -haemolysin,  $\gamma$ -haemolysin, toxins of the leucocidin family) are capable of forming pores in the host cell wall, causing the osmotic leakage of cell content and, therefore, lysis of the cell; cytolysis provides the required nutrients for further growth of the bacteria. The extracellular enzymes are produced by most staphylococcal isolates and aim to inactivate

**Table 2.** Virulence factors involved in the pathogenetic role of a typical *S. aureus* isolate

1. Cell wall associated factors	
1.i. Capsular polysaccharides	Impediment of phagocytosis by neutrophils, enhancement of bacterial colonisation and persistence on mucosal surfaces (O'Riordan and Lee, 2004)
1.ii. Microbial surface components recognising adhesive matrix molecules (MSCRAMMs)	
Clumping factor proteins ( <i>ClfA</i> and <i>ClfB</i> )	Regulation of adherence to host tissues and clumping of blood plasma (Lacey <i>et al.</i> , 2017)
Collagen-binding protein ( <i>Cna</i> )	Regulation of bacterial adhesion to host collagenous tissues (Madani <i>et al.</i> , 2017)
Fibronectin-binding proteins ( <i>FnbpA</i> , <i>FnbpB</i> )	Facilitation of attachment to fibronectin and plasma clot (Menzies, 2003)
Staphylococcal protein A ( <i>SpA</i> )	Inhibition of phagocytosis, promotion of immune evasion (Kobayashi and DeLeo, 2013)
1.iii. Staphyloxanthin	Promotion of resistance to reactive oxygen species generated by host neutrophils (Lang <i>et al.</i> , 2000)
2. Secreted factors	
2.i. Cytolytic factors	
Cytolysins	
$\alpha$ -Haemolysin ( $\alpha$ -toxin)	Induction of lysis on monocytes, platelets and brain cells (Berube and Wardenburg, 2013)
$\beta$ -Haemolysins	Induction of lysis of erythrocytes, neutrophils and lymphocytes (Larsen <i>et al.</i> , 2002)
Leucocidin-family of bi-component pore-forming toxins	Disruption and killing of a wide spectrum of leucocytes (Spaan <i>et al.</i> , 2017)
2.ii. Exoenzymes	
Hyaluronidase	Degradation of hyaluronic acid, altering penetration of host cell structures (Ibberson <i>et al.</i> , 2014)
Lipases	Inactivation of fatty acids promoting biofilm formation (Hu <i>et al.</i> , 2012)
Nucleases (Nuc enzymes)	Cleavage of single- or double-stranded DNA and RNA (Kiedrowski <i>et al.</i> , 2014)
Proteases	Inactivation of antimicrobial molecules (Dubin, 2002)
Staphylokinase (SAK)	Inactivation of antimicrobial molecules, promotion of bacterial invasion (Wieckowska-Szakiel <i>et al.</i> , 2007)
2.iii. Superantigens	
Enterotoxins (SE A-G, SEQ)	Stimulation of large populations of T-cells (Pinchuk <i>et al.</i> , 2010)
Toxic shock syndrome toxin-1 (TSST-1)	Stimulation of large populations of T-cells (Silversides <i>et al.</i> , 2010)
2.iv. Miscellaneous proteins	
Chemotaxis inhibitory protein (CHIPS)	Inhibition of chemotaxis of neutrophils (de Haas <i>et al.</i> , 2004)
Extracellular adherence protein (Eap)	Inhibition of neutrophil migration and proliferation (Eisenbeis <i>et al.</i> , 2017)
Extracellular fibrinogen binding protein (Efb)	Inhibition of complement activation and protection against phagocytosis (Ko <i>et al.</i> , 2011)
Formyl peptide receptor-like inhibitory protein (FLIPr)	Inhibition of chemotaxis of neutrophils (Stemerding <i>et al.</i> , 2013)
Staphylococcal complement inhibitor (SCIN)	Inhibition of complement activation (Rooijackers <i>et al.</i> , 2007)

host antimicrobial mechanisms, allowing bacterial dissemination. These exoenzymes include various lipases, nucleases and proteases (i.e. hyaluronidase, serine, cysteine, staphylokinase). Finally, many other proteins have been shown to have a further impact on the host immune system; staphylococcal complement inhibitor protein, extracellular fibrinogen binding protein, chemotaxis inhibitory protein and formyl peptide receptor-like protein-1 inhibitory protein are the ones most frequently detected (Otto, 2013).

Synthesis of these factors occurs during the two growth phases of the bacteria. During the early phase, cell wall-associated factors facilitate tissue attachment and evasion of host defence system, allowing staphylococci to accumulate and, possibly, also form a biofilm. and the late phase, during which bacteria secrete a

spectrum of exoproteins including proteinases, haemolysins and super-antigens, whilst, at the same time, cell wall-associated factors are downregulated, leading to enhancement of the biofilm and bacterial dissemination within the mammary gland (Novick, 2003). The production of the various virulence factors of the bacteria is controlled by various mechanisms, following the general strategy of the microbial adhesion, invasion and proliferation. The function of regulation systems is in response of bacterial cell density (quorum-sensing) and environmental factors (e.g. nutrient availability, pH, temperature, oxygen tension). Moreover, it is also noteworthy that a virulence determinant may be under the influence of several regulatory systems that act synergistically to ensure the appropriate conditions for bacterial survival (Wang and Muir, 2016).

*In vitro* studies have shown that regulation mechanisms can be classified into two major groups: (i) the two-component regulatory systems and (ii) the global transcriptional regulators (Cheung *et al.*, 2004). At nucleotide level, the two-component regulatory systems include the staphylococcal accessory element (*sae* locus) and the accessory gene regulator (*agr* locus). The *sae* locus codes the expression of several virulence determinants, mainly contributing to bacterial adhesion and host immune evasion. The *agr*-coded genes seem to promote the expression of bacterial exoproteins (e.g. TSST-1, enterotoxins, serine proteinase), simultaneously reducing the synthesis of cell-wall associated proteins (e.g. SpA, FnbpA, FnbB). This system was initially described in *S. aureus*, in which four distinct allelic variants, *agrA/B/C/D*, have been sequenced. Subsequently, presence of homologous *agr*-like loci in other staphylococcal species have been detected (Dufour *et al.*, 2002). Most *S. aureus* isolates have a third significant group of virulence genes regulators, usually referred as sigma factors ( $\sigma$ ).

### Mechanisms of resistance to antibacterial agents

A major attribute of most staphylococcal species (including *S. aureus*) is their extended capacity to develop, fairly rapidly, resistance to antimicrobial agents. Nowadays, multi-drug resistant staphylococci (e.g. *S. aureus*, *S. epidermidis*, *S. intermedius*) are commonly recovered from human or animal clinical samples (Mediavilla *et al.*, 2012).

Antimicrobial tolerance or resistance is linked to the genetic background of each individual isolate and develops through mutations and rearrangements within the staphylococcal genome or by acquisition of resistance determinants. The wide spectrum of staphylococcal genetic variants and the increased antibiotic pressure contribute significantly in antibiotic resistance formation. Most resistance determinants of staphylococcal genetic material are in highly volatile areas (e.g. genomic islands, plasmids) promoting the occurrence of mutations leading to antibiotic resistance (Foster, 2017). Further, during co-infections, genetic transfer may also take place and essential resistant components may easily be transferred horizontally. Finally, genetic transfer of resistant genetic elements between bacteria belonging to different species has also been recorded (Haaber *et al.*, 2017).

In general, mechanisms of resistance against antibiotics developed by staphylococci are: (i) relevant modulations of cell wall permeability, (ii) enzymatic inactivation of the antimicrobial agent, (iii) modification of the antibiotic target and (iv) activation of relevant bacterial efflux pumps (McCallum *et al.*, 2010). Depending on specific antimicrobial agents, one or more of these mechanisms may be involved (Pantosti *et al.*, 2007).

$\beta$ -Lactams and glycopeptides are antimicrobial agents that inhibit the formation of the staphylococcal cell wall. The penicillin-binding protein (PBP) 1 and PBP 2 are the target of  $\beta$ -lactams, which inhibit the function of PBPs and, therefore, the formation of an intact cell wall. To overcome the effects of  $\beta$ -lactams, methicillin-resistant *S. aureus* (MRSA) isolates produce a fifth additional PBP named PBP 2a, encoded by the *mecA* gene, which has reduced affinity with  $\beta$ -lactams and remains active in the presence of  $\beta$ -lactams (Lovering *et al.*, 2012). Glycopeptides (e.g. vancomycin) attach to the dipeptide D-ala/D-Ala and inhibit the function of PBP. *S. aureus* isolates with intermediate susceptibility to vancomycin have a remarkably modified architecture on their cell wall, lacking most of the crucial targets of glycopeptides (McGuinness *et al.*, 2017). Regarding resistance to tetracyclines,

the main mechanism is associated with the energy-dependent efflux of tetracycline encoded by the *tetA(K)* and *tetA(L)* genes (Khosravi *et al.*, 2017); recent findings have indicated an association between presence of genes encoding for tetracycline-resistance and for biofilm-formation in staphylococcal isolates (Vasileiou *et al.*, 2019b). With regards to acquisition of resistance to aminoglycosides, resistant isolates are capable to release cytoplasmic aminoglycoside modifying enzymes, which inhibit ribosome binding. Finally, resistance to macrolides and to lincosamides is mainly due to ribosomal modification encoded by the *erm* genes.

## Implication of staphylococci in mastitis in ewes

### Implication in clinical mastitis

*S. aureus* is the primary causal agent of clinical mastitis in ewes. The incidence risk is probably less than 7% across a lactation (Bergonier and Berthelot, 2003, Arsenault *et al.*, 2008). Field reports have indicated that staphylococci have been isolated from up to 70% of cases of clinical mastitis in field investigations in dairy production flocks (Bergonier and Berthelot, 2003, Mork *et al.*, 2007). In meat production flocks, staphylococci have been reported to be less frequent, but still responsible for up to 40% of cases of the disease (Arsenault *et al.*, 2008). Other coagulase-positive staphylococcal species associated with clinical mastitis in ewes include *S. anaerobius*, *S. hyicus*, *S. intermedius* and *S. schleiferi* (Table 3).

Coagulase-negative staphylococci have been isolated from cases of clinical mastitis, although much less frequently than *S. aureus*. These include *S. epidermidis*, *S. saprophyticus*, *S. simulans*, *S. xylosus* and *S. warneri* (Table 3). Further, Fthenakis and Jones (1990b) have reported experimentally induced clinical mastitis caused by *S. chromogenes*.

### Implication in subclinical mastitis

Subclinical mastitis is more common than clinical disease. Criteria employed for definition of subclinical mastitis are important in determining its prevalence; for example, in a field investigation, in which strict criteria had been used, prevalence of subclinical mastitis was 26% (Vasileiou *et al.*, 2018b, 2018c), whilst in other field studies, with less strict criteria, prevalence was found to be as high as 40%, although the accuracy of detection of cases has been questioned (Vasileiou *et al.*, 2018b). It is also noteworthy to cite a report of epidemic occurrence of subclinical mastitis in a flock, where prevalence of the disease has been reported to be 94%, with all cases caused by coagulase-negative staphylococci (Fthenakis *et al.*, 2004).

A clear consensus exists in the literature that coagulase-negative staphylococcal species are the primary aetiological agents of subclinical mastitis and can constitute up to 70% of bacterial isolates from cases of subclinical disease (Gelasakis *et al.*, 2015). The more frequent staphylococcal species recovered from cases of subclinical mastitis were *S. chromogenes*, *S. epidermidis*, *S. simulans* and *S. xylosus*. Other species recovered as aetiological agents of the disease less often include *S. auricularis*, *S. capitis*, *S. caprae*, *S. cohnii*, *S. equorum*, *S. haemolyticus*, *S. hominis*, *S. lentus*, *S. saprophyticus*, *S. sciuri*, *S. schleiferi*, *S. warneri* (Table 3).

*S. aureus* has also been recovered as the causative agent of subclinical mastitis, but much less frequently than from cases of clinical disease (Vasileiou *et al.*, 2018b). Sporadically, *S. aureus* may

**Table 3.** Selected references regarding implication of non-*S. aureus* staphylococcal strains in mastitis or mammary carriage in ewes

Staphylococcal species	References
<i>S. anaerobius</i>	de la Fuente <i>et al.</i> (1993)
<i>S. auricularis</i>	Fthenakis <i>et al.</i> (1994), Ariznabarreta <i>et al.</i> (2002), Kirecci <i>et al.</i> (2009)
<i>S. capitis</i>	Hariharan <i>et al.</i> (2004), Kirecci <i>et al.</i> (2009), Pilipincova <i>et al.</i> (2010)
<i>S. caprae</i>	Kirecci <i>et al.</i> (2009), Onni <i>et al.</i> (2010), Pilipincova <i>et al.</i> (2010)
<i>S. chromogenes</i>	Fthenakis and Jones (1990b), Fthenakis <i>et al.</i> (1994), Winter <i>et al.</i> (2002), Leitner <i>et al.</i> (2003), Mavrogianni <i>et al.</i> (2004), Cuccuru <i>et al.</i> (2011), Persson <i>et al.</i> (2017), Vasileiou <i>et al.</i> (2018a, 2018b)
<i>S. cohnii</i>	Kirecci <i>et al.</i> (2009), Vasileiou <i>et al.</i> (2018a, 2018b)
<i>S. epidermidis</i>	Fthenakis and Jones (1990b), Burriel (1997), Saratsis <i>et al.</i> (1998), Winter and Colditz (2002), Winter <i>et al.</i> (2002), Leitner <i>et al.</i> (2003), Cuccuru <i>et al.</i> (2011), Vasileiou <i>et al.</i> (2018a, 2018b)
<i>S. equorum</i>	Deinhofer and Pernthaner (1993), Hariharan <i>et al.</i> (2004), Persson <i>et al.</i> (2017)
<i>S. haemolyticus</i>	Deinhofer and Pernthaner (1993), Winter and Hofer (1996), Kirecci <i>et al.</i> (2009), Persson <i>et al.</i> (2017)
<i>S. hominis</i>	Ariznabarreta <i>et al.</i> (2002), Pilipincova <i>et al.</i> (2010), Vasileiou <i>et al.</i> (2018a, 2018b)
<i>S. hyicus</i>	Fthenakis <i>et al.</i> (1994), Winter <i>et al.</i> (1999), Ozenc <i>et al.</i> (2011)
<i>S. intermedius</i>	Ariznabarreta <i>et al.</i> (2002), Ergun <i>et al.</i> (2009), Kirecci <i>et al.</i> (2009)
<i>S. lentus</i>	Deinhofer and Pernthaner (1993), Winter and Hofer (1996), Ariznabarreta <i>et al.</i> (2002), Tejada <i>et al.</i> (2012), Vasileiou <i>et al.</i> (2018a, 2018b)
<i>S. lugdunensis</i>	Deinhofer and Pernthaner (1993)
<i>S. saprophyticus</i>	Fthenakis <i>et al.</i> (1994), Ergun <i>et al.</i> (2009), Kirecci <i>et al.</i> (2009)
<i>S. schleiferi</i>	Vasileiou <i>et al.</i> (2018a, 2018b)
<i>S. sciuri</i>	Kirecci <i>et al.</i> (2009), Tejada <i>et al.</i> (2012), Vasileiou <i>et al.</i> (2018a, 2018b)
<i>S. simulans</i>	Fthenakis and Jones (1990a, 1990b); Fragkou <i>et al.</i> (2007), Onni <i>et al.</i> (2010), Cuccuru <i>et al.</i> (2011), Vasileiou <i>et al.</i> (2018a, 2018b)
<i>S. vitulinus</i>	Vasileiou <i>et al.</i> (2018a, 2018b)
<i>S. warneri</i>	Fthenakis <i>et al.</i> (1994), Winter and Hofer (1996), Rupp <i>et al.</i> (2009), Vasileiou <i>et al.</i> (2018a, 2018b)
<i>S. xyloso</i>	Fthenakis and Jones (1990b), Fthenakis <i>et al.</i> (1994), Deinhofer and Pernthaner (1993), Ozenc <i>et al.</i> (2011), Persson <i>et al.</i> (2017)

account for as many as 40% of isolates from cases of clinical mastitis (Al-Majali and Jawabreh, 2003).

### Mammary carriage

The term mammary ‘carriage’ (or ‘carrier state’) (Verhoeven *et al.*, 2014) describes the presence of bacteria in the udder with no increased somatic cell numbers (i.e. in the absence of inflammation). The term refers to bacterial flora present in the teat duct or teat cistern (Fragkou *et al.*, 2007, Mavrogianni *et al.*, 2007). By using culture-independent methods of microbial identification (e.g. MALDI-TOF), recent work performed in cows has suggested the existence of a microbiota in the teat and the lactiferous ducts; a possible source of these bacteria can be the intestine, as it has been found that bacteria from the gut microbiota reach the mammary gland within leucocytes (e.g. dendritic cells, macrophages, lymphocytes) (Rainard, 2017). Vasileiou *et al.* (2018b) have indicated that the prevalence of staphylococcal mammary carriage in ewes was 6.5%. The significance of mammary carriage is that the microorganisms might become pathogenic under the effect of various factors, which decrease defensive efficacy of hosts or promote pathogenicity of bacteria. In a previous experimental study, Fragkou *et al.* (2007) have shown that, under the effect of factors

reducing efficacy of teat defences, staphylococcal carriage could multiply, ascend to the mammary parenchyma and, ultimately, cause clinical mastitis.

Cases of false positive mammary carriage refer to isolation of bacteria at the very early stage of infection before an inflammatory reaction would be elicited (Fthenakis and Jones, 1990b), as well as in cases of contamination of milk samples during collection. Nevertheless, with experienced staff and strict maintenance of aseptic sampling, contamination should be minimal; for example, Rovai *et al.* (2014), in a field investigation, found that only 2% of milk samples from ewes were contaminated.

### Characteristics of isolates with regards to potential virulence factors

Typing of *S. aureus* from cases of clinical mastitis has produced varying results regarding the presence of *agr* locus. Vautor *et al.* (2007) indicated that 70% of isolates belonged to the *agr* group III and 13% to the *agr* group I. In contrast, de Almeida *et al.* (2013) indicated that isolates from clinical mastitis belonged mostly to the *agr* group I (38%) and less often to the *agr* group II (19%), whilst isolates from subclinical mastitis were equally distributed to these two groups (38% in each). Finally, Bar-Gal *et al.*

(2015) reported that all *S. aureus* isolates from mastitis, clinical or subclinical, belonged to the *agr* group I.

Further, there is disagreement regarding the significance of biofilm-forming staphylococci from cases of mastitis in ewes varying from 0% (Azara *et al.*, 2017) to 26% (Vautor *et al.*, 2007) to 40–43% (Ergun *et al.*, 2012) to 91–98% (Tel *et al.*, 2012) of relevant isolates found to form biofilms. However, phenotypic results of biofilm formation depend upon the criteria employed for assessment and, moreover, isolates may not always express a biofilm-forming phenotype, despite carrying genes associated with slime production and biofilm-formation (Vasileiou *et al.*, 2018a). The prevalence of subclinical mastitis caused specifically by biofilm-forming staphylococci (independently of species) was found to be 15.5%, with hand-milking recognised as the most important risk factor (Vasileiou *et al.*, 2018a).

Examination of staphylococci by Multi Locus Sequence Typing also revealed varying and inconsistent findings. Porrero *et al.* (2012) examined *S. aureus* strains from clinical mastitis and found that they belonged to types ST9, ST133, ST1739 and ST2011. de Almeida *et al.* (2013) reported that *S. aureus* strains from clinical mastitis belonged to types ST750, ST1728, ST1729 and ST1730, whilst those from subclinical mastitis to types ST750, ST1728 and ST1729. Finally, Bar-Gal *et al.* (2015) reported that *S. aureus* strains from clinical or subclinical mastitis belonged to types ST133 and ST522. In general, *S. aureus* ST133 is most often isolated from samples of sheep origin (McMillan *et al.*, 2016).

In studies which had investigated production of virulence factors by staphylococci (*S. aureus* or coagulase-negative isolates) from cases of mastitis, various factors or genes encoding for shock syndrome toxin-1 (Orden *et al.*, 1992b, Scherrer *et al.*, 2004), leucocidin (Burriel and Dagnall, 1997), Panton-Valentine toxin (Unal and Cinar, 2012), exfoliative toxins (Mariutti *et al.*, 2015), haemolysins (Azara *et al.*, 2017) and autolysin (Azara *et al.*, 2017) have been detected. The presence of these factors was, in general, more prevalent in *S. aureus* than in coagulase-negative isolates, which can explain the increased pathogenicity of the former.

### Resistance to antibacterial agents of isolates from cases of mastitis in ewes

There is one rule for the effective treatment of mastitis: the combination of speed and efficacy. As soon as mastitis is diagnosed, effective antimicrobial agents should be administered (Mavrogianni *et al.*, 2011). Ideally, and to preserve susceptibility of pathogens to available drugs, treatment should be a narrow spectrum antibiotic known to be effective against staphylococci.

Recent evidence from around Europe does not indicate significant problems of resistance to antibiotics commonly used for cases of mastitis in sheep. Vautor *et al.* (2007) reported only sporadic resistance in *S. aureus* isolated in France. Onni *et al.* (2011), in Italy, also found limited resistance in *S. epidermidis*, except to penicillin, for which the resistance rate was 38%. Similar results have been observed in Turkey, where in coagulase-negative isolates from subclinical mastitis only resistance to  $\beta$ -lactams was noteworthy (43%), whilst there was much smaller frequency of resistance to tetracycline (11%) and even less to other agents (Ergun *et al.*, 2012). Further work in Turkey corroborated those findings, the rate of resistance to penicillin was 27% and to tetracycline 8% (Unal *et al.*, 2012). Martins *et al.* (2017) published similar results; 17% of isolates were resistant to penicillin and

11% to tetracycline. Finally, evidence from Greece was consistent with the above, as the frequency of resistant isolates was <25% for all the antimicrobial agents evaluated (Vasileiou *et al.*, 2019b). Different findings have been reported by Azara *et al.* (2017), who found greater resistance to tetracycline (50%) of *S. aureus* from clinical mastitis. In contrast to the above results, in Brazil, Franca *et al.* (2012) determined a higher resistance to amoxicillin, erythromycin, lincomycin, streptomycin and tetracycline (>35% of staphylococcal isolates tested).

Whilst the above results are indicative of the possibility for effective treatment of clinical mastitis, *in vitro* results of antimicrobial susceptibility do not always correspond with results of *in vivo* administration of an antibacterial agent. Various reasons may account for this discrepancy, e.g. pharmacokinetic limitations during clinical application, inappropriate treatment regime or iatrogenic infection occurring during treatment (Mavrogianni *et al.*, 2011).

## Staphylococcal mastitis in sheep

### Sources of infection

Staphylococci usually disseminate into the mammary gland from the hands of milkers (Marco Melero, 1994, Vasileiou *et al.*, 2018a). The use of bare hands during milking contributes to transfer of bacteria to the teat, when the duct is open.

Other potential sources of bacteria include the nasopharynx of sucking lambs (Gougoulis *et al.*, 2008), staphylococci already present in the teat duct (Fragkou *et al.*, 2007) and staphylococci present on the udder skin (Mavrogianni *et al.*, 2007). Albenzio *et al.* (2003) considered that lambs' mouths and milkers' hands were the major sources of ewe udder and milk contamination, whilst few staphylococci and streptococci were isolated from the teat cups. During suckling, the teat orifice comes into contact with the wall of the buccal cavity of lambs. As Laukova and Marounek (1992) have isolated staphylococci from the upper alimentary tract of lambs, it is evident that the bacteria can transmit to the teat during sucking. As the lower part of the teat comes into contact with the pharynx of the lamb (Titchen, 1977) the bacteria subsequently enter the duct; perhaps; the tongue of the lamb may 'push' the bacteria upwards into the duct. Isolation of the micro-organism after only a short (1 min) sucking activity indicates the speed by which the whole process can take place (Gougoulis *et al.*, 2008). Staphylococci have been found as resident flora of the teat duct of healthy ewes (Fragkou *et al.*, 2007, Mavrogianni *et al.*, 2007). When the microbial equilibrium is disrupted for any reason, it is possible that pathogenicity of the flora isolates increases, leading to invasion of the mammary parenchyma and disease. Further, physicochemical changes occurring of udder skin, e.g. in response to bad weather conditions or harsh teat disinfectants, may create chapping increasing the susceptibility of the mammary gland. In the epidermis, drying results in a lower lipid content thus less antibacterial fatty acids, bacteriostatic salts and proteins, as well as immunoglobulins (Noble and Somerville, 1974). Additionally, the reduced hydration of chapped skin, alters the skin microflora, consequently decreasing resistance to bacterial colonisation (Fox *et al.*, 1991). Chapping removes the acid mantle and increases teat surface area, due to excoriations and fissuring, thus providing additional surface for bacterial attachment. All these increase bacterial populations on the udder skin, thus increasing risk of infection of the mammary parenchyma (Mavrogianni *et al.*, 2007).

Giannakopoulos *et al.* (2019) have modelled for the first time areas of high risk for the development of staphylococcal mastitis, taking into account data regarding prevalence of the disease in the field and environmental and locality conditions, using the ecological niche modelling approach. Whilst the work referred to Greece, there is the possibility to extend the findings to cover other parts of the world.

### Course of the disease

The general principles of mammary defences apply for staphylococcal infections. After successful ascent of staphylococci into the teat cistern and the mammary parenchyma, leucocytes constitute the main line of defence against staphylococci. These are developed immediately upon bacterial entry into the mammary gland, 2–4 h after that and 3–4 d later, respectively (Fthenakis and Jones, 1990b, Fragkou *et al.*, 2010). An increased diameter of mammary vessels, a greater blood volume of blood passing through the gland increase soon after infection, and an increased permeability of the blood–milk barrier, by various mechanisms (e.g. by modulating claudins at the tight mammary junctions), lead to the transportation of an increased amount of blood constituents into the mammary gland (Barbagianni, 2016). Thus, the inflammatory response is developed and sustained. Neutrophils phagocytose bacteria and perform intracellular killing by the rapid release of reactive oxygen species: superoxide radicals and hydrogen peroxide ('respiratory burst') (Van Oostveldt *et al.*, 2002). Neutrophils also release various antibacterial proteins, e.g. cathelicidins, which become available at the mammary parenchyma (Katsafadou *et al.*, 2019).

Neutrophils can also kill staphylococci extracellularly, by means of extracellular traps, which have been described for the first time in sheep by Pisanu *et al.* (2015). These represent a mesh of DNA, histones, antimicrobial proteins and proteinases that entrap and inactivate the invading staphylococci with no direct contact or engulfment necessary (Phillipson and Kubes, 2011).

Interleucins play an important role in the host response, as they regulate the influx of leucocytes in milk after bacterial invasion. Albenzio *et al.* (2012) have reported the relationships between bacteria, leucocytes and interleucins produced after bacterial invasion (TNF- $\alpha$ , IL-8, IL-1 $\beta$ , IL-10, IL-12). Concentrations of TNF- $\alpha$  and IL-12 were higher after invasion of pathogenic bacteria (including staphylococci); IL-8 was associated with somatic cell counts and pathogenic bacteria. Regarding IL-1 $\beta$ , Albenzio *et al.* (2012) have not observed any differences after intramammary staphylococcal challenge, whilst other authors have (Winter and Colditz, 2002, Winter *et al.*, 2003).

Production of virulence factors by the invading staphylococci leads, principally, to destruction of mammary epithelial cells and leucocytes, as well as blood vessels within the mammary parenchyma and consequentially results in intramammary haemorrhages (Fthenakis and Jones, 1990b). Further, biofilm formation allows rapid diffusion of the bacteria, which thus become difficult to control. Occasionally, pathogens that had not been eliminated by the mammary defences pass into the blood circulation. Further, staphylococci can survive in leucocytes, especially if these are not fully functioning, e.g. due to lack of energy in the immediately post-partum period (Fthenakis *et al.*, 2015). This could lead to bacterial accumulations and abscessation within the mammary parenchyma, whence a recrudescence of the infection can ensue (Fthenakis and Jones, 1990b).

The various virulence factors in mastitis-associated staphylococci have not been detected consistently in all isolates tested and there are significant differences even within the various species of the microorganism. This underlines the importance of the host defences in the development and outcome of an intramammary infection. Potential development of clinical mastitis or limitation of the infection to subclinical mastitis depends upon the combination of virulence factors of the invading bacteria and the efficacy of the defence mechanisms of the host. There is now evidence confirming that cellular defences are the determining host factor that may limit a virulent isolate to only cause subclinical mastitis (Barbagianni *et al.*, 2015, Mavrogianni *et al.*, 2017). Cell defences are non-specific, acting against all invading bacteria, including staphylococci. A factor that can affect efficacy of cell defences is the breed of sheep (Riggio and Portolano, 2015). Potentially, subclinical mastitis can convert to clinical, if defences of the host are impaired. Complete recovery requires a fully functioning defence system of the animals, coupled with an effective course of treatment. Other possible outcomes include the development of a long-standing ('chronic') infection with periodic flare-ups of clinical disease, the necrosis of mammary parenchyma, leading to partial or extensive tissue slough-off, the formation of intramammary abscesses with presence of staphylococci therein and the recrudescence of disease. The role of humoral defences has not been studied in depth. Nevertheless, in a recent study, anti-staphylococcal antibodies detected after vaccination of ewes have been found to contribute to improved clearance of staphylococci, but not to fully prevent establishment of the infection (Vasileiou *et al.*, 2019a).

### Vaccination against the disease

As part of udder health management, various measures can be implemented for prevention of mastitis. Most of these have a broad spectrum of efficacy and are relevant for prevention of staphylococcal mastitis as well. Such measures include various management practices, e.g. application of post-milking teat disinfection and correct use and maintenance of milking systems.

Vaccination is a targeted means for prevention of staphylococcal mastitis. In the past, inactivated vaccines with whole staphylococcal cells and/or toxoids have been developed against mastitis (Watson, 1988), offering mainly reduction in severity of clinical signs (not reduction in incidence risk). A vaccine with inactivated *S. aureus*–*S. simulans* whole cells and *S. aureus* exopolysaccharide antigens within liposomes reduced the incidence risk of the disease (Amorena *et al.*, 1994). More recently, Perez *et al.* (2009) described the induction of antibodies against the poly-N-acetyl  $\beta$ -1,6 glucosamine exopolysaccharide, the main component of the extracellular matrix of staphylococcal biofilms. A vaccine has been produced which includes a *S. aureus* bacterin containing bacterial cells surrounded by components of the biofilm matrix. The vaccine elicits an exopolysaccharide specific antibody response offering some protection against *S. aureus* mastitis (Prenafeta *et al.*, 2010) and has contributed to prevention of slime production by staphylococcal isolates, thus limiting their growth and dissemination within and outside the mammary gland. Vasileiou *et al.* (2019a) described the efficacy of this vaccine in reducing the incidence risk of staphylococcal mastitis in an extensive field study, offering protection against *S. aureus* and coagulase-negative staphylococci. Autochthonous staphylococcal vaccines are of limited use in specific flocks and do not have a wide applicability. There are no reports of their efficacy, as they



are produced for 'tailor-made' administration in flocks with perceived problem of staphylococcal mastitis, without first being evaluated for efficacy.

### Public health considerations

Staphylococcal enterotoxins are in a family of over 20 exotoxins, which are related and have a sequence homology (Pinchuk *et al.*, 2010). The enterotoxins can cause significant diseases in people, among these food poisoning and toxic shock syndrome. Enterotoxins are mainly produced by *S. aureus*, although other staphylococcal species may also show enterotoxigenic properties. Staphylococcal food poisoning is an intoxication occurring consequently to consumption of foods containing sufficient amounts of one (or more) pre-formed enterotoxins (Angeles Argudin *et al.*, 2010). Hence, it becomes evident that if milk collected from sheep was subjected to appropriate thermal processing leading to staphylococcal killing there would be no real danger of enterotoxin poisoning from dairy products from that milk. In contrast, the danger would increase sharply with consumption of unprocessed milk, given the frequency of staphylococcal mammary infections, especially as production of enterotoxin has been detected in *S. aureus* isolated from intramammary infections (Orden *et al.*, 1992a, 1992b) or the farm milk tank (Scherrer *et al.*, 2004).

For these same reasons, dairy products have not been considered to be implicated in the dissemination of antimicrobial resistance to consumers of milk. Nevertheless, recent studies have documented that cell-free genetic material from staphylococci resistant to antimicrobial agents, not destroyed during thermal processing of milk, could be transferred to humans (Wang *et al.*, 2012, Schwarz *et al.*, 2017). Given that resistance genes could be incorporated in other bacterial species (e.g. *Streptococcus* spp., *Acinetobacter* spp.), which are part of the normal bacterial flora of humans, dissemination of resistance genes can occur, potentially making staphylococci 'stores' of resistant genes and dairy products a means for their transfer.

### Concluding remarks

Staphylococci are the most frequent mastitis pathogens in ewes. Extensive research has clarified various facets of their pathogenicity for the mammary gland of sheep. Some of that knowledge has been based in relevant work performed in cattle. However, the situation with cattle and sheep differs significantly, not only in terms of differences between the animal species but also between management practices in the respective industries. In relation to ovine mastitis, there are still areas, which have not been fully clarified, whilst further questions have arisen from the findings of recent work.

The identification of a supposed microbiota within the mammary gland has implications in the interpretation of the results of bacteriological testing of milk samples from cases of mastitis; much more work is needed to confirm any, or the absence of a, pathogenic role of bacteria. The microbiota has implications for the immune response of the mammary gland as these bacteria may contribute to a defensive role of the gland.

The recent detection of anti-staphylococcal antibodies in cases of mastitis raises questions regarding their significance in contributing to immunological enhancement of the defence response.

Further, the identification of biofilm-forming staphylococci (independently of species) as causal agents of mastitis, i.e. as an

independent disease entity beyond staphylococcal species, raises issues regarding the dynamics of infection by these organisms. Their presence in milking parlours and relevant factors contributing in their dissemination have not been studied.

Finally, the identification, in dairy products, of cell-free genetic material of strains resistant to antimicrobial agents adds another public health concern. Pasteurised milk had previously been considered of low risk for the transfer of resistance genes, but the new findings indicate a further route for dissemination of such genes. This puts further pressure in maintaining udder health and limiting staphylococcal involvement as a mammary pathogen, with a view to minimising public health concerns.

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### References

- Albenzio M, Taibi L, Caroprese M, De Rosa G, Muscio A and Sevi A (2003) Immune response, udder health and productive traits of machine milked and suckling ewes. *Small Ruminant Research* **48**, 189–200.
- Albenzio M, Caroprese M, Santillo A, Ruggieri D, Ciliberti MG and Sevi A (2012) Immune competence of the mammary gland as affected by somatic cell and pathogenic bacteria in ewes with subclinical mastitis. *Journal of Dairy Science* **95**, 3877–3887.
- Al-Majali AM and Jawabreh S (2003) Period prevalence and aetiology of sub-clinical mastitis in Awassi sheep in southern Jordan. *Small Ruminant Research* **47**, 243–248.
- Amorena B, Baselga R and Albizu I (1994) Use of liposome-immunopotentiated exopolysaccharide as a component of an ovine mastitis staphylococcal vaccine. *Vaccine* **12**, 243–249.
- Angeles Argudin M, Carmen Mendoza M and Rosario Rodicio M (2010) Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins* **2**, 1751–U342.
- Ariza-Miguel J, Oniciuc EA, Sanz I, Fernandez-Natal I, Hernandez M and Rodriguez-Lazaro D (2015) Evaluation of two commercially available chromogenic media for confirmation of methicillin-resistant *Staphylococcus aureus* from human, animal, and food samples. *International Journal of Food Microbiology* **209**, 26–28.
- Ariznabarreta A, Gonzalo C and San Primitivo F (2002) Microbiological quality and somatic cell count of ewe milk with special reference to staphylococci. *Journal of Dairy Science* **85**, 1370–1375.
- Arsenault J, Dubreuil P, Higgins R and Belanger D (2008) Risk factors and impacts of clinical and subclinical mastitis in commercial meat-producing sheep flocks in Quebec, Canada. *Preventive Veterinary Medicine* **87**, 373–393.
- Azara C, Longheu G, Sanna G and Tola S (2017) Biofilm formation and virulence factor analysis of *Staphylococcus aureus* isolates collected from ovine mastitis. *Journal of Applied Microbiology* **123**, 372–379.
- Barbagianni MS (2016) *Experimental Study of Pregnancy Toxaemia in Ewes and its Association with Mastitis in the Post-Partum Period* (PhD thesis). University of Thessaly.
- Barbagianni MS, Mavrogianni VS, Katsafadou AI, Spanos AS, Tsioli V, Galatos A, Nakou M, Valasi I, Gouletsou PG and Fthenakis GC (2015) Pregnancy toxaemia as risk factor for development of mastitis in sheep during the immediately post-partum period. *Small Ruminant Research* **130**, 246–251.
- Bar-Gal GK, Blum SE, Hadas L, Ehrlich R, Monecke S and Leitner G (2015) Host-specificity of *Staphylococcus aureus* causing intramammary infections in dairy animals assessed by genotyping and virulence genes. *Veterinary Microbiology* **176**, 143–154.
- Bergonier D and Berthelot X (2003) New advances in epizootiology and control of ewe mastitis. *Livestock Production Science* **79**, 1–16.
- Berube BJ and Wardenburg JB (2013) *Staphylococcus aureus*  $\alpha$ -toxin: nearly a century of intrigue. *Toxins (Basel)* **6**, 1140–1166.

- Bridle J** (1907) La mammite gangreneuse des brebis laitières: pathogenie et vaccination. *Bulletin de la Societe Centrale de Medecine Veterinaire* **61**, 500–506.
- Burriel AR** (1997) Resistance of coagulase-negative staphylococci isolated from sheep to various antimicrobial agents. *Research in Veterinary Science* **63**, 189–190.
- Burriel AR and Dagnall GJR** (1997) Leukotoxic factors produced by staphylococci of ovine origin. *Microbiological Research* **152**, 247–250.
- Cameron M, Perry J, Middleton JR, Chaffer M, Lewis J and Keefe GP** (2018) Short communication: evaluation of MALDI-TOF mass spectrometry and a custom reference spectra expanded database for the identification of bovine-associated coagulase-negative staphylococci. *Journal of Dairy Science* **101**, 590–595.
- Carneiro CR, Postol E, Nomizo R, Reis LF and Brentani RR** (2004) Identification of enolase as a laminin-binding protein on the surface of *Staphylococcus aureus*. *Microbes and Infection* **6**, 604–608.
- Cerca N, Jefferson KK, Maira-Litran T, Pier DB, Kelly-Quintos C, Goldmann DA, Azeredo J and Pier GB** (2007) Molecular basis for preferential protective efficacy of antibodies directed to the poorly acetylated form of staphylococcal poly-N-acetyl-beta-(1–6)-glucosamine. *Infection and Immunity* **75**, 3406–3413.
- Cheung AL, Bayer AS, Zhang G, Gresham H and Xiong YQ** (2004) Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunology & Medical Microbiology* **40**, 1–9.
- Cramton SE, Gerke C, Schnell NF, Nichols WW and Götz F** (1999) The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infection and Immunity* **67**, 5427–5433.
- Cramton SE, Ulrich M, Götz F and Doring G** (2001) Anaerobic conditions induce expression of polysaccharide intercellular adhesion in *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Infection and Immunity* **69**, 4079–4085.
- Cuccuru C, Meloni M, Sala E, Scaccabarozzi L, Locatelli C, Moroni P and Bronzo V** (2011) Effects of intramammary infections on somatic cell score and milk yield in Sarda sheep. *New Zealand Veterinary Journal* **59**, 128–131.
- de Almeida LM, de Almeida MZPRB, de Mendonça CL and Mamizuka EM** (2013) Comparative analysis of agr groups and virulence genes among sub-clinical and clinical mastitis *Staphylococcus aureus* isolates from sheep flocks of the Northeast of Brazil. *Brazilian Journal of Microbiology* **44**, 493–498.
- de Haas CJ, Veldkamp KE, Peschel A, Weerkamp F, Van Wamel WJ, Heezius EC, Poppelier MJ, Van Kessel KP and van Strijp JA** (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial anti-inflammatory agent. *The Journal of Experimental Medicine* **199**, 687–695.
- Deinhofer M and Pernthaner A** (1993) Differentiation of staphylococci from ewe and goat milk samples. *Deutsche Tierärztliche Wochenschrift* **100**, 234–236.
- de la Fuente R, Ruiz Santa Quiteria JA, Cid D, Domingo M and Suarez G** (1993) Experimental intramammary infection of ewes with *Staphylococcus aureus* subsp *anaerobius*. *Research in Veterinary Science* **54**, 221–226.
- Donay JL, Mathieu D, Fernandes P, Pregermain C, Bruel P, Wargnier A, Casin I, Weill FX, Lagrange PH and Herrmann JL** (2004) Evaluation of the automated phoenix system for potential routine use in the clinical microbiology laboratory. *Journal of Clinical Microbiology* **42**, 1542–1546.
- Dubin G** (2002) Extracellular proteases of *Staphylococcus* spp. *Biological Chemistry* **383**, 1075–1086.
- Dufour P, Gillet Y, Bes M, Lina G, Vandenesch F, Floret D, Etienne J and Richet H** (2002) Community-acquired methicillin-resistant *Staphylococcus aureus* infections in France: emergence of a single clone that produces Panton-Valentine leucocidin. *Clinical Infectious Diseases* **35**, 819–824.
- Ehrlich GD, Demeo PJ, Palmer MP, Sauber TJ, Altman DS, Altman G, Sotereanos N, Conti SF, Baratz M, Maale GE, Hu FZ, Post JC, Nistico L, Krefit R, Hall-Stoodley L, Costerton JW and Stoodley P** (2014) Culture-negative infections in orthopedic surgery. In Ehrlich G, DeMeo P, Costerton J, Winkler H (eds), *Culture Negative Orthopedic Biofilm Infections*. Springer Series on Biofilms, vol 7. Springer, Berlin pp. 17–27. *Springer Series on Biofilms* **7**.
- Eisenbecker J, Peisker H, Backes CS, Bur S, Hölters S, Thewes N, Greiner M, Junker C, Schwarz EC, Hoth M, Junker K, Preissner KT, Jacobs K, Herrmann M and Bischoff M** (2017) The extracellular adherence protein (Eap) of *Staphylococcus aureus* acts as a proliferation and migration repressing factor that alters the cell morphology of keratinocytes. *International Journal of Medical Microbiology* **307**, 116–125.
- Ergun Y, Aslantas O, Dogruer G, Kirecci E, Saribay MK, Ates CT, Ulku A and Demir C** (2009) Prevalence and etiology of subclinical mastitis in Awassi dairy ewes in southern Turkey. *Turkish Journal of Veterinary & Animal Sciences* **33**, 477–483.
- Ergun Y, Aslantas O, Kirecci E, Ozturk F, Ceylan A and Boyar Y** (2012) Antimicrobial susceptibility, presence of resistance genes and biofilm formation in coagulase negative staphylococci isolated from subclinical sheep mastitis. *Kafkas Universitesi Veteriner Fakultesi Dergisi* **18**, 449–456.
- European Food Safety Authority** (2014) Scientific opinion on the welfare risks related to the farming of sheep for wool, meat and milk production. *EFSA Journal* **12**, 3933–4060.
- Euzeby JP** (1997) List of bacterial names with standing in nomenclature: a folder available on the Internet. *International Journal of Systematic Bacteriology* **47**, 590–592 (list of prokaryotic names with standing in nomenclature, Available at <http://www.bacterio.net>).
- Foster TJ** (2017) Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. *FEMS Microbiology Reviews* **41**, 430–444.
- Foster TJ and Höök M** (1998) Surface protein adhesins of *Staphylococcus aureus*. *Trends in Microbiology* **6**, 484–488.
- Fox LK, Nagy JA, Hillers JK, Cronrath JD and Ratkowsky DA** (1991) Effects of postmilking teat treatment on the colonization of *Staphylococcus aureus* on chapped teat skin. *American Journal of Veterinary Research* **52**, 799–802.
- Fragkou IA, Mavrogianni VS, Cripps PJ, Gougoulis DA and Fthenakis GC** (2007) The bacterial flora in the teat duct of ewes can protect against and can cause mastitis. *Veterinary Research* **38**, 525–545.
- Fragkou IA, Dagleish MP, Papaioannou N, Cripps PJ, Boscos CM, Ververidis HN, Orfanou DC, Solomakos N, Finlayson J, Govaris A, Kyriazakis I and Fthenakis GC** (2010) The induction of lymphoid follicle-like structures in the ovine teat duct following experimental infection with *Mannheimia haemolytica*. *Veterinary Journal* **184**, 194–200.
- Fragkou IA, Boscos CM and Fthenakis GC** (2014) Diagnosis of clinical or subclinical mastitis in ewes. *Small Ruminant Research* **118**, 86–92.
- Franca CA, Peixoto RM, Cavalcante MB, Melo NE, Oliveira CJB, Veschi JA, Mota RA and Costa MM** (2012) Antimicrobial resistance of *Staphylococcus* spp. from small ruminant mastitis in Brazil. *Pesquisa Veterinaria Brasileira* **32**, 747–753.
- Fthenakis GC and Jones JET** (1990a) The effect of experimentally induced subclinical mastitis on the milk yield of ewes and on the growth of lambs. *British Veterinary Journal* **146**, 43–49.
- Fthenakis GC and Jones JET** (1990b) The effect of inoculation of coagulase negative staphylococci into the ovine mammary gland. *Journal of Comparative Pathology* **102**, 211–219.
- Fthenakis GC, Marples RR, Richardson JF and Jones JET** (1994) Some properties of coagulase-negative staphylococci isolated from cases of ovine mastitis. *Epidemiology and Infection* **112**, 171–176.
- Fthenakis GC, Leontides L, Skoufos J, Taitzoglou IA and Tzora A** (2004) Case report: high prevalence rate of ovine mastitis, caused by coagulase-negative staphylococci and predisposed by increased gossypol consumption. *Small Ruminant Research* **52**, 185–189.
- Fthenakis GC, Mavrogianni VS, Gallidis E and Papadopoulos E** (2015) Interactions between parasitic infections and reproductive efficiency in sheep. *Veterinary Parasitology* **208**, 56–66.
- Gelasakis AI, Mavrogianni VS, Petridis IG, Vasileiou NGC and Fthenakis GC** (2015) Mastitis in sheep – the last 10 years and the future of research. *Veterinary Microbiology* **185**, 136–146.
- Gerke C, Kraft A, Sussmuth R, Schweitzer O and Götz F** (1998) Characterization of the N-acetyl-glucosaminyl-transferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. *Journal of Biological Chemistry* **273**, 18586–18593.
- Giannakopoulos A, Vasileiou NGC, Gougoulis DA, Cripps PJ, Ioannidi KS, Chatzopoulos DC, Billinis C, Mavrogianni VS, Petinaki E and Fthenakis GC** (2019) Use of geographical information system and ecological niche modelling for predicting potential space distribution of sub-clinical mastitis in ewes. *Veterinary Microbiology* **228**, 119–128.
- Gougoulis DA, Kyriazakis I, Tzora A, Taitzoglou IA, Skoufos J and Fthenakis GC** (2008) Effects of lamb sucking on the bacterial flora of

- teat duct and mammary gland of ewes. *Reproduction in Domestic Animals* **43**, 22–26.
- Haaber J, Penadés JR and Ingmer H** (2017) Transfer of antibiotic resistance in *Staphylococcus aureus*. *Trends in Microbiology* **25**, 893–905.
- Hariharan H, Donachie W, Macaldowie C and Keefe G** (2004) Bacteriology and somatic cell counts in milk samples from ewes on a Scottish farm. *Canadian Journal of Veterinary Research* **68**, 188–192.
- Heying R, van de Gevel J, Que YA, Moreillon P and Beekhuizen H** (2007) Fibronectin-binding proteins and clumping factor A in *Staphylococcus aureus* experimental endocarditis: FnBPA is sufficient to activate human endothelial cells. *Thrombosis and Haemostasis* **97**, 617–626.
- Hu C, Xiong N, Zhang Y, Rayner S and Chen S** (2012) Functional characterization of lipase in the pathogenesis of *Staphylococcus aureus*. *Biochemical and Biophysical Research Communications* **419**, 617–620.
- Hulland C, Dufour S and Munoz M** (2018) *Milk Bacteriological Analysis Using MALDI-TOF Technology*. New Prague, USA: National Mastitis Council.
- Ibberson CB, Jones CL, Singh S, Wise MC, Hart ME, Zurawski DV and Horswill AR** (2014) *Staphylococcus aureus* hyaluronidase is a CodY-regulated virulence factor. *Infection and Immunity* **82**, 4253–4264.
- Katsafadou AI, Tsangaris GT, Billinis C and Fthenakis GC** (2015) Use of proteomics in the study of microbial diseases of small ruminants. *Veterinary Microbiology* **181**, 27–33.
- Katsafadou AI, Tsangaris GT, Anagnostopoulos AK, Billinis C, Barbogianni MS, Vasileiou NGC, Spanis SA, Mavrogianni and Fthenakis GC** (2019) Differential quantitative proteomics study of experimental *Mannheimia haemolytica* mastitis in sheep. *Journal of Proteomics* **205**, 103393.
- Khosravi AD, Jenabi A and Montazeri EA** (2017) Distribution of genes encoding resistance to aminoglycoside modifying enzymes in methicillin-resistant *Staphylococcus aureus* (MRSA) strains. *Kaohsiung Journal of Medical Sciences* **33**, 587–593.
- Kiedrowski MR, Crosby HA, Hernandez FJ, Malone CL, McNamara JO and Horswill RA** (2014) *Staphylococcus aureus* Nuc2 is a functional, surface-attached extracellular nuclease. *PLoS One* **9**, e95574.
- Kirecci E, Ergun Y, Dogruer G and Saribay MK** (2009) Usefulness of the e-test for the determination of the susceptibility of *Staphylococcus* sp. isolated from milk of sheep and goats with subclinical mastitis to amikacin and amoxicillin-clavulanic acid. *Bulletin of the Veterinary Institute in Pulawy* **53**, 401–405.
- Ko YP, Liang X, Smith CW, Degen JL and Höök M** (2011) Binding of Efb from *Staphylococcus aureus* to fibrinogen blocks neutrophil adherence. *Journal of Biological Chemistry* **286**, 9865–9874.
- Kobayashi SD and DeLeo FR** (2013) *Staphylococcus aureus* protein A promotes immune suppression. *mBio*, e00764–13.
- Lacey KA, Leech JM, Lalor SJ, McCormack N, Geoghegan JA and McLoughlin RM** (2017) The *Staphylococcus aureus* cell wall-anchored protein clumping factor A is an important T cell antigen. *Infection and Immunity* **85**, e00549–17.
- Lan L, Cheng A, Dunman PM, Missiakas D and He C** (2010) Golden pigment production and virulence gene expression are affected by metabolisms in *Staphylococcus aureus*. *Journal of Bacteriology* **192**, 3068–3077.
- Lang S, Livesley MA, Lambert PA, Littler WA and Elliott TS** (2000) Identification of a novel antigen from *Staphylococcus epidermidis*. *FEMS Immunology and Medical Microbiology* **29**, 213–220.
- Larsen HD, Aarestrup EM and Jensen NE** (2002) Geographical variation in the presence of genes encoding superantigenic exotoxins and beta-hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe and USA. *Veterinary Microbiology* **85**, 61–67.
- Latasa C, Solano C, Penadés JR and Lasa I** (2006) Biofilm-associated proteins. *Comptes Rendus Biologies* **329**, 849–857.
- Laukova A and Marounek M** (1992) Physiological and biochemical characteristics of staphylococci isolated from the rumen of young calves and lambs. *Zentralblatt für Mikrobiologie* **147**, 489–494.
- Leitner G, Chaffer M, Caraso Y, Ezra E, Kababea D, Winkler M, Glickman A and Saran A** (2003) Udder infection and milk somatic cell count, NAGase activity and milk composition-fat, protein and lactose-in Israeli-Assaf and Awassi sheep. *Small Ruminant Research* **49**, 157–164.
- Lovering AL, Gretes MC, Safadi SS, Danel F, De Castro L, Page MG and Strynadka NC** (2012) Structural insights into the anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity of ceftobiprole. *Journal of Biological Chemistry* **287**, 32096–32102.
- Lowy FD** (1998) Medical progress – *Staphylococcus aureus* infections. *New England Journal of Medicine* **339**, 520–532.
- Madani A, Garakani K and Mofrad RKM** (2017) Molecular mechanics of *Staphylococcus aureus* adhesin, CNA, and the inhibition of bacterial adhesion by stretching collagen. *PLoS One* **12**, e0179601.
- Marco Melero JC** (1994) *Mastitis in Lactating Sheep: Epidemiology, Diagnosis and Control* (Doctoral thesis). University of Zaragoza.
- Mariutti RB, Souza TACB, Ullah A, Caruso IP, de Moraes FR, Zaphorlin LM, Tartaglia NR, Seyffert N, Azevedo VA, Le Loir Y, Murakami MT and Arni RK** (2015) Crystal structure of *Staphylococcus aureus* exfoliative toxin D-like protein: structural basis for the high specificity of exfoliative toxins. *Biochemical and Biophysical Research Communications* **467**, 171–177.
- Martins KB, Faccioli PY, Bonesso MF, Fernandes S, Oliveira AA, Dantas A, Zafalon LF and Cunha MDRS** (2017) Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis. *Journal of Dairy Science* **100**, 2184–2195.
- Mavrogianni VS, Fthenakis GC, Burriel AR, Gouletsou P, Papaioannou N and Taitzoglou IA** (2004) Experimentally induced teat stenosis in dairy ewes: clinical, pathological and ultrasonographic features. *Journal of Comparative Pathology* **130**, 70–74.
- Mavrogianni VS, Cripps PJ and Fthenakis GC** (2007) Bacterial flora and risk of infection of the ovine teat duct and mammary gland throughout lactation. *Preventive Veterinary Medicine* **79**, 163–173.
- Mavrogianni VS, Menzies PI, Fragkou IA and Fthenakis GC** (2011) Principles of mastitis treatment in sheep and goats. *Veterinary Clinics of North America Food Animal Practice* **27**, 115–120.
- Mavrogianni VS, Papadopoulos E, Gougoulis DA, Gallidis E, Ptochos S, Fragkou IA, Orfanou DC and Fthenakis GC** (2017) Gastrointestinal trichostromylosis can predispose ewes to clinical mastitis after experimental mammary infection. *Veterinary Parasitology* **245**, 71–77.
- McCallum N, Berger-Bächi B and Senn MM** (2010) Regulation of antibiotic resistance in *Staphylococcus aureus*. *International Journal of Medical Microbiology* **300**, 118–129.
- McGuinness WA, Malachowa N and DeLeo FR** (2017) Vancomycin resistance in *Staphylococcus aureus*. *Yale Journal of Biological Medicine* **902**, 269–281.
- McMillan K, Moore SC, McAuley CM, Fegan N and Fox EM** (2016) Characterization of *Staphylococcus aureus* isolates from raw milk sources in Victoria, Australia. *BMC Microbiology* **16**, 169.
- Mediavilla JR, Chen L, Mathema B and Kreiswirth BN** (2012) Global epidemiology of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA). *Current Opinion in Microbiology* **15**, 588–595.
- Menzies BE** (2003) The role of fibronectin binding proteins in the pathogenesis of *Staphylococcus aureus* infections. *Current Opinion in Infectious Diseases* **16**, 225–229.
- Mork T, Waage S, Tollersrud S, Kvitle B and Sviland S** (2007) Clinical mastitis in ewes; bacteriology, epidemiology and clinical features. *Acta Veterinaria Scandinavica* **49**, 23–30.
- Murray RJ, Pearson JC, Coombs GW, Flexman JP, Gollidge CL, Speers DJ, Dyer JR, McLellan DG, Reilly M, Bell JM, Bowen SF and Christiansen KJ** (2008) Outbreak of invasive methicillin-resistant *Staphylococcus aureus* infection associated with acupuncture and joint injection. *Infection Control and Hospital Epidemiology* **29**, 859–865.
- Noble WC and Somerville DA** (1974) Skin as a habitat. In Noble WC (ed.), *Microbiology of Human Skin*. Philadelphia: Saunders, pp. 3–78.
- Nocard E** (1887) Note sur la mammite gangreneuse des brebis laitières. *Annales de l'Institut Pasteur* **1**, 417–428.
- Novick RP** (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology* **48**, 1429–1449.
- Onni T, Sanna G, Cubeddu GP, Marogna G, Lollai S, Leori G and Tola S** (2010) Identification of coagulase-negative staphylococci isolated from

- ovine milk samples by PCR-RFLP of 16S rRNA and gap genes. *Veterinary Microbiology* **144**, 347–352.
- Onni T, Sanna G, Larsen J and Tola S** (2011) Antimicrobial susceptibilities and population structure of *Staphylococcus epidermidis* associated with ovine mastitis. *Veterinary Microbiology* **148**, 45–50.
- Orden JA, Cid D, Blanco ME, Quiteria JARS, Gomez Lucia E and de la Fuente R** (1992a) Enterotoxin and toxic shock syndrome toxin-one production by staphylococci isolated from mastitis in sheep. *APMIS* **100**, 132–134.
- Orden JA, Goyache J, Hernandez J, Domenech A, Suarez G and Gomez Lucia E** (1992b) Production of staphylococcal enterotoxins and TSST-1 by coagulase-negative staphylococci isolated from ruminant mastitis. *Journal of Veterinary Medicine B* **39**, 144–148.
- O'Riordan K and Lee JC** (2004) *Staphylococcus aureus* capsular polysaccharides. *Clinical Microbiology Reviews* **17**, 218–234.
- Otto M** (2013) *Staphylococcus aureus* toxins. *Current Opinion in Microbiology* **17**, 32–37.
- Ozenc E, Seker E, Acar DB, Birdane MK, Darbaz I and Dogan N** (2011) The importance of staphylococci and threshold value of somatic cell count for diagnosis of subclinical mastitis in Pirlak sheep at mid-lactation. *Reproduction in Domestic Animals* **46**, 970–974.
- Palmqvist N, Foster T, Tarkowski A and Josefsson E** (2002) Protein A is a virulence factor in *Staphylococcus aureus* arthritis and septic death. *Microbial Pathogenesis* **33**, 239–249.
- Pantosti A, Sanchini A and Monaco M** (2007) Mechanisms of antibiotic resistance in *Staphylococcus aureus*. *Future Microbiology* **2**, 323–334.
- Perez MM, Prenafeta A, Valle J, Penades J, Rota C, Solano C, Marco J, Grillo MJ, Lasa I, Irache JM, Maira-Litran T, Jimenez-Barbero J, Costa L, Pier GB, de Andres D and Amorena B** (2009) Protection from *Staphylococcus aureus* mastitis associated with poly-N-acetyl beta-1,6 glucosamine specific antibody production using biofilm-embedded bacteria. *Vaccine* **27**, 2379–2386.
- Persson Y, Nyman AK, Soderquist L, Tomic N and Waller KP** (2017) Intramammary infections and somatic cell counts in meat and pelt producing ewes with clinically healthy udders. *Small Ruminant Research* **156**, 66–72.
- Phillipson M and Kubes P** (2011) The neutrophil in vascular inflammation. *Nature Medicine* **17**, 1381–1390.
- Pilipincova I, Bhide M, Dudrikova E and Travnicek M** (2010) Genotypic characterization of coagulase-negative staphylococci isolated from sheep milk in Slovakia. *Acta Veterinaria Brno* **79**, 269–275.
- Pinchuk IV, Beswick EJ and Reyes VE** (2010) Staphylococcal enterotoxins. *Toxins (Basel)* **2**, 2177–2197.
- Pisanu S, Cubeddu T, Pagnozzi D, Rocca S, Cacciotto C, Alberti A, Marogna G, Uzzau S and Addis MF** (2015) Neutrophil extracellular traps in sheep mastitis. *Veterinary Research* **46**, 59.
- Porrero MC, Hasman H, Vela AI, Fernandez-Garayzabal JF, Dominguez L and Aarestrup FM** (2012) Clonal diversity of *Staphylococcus aureus* originating from the small ruminants goats and sheep. *Veterinary Microbiology* **156**, 157–161.
- Prenafeta A, March R, Foix A, Casals I and Costa L** (2010) Study of the humoral immunological response after vaccination with a *Staphylococcus aureus* biofilm-embedded bacterin in dairy cows: possible role of the exopolysaccharide specific antibody production in the protection from *Staphylococcus aureus* induced mastitis. *Veterinary Immunology Immunopathology* **134**, 208–217.
- Rainard P** (2017) Mammary microbiota of dairy ruminants: fact or fiction? *Veterinary Research* **48**, 25.
- Riggio V and Portolano B** (2015) Genetic selection for reduced somatic cell counts in sheep milk: a review. *Small Ruminant Research* **126**, 33–42.
- Rooijackers SH, Milder FJ, Bardoel BW, Ruyken M, van Strijp JA and Gros P** (2007) Staphylococcal complement inhibitor: structure and active sites. *Journal of Immunology* **179**, 2989–2998.
- Rovai M, Caja G, Salama AAK, Jubert A, Lázaro B, Lázaro M and Leitner G** (2014) Identifying the major bacteria causing intramammary infections in individual milk samples of sheep and goats using traditional bacteria culturing and real-time polymerase chain reaction. *Journal of Dairy Science* **97**, 5393–5400.
- Rupp R, Bergonier D, Dion S, Hygonenq MC, Aurel MR, Robert-Granic C and Foucras G** (2009) Response to somatic cell count-based selection for mastitis resistance in a divergent selection experiment in sheep. *Journal of Dairy Science* **92**, 1203–1219.
- Sahuquillo Arce JM, Yarad Auad F and Hernández Cabezas A** (2013) Biofilms: a biological antimicrobial resistance system. In Méndez-Vilas A (ed.), *Microbial Pathogens and Strategies for Combating them: Science, Technology and Education*. Badajoz: Formatex, pp. 61–67.
- Saratsis P, Leontides L, Tzora A, Alexopoulos C and Fthenakis GC** (1998) Incidence risk and aetiology of mammary abnormalities in dry ewes in 10 flocks in Southern Greece. *Preventive Veterinary Medicine* **37**, 173–183.
- Scherrer D, Corti S, Muehlherr JE, Zweifel C and Stephen R** (2004) Phenotypic and genotypic characteristics of *Staphylococcus aureus* isolates from raw bulk-tank milk samples of goats and sheep. *Veterinary Microbiology* **101**, 101–107.
- Schönborn S, Wente N, Paduch JH and Krömker V** (2017) In vitro ability of mastitis causing pathogens to form biofilms. *Journal of Dairy Research* **84**, 198–201.
- Schwarz S, Loeffler A and Kadlec K** (2017) Bacterial resistance to antimicrobial agents and its impact on veterinary and human medicine. *Veterinary Dermatology* **28**, 82–e19.
- Selvaggi M, D' Alessandro AG and Dario C** (2017) Environmental and genetic factors affecting milk yield and quality in three Italian sheep breeds. *Journal of Dairy Research* **84**, 27–31.
- Silversides JA, Lappin E and Ferguson AJ** (2010) Staphylococcal toxic shock syndrome: mechanisms and management. *Current Infectious Diseases Reports* **12**, 392–400.
- Skrlin J** (2016) Impact of biofilm on healing and a method for identifying it in the wound. *Acta Medica Croatica* **70**, 29–32.
- Spaan NA, van Strijp JAG and Torres VJ** (2017) Leukocidins: Staphylococcal bi-component pore-forming toxins find their receptors. *Nature Reviews Microbiology* **15**, 435–447.
- Stemmering AM, Köhl J, Pandey MK, Kuipers A, Leusen JH, Boross P, Nederend M, Vidarsson G, Weersink AY, van de Winkel JG, van Kessel KP and van Strijp JA** (2013) *Staphylococcus aureus* formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like are potent FcγR antagonists that inhibit IgG-mediated effector functions. *Journal of Immunology* **191**, 353–362.
- Stutz K, Stephan R and Tasara T** (2011) *Spa*, *ClfA*, and *FnbA* genetic variations lead to Staphaurex test-negative phenotypes in bovine mastitis *Staphylococcus aureus* isolates. *Journal of Clinical Microbiology* **49**, 638–646.
- Tejada TS, Silva DT, Dias PA, Conceicao RCS, Neto HM and Timm CD** (2012) Subclinical mastitis caused by *Staphylococcus* coagulase negative in meet-producing sheep. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia* **64**, 1074–1076.
- Tel OY, Aslantas O, Keskin O, Yilmaz ES and Demir C** (2012) Investigation of the antibiotic resistance and biofilm formation of *Staphylococcus aureus* strains isolated from gangrenous mastitis of ewes. *Acta Veterinaria Hungarica* **60**, 189–197.
- Titchen DA** (1977) Cineradiographic studies of swallowing in the suckled lamb. *Annales de Recherche Veterinaire* **8**, 483.
- Unal N and Cinar OD** (2012) Detection of staphylococcal enterotoxin, methicillin-resistant and Pantone-Valentine leukocidin genes in coagulase-negative staphylococci isolated from cows and ewes with subclinical mastitis. *Tropical Animal Health and Production* **44**, 369–375.
- Unal N, Askar S, Macun HC, Sakarya F, Altun B and Yildirim M** (2012) Pantone-Valentine leukocidin and some exotoxins of *Staphylococcus aureus* and antimicrobial susceptibility profiles of staphylococci isolated from milks of small ruminants. *Tropical Animal Health and Production* **44**, 573–579.
- Van Oostveldt K, Paape MJ, Dosogne H and Burvenich C** (2002) Effect of apoptosis on phagocytosis, respiratory burst and CD18 adhesion receptor expression of bovine neutrophils. *Domestic Animal Endocrinology* **22**, 37–50.
- Vasileiou NGC** (2019) *Mastitis in Ewes associated with Staphylococcus spp.: New Clinical, Epidemiological, Management, Microbiological and Zoonotic Findings and Evaluation of a Novel Vaccine against the Disease* (PhD thesis). University of Thessaly.
- Vasileiou NGC, Chatzopoulos DC, Gougoulis DA, Sarrou S, Katsafadou AI, Spyrou V, Mavrogianni VS, Petinaki E and**

- Fthenakis GC** (2018a) Slime-producing staphylococci as causal agents of subclinical mastitis in sheep. *Veterinary Microbiology* **224**, 93–99.
- Vasileiou NGC, Cripps PJ, Ioannidi KS, Chatzopoulos DC, Gougoulis DA, Sarrou S, Orfanou DC, Politis A, Calvo Gonzalez-Valerio T, Argyros S, Mavrogianni VS, Petinaki E and Fthenakis GC** (2018b) Extensive countrywide field investigation of subclinical mastitis in sheep in Greece. *Journal of Dairy Science* **101**, 7297–7310.
- Vasileiou NGC, Gougoulis DA, Riggio V, Ioannidi KS, Chatzopoulos DC, Mavrogianni VS, Petinaki E and Fthenakis GC** (2018c) Association of subclinical mastitis prevalence with sheep breeds in Greece. *Journal of Dairy Research* **85**, 317–320.
- Vasileiou NGC, Chatzopoulos DC, Cripps PJ, Ioannidi KS, Gougoulis DA, CHouzouris TM, Lianou DT, Calvo Gonzalez-Valerio T, Guix Vallverdu R, Argyros S, Cesio M, Font I, Mavrogianni VS, Petinaki E and Fthenakis GC** (2019a) Evaluation of efficacy of a biofilm-embedded bacteria-based vaccine against staphylococcal mastitis in sheep – a randomized, placebo-controlled field study. *Journal of Dairy Science*. in press.
- Vasileiou NGC, Sarrou S, Papagiannitsis C, Chatzopoulos DC, Malli E, Mavrogianni VS, Petinaki E and Fthenakis GC** (2019b) Antimicrobial agent susceptibility and typing of staphylococcal isolates from subclinical mastitis in ewes. *Microbial Drug Resistance*. doi: 10.1089/mdr.2019.0009
- Vautor E, Carsenti-Dellamonica H, Sabah M, Mancini G, Pepin M and Dellamonica P** (2007) Characterization of *Staphylococcus aureus* isolates recovered from dairy sheep farms (agr group, adherence, slime, resistance to antibiotics). *Small Ruminant Research* **72**, 197–199.
- Verhoeven PO, Gagnaire J, Botelho-Nevers E, Grattard F, Carricajo A, Lucht F, Pozzetto B and Berthelot P** (2014) Detection and clinical relevance of *Staphylococcus aureus* nasal carriage: an update. *Expert Review of Anti-Infective Therapy* **12**, 75–89.
- Wang B and Muir TW** (2016) Regulation of virulence in *Staphylococcus aureus*: molecular mechanisms and remaining puzzles. *Cell Chemical Biology* **23**, 214–224.
- Wang H, McEntire JC, Zhang L, Li X and Doyle M** (2012) The transfer of antibiotic resistance from food to humans: facts, implications and future directions. *Revue Scientifique et Technique – Office International des Epizooties* **31**, 249–260.
- Watson DL** (1988) Vaccination against experimental staphylococcal mastitis in ewes. *Research in Veterinary Science* **45**, 16–21.
- Wieckowska-Szakiel M, Sadowska B and Rózalska B** (2007) Staphylokinase production by clinical *Staphylococcus aureus* strains. *Polish Journal of Microbiology* **56**, 97–102.
- Winn WC and Koneman EW** (2006) *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th Edn. Philadelphia: Lippincott Williams & Wilkins.
- Winter P and Colditz IG** (2002) Immunological responses of the lactating ovine udder following experimental challenge with *Staphylococcus epidermidis*. *Veterinary Immunology and Immunopathology* **89**, 57–65.
- Winter P and Hofer E** (1996) Coagulase-negative staphylococci as a pathogen of subclinical and clinical mastitis in three flocks of milk sheep. *Tierärztliche Umschau* **51**, 222–226.
- Winter P, Hoflechner A and Baumgartner W** (1999) Resistance patterns of ovine mastitis pathogens. *Berliner und Münchener Tierärztliche Wochenschrift* **112**, 216–222.
- Winter P, Rammelmayr A and Baumgartner W** (2002) Udder health status of dairy flocks during one lactation period with special regards to infections caused by coagulase-negative staphylococci. *Wiener Tierärztliche Monatsschrift* **89**, 242–248.
- Winter P, Schilcher F, Fuchs K and Colditz IG** (2003) Dynamics of experimentally induced *Staphylococcus epidermidis* mastitis in East Friesian milk ewes. *Journal of Dairy Research* **70**, 157–164.