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# Mastitis in sheep – The last 10 years and the future of research

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ABSTRACT

Bacterial mastitis is a significant welfare and financial problem in sheep flocks. This paper reviews the recently published literature, including publications that highlight the significance and virulence factors of the causal agents, especially *Staphylococcus aureus* and *Mannheimia haemolytica*, the primary causes of the disease. Research has also contributed to the understanding of risk factors, including genetic susceptibility of animals to infections, supporting future strategies for sustainable disease control. Pathogenetic mechanisms, including the role of the local defenses in the teat, have also been described and can assist formulation of strategies that induce local immune responses in the teat of ewes. Further to well-established diagnostic techniques, i.e., bacteriological tests and somatic cell counting, advanced methodologies, e.g., proteomics technologies, will likely contribute to more rapid and accurate diagnostics, in turn enhancing mastitis control efforts.



## 1. Introduction

Mammary infections can lead to various clinical or subclinical diseases in sheep. These are bacterial mastitis ('mastitis'), mycoplasma mastitis (contagious agalactia) and lentiviral mammary infection. The objective of the present review is to discuss bacterial mastitis, as contagious agalactia (Corrales et al., 2007; Gomez-Martin et al., 2013) and lentiviral infections (Minguijón et al., 2015) have been recently reviewed. The term 'mammary infection' includes only microbial diseases of the mammary parenchyma, hence infections of the teats and the udder skin, e.g., *Papilloma virus* infection of teats, contagious echtyma (orf), impetigo (staphylococcal dermatitis) or sheep pox, are not discussed in the present review.

Mammary infections are the primary causes of 'Milk-drop syndrome in ewes' (>85% of all causes) (Giadinis et al., 2012). The syndrome has been defined as a pathological entity at flock level, characterised by reduced milk yield of lactating ewes, with no clinical signs specific to a disease (Giadinis et al., 2012).

In dairy-type flocks, mammary infections have an obvious financial significance, due to the reduction in milk yield, the downgrading of milk quality and the rejection of milk after antibiotic administration. Nevertheless, 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 ewes and the relevant veterinary expenses.

In sheep, mammary infections are also of great welfare concern (European Food Safety Authority, 2009). Clinical mastitis is a disease that leads to anxiety, restlessness, changes in feeding behavior and pain in affected ewes (Fthenakis and Jones, 1990b). Even in subclinical mastitis, normal behavioral patterns of sheep are modified (Gougoulis et al., 2008a, 2010), hence raising potential welfare concerns.

In recent years, there has been an increased interest, internationally, in the study of ovine mastitis, with increasing number of doctoral theses published at various universities around Europe. Moreover, the European Commission has awarded a research grant ('3SR–Sustainable solutions for small ruminants') to an international consortium, which aimed to identify and promote means of sustainable control of the disorder; some of the findings of that project will be reviewed in this paper. Finally, the conclusions of a recent meeting of a working group in 'Welfare of sheep' of the Animal Health and Welfare Panel of the European Food Safety Authority, of which one of the authors (GCF) was an invited expert member, indicated that mastitis is one of the three most important problems adversely affecting welfare of sheep across the range of sheep production and management systems (European Food Safety Authority, 2014).

Several review papers have discussed earlier research in ovine mastitis (Watson and Buswell, 1984; Menzies and Ramanoon, 2001; Bergonier et al., 2003; Contreras et al., 2007). This paper focuses in research findings that have been published in the last 10 years and discusses potential opportunities for future research.

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## 2. Methodology

The review includes primarily references published in journals cited at the Web of Knowledge database (wok.mimas.ac.uk); 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*', '*Mannheimia*', '*teat*'). Subsequently, the full papers have been retrieved through the websites of the respective journals.

Moreover, selected papers from the proceedings of scientific meetings, mainly held in 2012 to 2014, are also discussed. Those present a means for quick publication of recent, relevant research in the field and the contents of proceedings of these meetings have been edited before publication.

## 3. Aetiological agents

Several bacterial agents as detailed below, have been found to be associated with clinical or subclinical mastitis in ewes (Contreras et al., 2007). Most often, these have been identified based in the use of conventional phenotypic identification systems. Differences have been identified in the aetiological agents of the disease related to the production system. In dairy production systems, coagulase-negative staphylococci or *Staphylococcus aureus* are the principal causes of subclinical or clinical mastitis, respectively (Bergonier et al., 2003; Mørk et al., 2005; Contreras et al., 2007). In meat production systems, most cases of clinical mastitis are associated with *Mannheimia haemolytica* or *S. aureus* (Mavrogianni et al., 2007; Arsenault et al., 2008; Koop et al., 2010; Omaleki et al., 2010). An overview of recent studies regarding incidence and etiology of ovine mastitis is presented in Table 1.

### *3.1. Staphylococcus aureus*

*S. aureus* is the most common mastitis-related pathogen in sheep (Mørk et al., 2005; Mavrogianni et al., 2011). Bergonier et al. (2003) indicated that *S. aureus* was the major mastitis-causing agent, isolated from sporadic cases or outbreaks of the disease. The organism is responsible for about 40% of cases in ewes suckling lambs and 80% of cases in milking ewes (Mørk et al., 2007; Koop et al., 2010; Mavrogianni et al., 2011).

*S. aureus* strains are divided in four *agr* (accessory gene regulator) groups (I, II, III, IV), according to the *agr* locus, which regulates production of virulence factors (Novick et al., 1995; Vautor et al., 2007). Based on this classification, rapid discrimination of *S. aureus* strains has been proposed (Goerke et al., 2005). Vautor et al. (2007) have used this classification to study the distribution of *S. aureus* strains from milk of ewes with mastitis and from nostrils of sheep, into the four *agr* groups; they found that most isolates could be assigned to groups I (44%) or III (46%).

In recent years, antibiotic resistant *S. aureus* strains have been recognised as an emerging threat for public health. In several studies, the antibiotic resistance patterns of different *S. aureus* isolates from cases of ovine mastitis have been described (Fthenakis, 1998; Goñi et al., 2004; Mørk et al., 2005; Vautor et al., 2007); moreover, the potential of resistant strains of the organisms in dairy products from sheep milk and the potential consequences for public health have been discussed (Normanno et al., 2007). Lollai et al. (2008) evaluated antibiotic susceptibility of 1284 strains of *S. aureus* from ovine mastitis cases and found increased resistance rate against streptomycin (48–87%), with smaller resistance rates found against penicillin or ampicillin (2–12% or 0–12%, respectively). Other studies have nevertheless grouped staphylococcal strains from mastitis independently of coagulase-production type and have reported increased resistance to penicillin G (up to 31%) or to ampicillin (up to 30%) (Corrente et al., 2003; Kunz et al., 2011; Onni et al., 2011).

### *3.2. Other staphylococci*

Coagulase-negative staphylococci are pathogens of smaller virulence, associated mostly with subclinical intramammary infections (Bonnefont et al., 2011), although they may also cause clinical disease (Fthenakis and Jones, 1990b). *Staphylococcus epidermidis* is the most common species associated with ovine mastitis (Onni et al., 2010), followed by *Staphylococcus chromogenes*, *Staphylococcus simulans* and *Staphylococcus xylosus*; less prevalent species include *Staphylococcus auricularis*,*Staphylococcus capitis*, *Staphylococcus caprae, Staphylococcus cohnii, Staphylococcus equorum, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus lentus, Staphylococcus muscae, Staphylococcus saprophyticus, Staphylococcus sciuri* and *Staphylococcus warneri*. Table 2 summarises the coagulase-negative staphylococcal species reported in various studies in the last 10 years. The general findings are in accord with results of earlier studies (Fthenakis, 1994; Bergonier et al., 2003) regarding the role of coagulase-negative staphylococci in subclinical ovine mastitis.

Recently, it has been recognised that antimicrobial susceptibility of coagulase-negative staphylococci, which represent the majority of organisms isolated from ovine milk, is important for the early recognition of newly emerging resistant milk-borne bacterial agents (Onni et al., 2011).

Additionally, *Staphylococcus hyicus*, *Staphylococcus intermedius* and *Staphylococcus schleiferi* (all coagulase-positive species) have been isolated, with a low frequency, from cases of clinical mastitis in sheep.

### *3.3.* Mannheimia *spp.*

*M. haemolytica*, *M. glucosida* and *M. ruminalis* have been isolated from cases of acute mastitis in sheep (Omaleki et al., 2010, 2011), with *M. haemolytica* confirmed as the most common cause of mastitis in flocks in meat type producing systems (Arsenault et al., 2008; Koop et al., 2010; Omaleki et al., 2010). In contrast, in dairy sheep, only 11% of cases of intramammary infections were found to be caused by *M. haemolytica* (Mavrogianni et al., 2007).

### *3.4.* Streptococcus *spp.*

*Streptococcus* spp. are sporadic pathogens of ovine mastitis (Zdragas et al., 2005; Contreras and Rodríguez, 2011). Increased incidence of mastitis caused by these organisms is usually associated with inappropriate housing conditions or milking practices, as found in studies of the disease in Italian or German flocks, where incidence risks of 23–31% have been reported (Marogna et al., 2010; Cuccuru et al., 2011; Kern et al., 2013).

### *3.5. Other bacteria*

Other Gram-positive bacteria associated with intramammary infections include *Bacillus cereus* (usually, after intramammary administration of antibiotic tubes performed under suboptimal conditions of hygiene), *Clostridium* spp. (Mørk et al., 2007; Fotou et al., 2011), *Corynebacterium* spp. (Spanu et al., 2011), *Enterococcus* spp. (*Enterococcus faecalis*, *E. faecium*, *E. durans*; Marogna et al., 2010), *Listeria monocytogenes* (Winter et al., 2004; Brugère-Picoux, 2008), *Micrococcus* spp. (Ariznabarreta et al., 2002), *Mycobacterium* spp. (including *Mycobacterium avium* subsp. *paratuberculosis*; Nebbia et al., 2006) and *Trueperella pyogenes* (Saratsis et al., 1998; Hadimli et al., 2010).

**Table 1**
Bacterial agents isolated from ewes with clinical or subclinical mastitis (2004–2014).

| Country | | Animals | Flocks | Samples | Frequency (%) of bacterial isolation | Frequency (%) of bacterial isolates | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *Staphylococcus* spp. | *S. aureus* | *Streptococcus* spp. | *Mannheimia* spp. | Enterobacteriaceae | *Bacillus* spp. | Coryne-bacteria | Other | |
| Greece | | 32 | 1 | 64 | 94 | 78 | 7 | | | 4 | 7 | 3 | | Fthenakis et al. (2004) |
| UK | | 50 | 1 | 467 | 17[a] | 68 | 1 | 10 | 3 | | 10 | 1 | 6 | Hariharan et al. (2004) |
| Greece | Suckling period | 21 | 3 | 244 | 8 | 90 | [b] | | | | | | 11 | Mavrogianni et al. (2007) |
| | Milking period | 21 | 3 | 156 | 6 | 78 | [b] | | 11 | | | | 11 | |
| Norway | | 509 | 353 | 547 | 85[c] | 3 | 77 | 5 | 2 | 9 | | 1 | 3 | Mørk et al. (2007) |
| Canada | Subclinical mastitis | 354 | 27 | 696 | 18[d] | 28 | 29 | 5 | 2 | 7 | 20 | 7 | 3 | Arsenault et al. (2008) |
| | Clinical mastitis | 35 | 17 | 35 | 63 | 14 | 32 | | 32 | | 5 | 0 | 18 | |
| Netherlands | | 350 | 1 | 31 | 94 | | 41 | | 52 | | | | 7 | Koop et al. (2010) |
| Italy | | 2198 | 15 | 2198 | 50 | 28 | 14 | 29 | | | | | 30 | Marogna et al. (2010) |
| Italy | | 2201 | 15 | 2201 | 10[e] | 100 | | | | | | | | Onni et al. (2010) |
| Italy | | 1120 | 4 | 8843 | 32 | 84 | 11 | 5 | 1 | | | 0 | 0 | Riggio et al. (2010) |
| Greece | | 240 | 25 | 240 | 15[f] | | 16 | | | 7 | 20 | 7 | 51 | Fotou et al. (2011) |
| Italy | | 202 | 1 | 2828 | 42 | 69 | | 31 | | | | | | Cuccuru et al. (2011) |
| Turkey | | 232 | | 464 | 11 | 64 | 25 | | | 11 | | | | Ozenc et al. (2011) |
| USA | At lambing | 245 | 1 | 435 | 22 | 45 | 1 | 6 | | 7 | 6 | 11 | 24 | Spanu et al. (2011) |
| | 2–3 weeks after lambing | 214 | 1 | 426 | 10 | 48 | 2 | 5 | | 3 | 5 | 3 | 34 | |
| Germany | | 614 | 20 | 1228[j] | 75 | 49 | 6 | 23 | | 6 | | | 16 | Kern et al. (2013) |
| Brazil | | 275 | 17 | 550 | 24 | 93 | [b] | 5 | | | | | 2 | Blagitz et al. (2014) |

[a] Isolates from mixed cultures and from cultures that yielded *Bacillus* not considered.
[b] *S. aureus* isolates included in the column of *Staphylococcus* spp.
[c] Frequency calculated after exclusion of samples with no bacterial growth (13%) and of contaminated samples (2%).
[d] Refers to proportion of mammary glands, from which bacteria were isolated.
[e] Work studied only coagulase-negative staphylococci.

Other Gram-negative bacteria isolated from intramammary infections include *Citrobacter* spp., *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Pasteurella multocida*, *Proteus* spp., *Pseudomonas aeruginosa* (Leitner and Krifucks, 2007), *Salmonella* spp., *Serratia* spp. (Contreras and Rodríguez, 2011) and *Yersinia pseudotuberculosis* (Juste et al., 2009), representing about 3% of all organisms isolated from dairy sheep milk (Bergonier et al., 2003). These are considered to be environmental organisms, capable of causing clinical mastitis depending on the immunological status of exposed animals (Contreras and Rodríguez, 2011).

Concerns regarding infection with some of the above organisms (e.g., *L. monocytogenes*, *M. avium* subsp. *paratuberculosis*, *Salmonella* spp.) have been expressed about their potential public health significance, especially when sheep milk is produced for human consumption. Finally, cases of mastitis caused by fungi or yeast have been reported occasionally.

## 4. Risk factors

Various factors associated with management of ewes have been reported as possible risk factors for mastitis. Of these, some can be related to specific causative bacterial agents. Moreover, as general knowledge has increased, new insights have become available regarding previously unknown facets of the disease.

### 4.1. Hygiene of the sheep shed

Substandard hygiene in sheep shed predisposes to intramammary infections. Relevant issues include high stocking density, insufficient straw bedding and limited ventilation (Caroprese, 2008). These can result in increased concentrations of bacteria inside the shed, which can enhance risk of intramammary infections (Bergonier et al., 2003). Lack of regular and efficient manure removal and disinfection can compromise further hygiene status in sheds.

### 4.2. Milking practices

Incorrect milking practices performed by untrained personnel, irrespectively of machine- or hand-milking, predispose to mastitis. In either case, over-milking and/or milk retention in the gland cistern should be avoided, as they promote bacterial multiplication. Likewise, during machine-milking, inappropriate regulation and/or malfunction of the milking system (e.g., incorrect vacuum level, pulsation rate and ratio, vacuum fluctuations) are considered, in general, to predispose to intramammary infections (Albenzio et al., 2003; Contreras et al., 2007). However, Peris et al. (2003a,b) have not been able to show an association between vacuum level, pulsation rate and overmilking on udder health status in ewes. Inefficient cleaning and disinfection of the milking system, poor water hygiene and over-use of liners can lead to accumulation of pathogens, which may then ascend through the teat duct into the mammary cistern. On the other hand, hand-milking of sheep will favor intramammary infections with pathogens transmitted from the hands of milkers, more often staphylococcal isolates (Marco Melero, 1994). If teat-dipping, which protects against transmission of mammary pathogens (Bergonier and Berthelot, 2003), is not practiced as part of the milking routine, then rate of new infections will increase (Contreras et al., 2007). However, occasionally, teat-dipping solutions contaminated with environmental pathogens can lead to intramammary infections (Tzora and Fthenakis, 1998; Contreras and Rodríguez, 2011).

### 4.3. Feeding practices

Inappropriate feeding may lead to clinical and subclinical mastitis. Increased incidence risk of clinical and subclinical mastitis in ewes with vitamin A deficiency has been reported, as the result of reduced integrity and functionality of the epithelial defenses of the mammary gland in affected animals (Koutsoumpas et al., 2013). Similarly, selenium deficiency (Giadinis et al., 2011) or increased consumption of cottoncake meal (containing increased gossypol concentration) (Fthenakis et al., 2004) have been reported to contribute to development of mastitis in ewes and attributed to impeded cellular defenses of the affected ewes. Finally, recently, reduced feed-energy availability has been recognised as a risk factor for mastitis in ewes (Barbagianni et al., 2015).

### 4.4. Udder conformation

Teat placement and udder conformation may also predispose to intramammary infections. Udders with small and horizontally

**Table 2**
Frequency of *Staphylococcus* spp. other than *S. aureus*, from reports of intramammary infections.

| *Staphylococcus* spp. | Fthenakis et al. (2004) *n* = 43 | Hariharan et al. (2004) *n* = 33 | Leitner et al. (2004) *n* = 36 | Kiossis et al. (2007) *n* = 26 | Kiossis et al. (2007) *n* = 47 | Marogna et al. (2010) *n* = 224 | Marogna et al. (2010) *n* = 59 | Onni et al. (2010) *n* = 226 | Onni et al. (2010) *n* = 226 | Cuccuru et al. (2011) *n* = 820 | Spanu et al. (2011) *n* = 45 | Spanu et al. (2011) *n* = 23 | Overall frequency (all studies) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. auricularis* | | | | | | 1 | | | | | 9 | 30 | 13 |
| *S. capitis* | 12 | 3 | | | | 1 | 3 | 2 | | | 2 | | 4 |
| *S. caprae* | 14 | | | | | 10 | 5 | 13 | 15 | 5 | | 4 | 9 |
| *S. chromogenes* | 12 | | 32 | 8 | | 14 | 12 | 15 | 13 | 21 | 22 | 9 | 16 |
| *S. cohnii* | | | | | | | | | | | 2 | 9 | 6 |
| *S. epidermidis* | 26 | | 14 | 58 | 62 | 62 | 66 | 56 | 58 | 58 | 22 | 9 | 45 |
| *S. equorum* | | 58 | | | | | | | 1 | | | | 30 |
| *S. fleurettii* | 5 | | | | | | | | | | | | 5 |
| *S. haemolyticus* | | | 14 | | | | | | 4 | | | | 9 |
| *S. hominis* | | | | | | 1 | | 2 | | | 2 | | 2 |
| *S. hyicus* | 5 | | | 12 | 6 | 2 | 3 | | | | | | 6 |
| *S. intermedius* | | | | | | 1 | | | | | | | 1 |
| *S. lentus* | | | | | 11 | | | | | | 2 | | 7 |
| *S. muscae* | | | | | | | | | 1 | | | | 1 |
| *S. saprophyticus* | 7 | | | | | | | | 1 | | | | 4 |
| *S. schleiferi* | 5 | | | | | | | | 1 | | | | 3 |
| *S. sciuri* | | | | | | 1 | 2 | 3 | 3 | | | | 2 |
| *S. simulans* | 16 | 18 | 28 | | | 2 | | 2 | 2 | 16 | 9 | 9 | 11 |
| *S. warneri* | | | | | | 1 | 2 | 2 | | | | 9 | 4 |
| *S. xylosus* | | 22 | 14 | 23 | 21 | 5 | 7 | 6 | 2 | | 29 | 22 | 15 |

placed teats together with inappropriate udder conformation (deep and pendulous udder) may predispose to mastitis (Casu et al., 2010; Gelasakis et al., 2012). Machine milkability of such udders is poor, due to the frequent falling of the clusters and the relatively high quantity of retained milk in the udder cistern, thus requiring further stripping (Gelasakis et al., 2012).

### *4.5. Prolificacy and suckling of lambs*

In meat production flocks, a significant positive association between prolificacy and incidence of clinical mastitis has been recorded by Waage and Vatn (2008), with odds of developing the disease increased for ewes with at least two lambs. Increased incidence risk of clinical mastitis in ewes suckling multiple lambs has also been reported (Arsenault et al., 2008; Koop et al., 2010). Sucking by lambs contributes to transmission of *M. haemolytica* from the tonsils of the lamb to the teat duct of ewes (Fragkou et al., 2011). High numbers of suckling lambs per ewe is usually accompanied by more frequent sucking events and longer total sucking periods, promoting teat bites and subsequent development of teat lesions (Waage and Vatn, 2008). These have been well-documented to predispose to colonisation of the teat duct by bacteria and subsequent intramammary infection and mastitis (Mavrogianni et al., 2006b; Fragkou et al., 2007a; Koop et al., 2010). Perhaps, therefore, cross-sucking of lambs to ewes other than their dam can lead to transmission of bacteria among animals in the same flock (Bergonier et al., 2003).

### *4.6. Health status*

Immunocompromised ewes are more susceptible to diseases, including mastitis. Immunological stress can be the effect of health problems or of increased production. High production may lead to increased nutritional demands and consequential reduction in immunological competence (Houdijk et al., 2003), accounting, at least partly, for the increased incidence of intramammary infections during the initial stage of a lactation period (Bergonier et al., 2003). The peri-parturient relaxation of immunity that occurs in ewes (Barger, 1993; Coop and Kyriazakis, 1999) may be responsible for the increased incidence risk of the disease observed in the immediate post-partum period (Mavrogianni et al., 2014).

In general, various systemic or local health problems of ewes can be associated with development of mastitis. Waage and Vatn (2008) reported that dystocia was followed by an increased incidence of mastitis. In two recent studies, Mavrogianni et al. (2012,2014) have proposed that parasitic infections predisposed ewes to mastitis: Mavrogianni et al. (2012) described that nematode infections during lactation predisposed ewes to mastitis, whilst Mavrogianni et al. (2014) reported that trematode infections of pregnant ewes would support development of mastitis in the subsequent lactation period; proposed pathogenetic mechanisms were that depletion of energy by helminthes would affect leucocytic function in the affected animals, thus impeding efficient mammary defenses, whereas in trematode infections, increased β-hydroxybuturate concentrations had a direct effect in function of leucocytes (Mavrogianni et al., 2014). Also, recently, pregnancy-toxaemia has been recognised as a risk factor for mastitis in ewes (Barbagianni et al., 2015).

Teat lesions can also have an effect in intra-mammary infections. Increased bacterial accumulation in the skin of chapped teats, resulting from use of dense teat disinfectants or exposure to cold weather, may facilitate intramammary bacterial invasion and subsequent mastitis (Fragkou et al., 2007a). Ewes with lesions from epitheliotropic *Orf Virus* infection in the teats also predisposed to clinical mastitis (Mavrogianni et al., 2006a; Mavrogianni and Fthenakis 2007), considered as the result of depletion of local teat defenses (Billinis et al., 2012). Melchior et al. (2006) have suggested that recurrent intramammary infections in ewes may be associated with increased biofilm formation ability of the causative bacteria in the mammary glands of ewes with chronic mastitis; this might explain the previously reported positive correlation between parity number and mastitis prevalence (Fthenakis, 1994, 1996; Bergonier et al., 2003).

### *4.7. Genetic factors*

Genetic factors may possibly be involved in increased susceptibility of ewes to mastitis. Higher resistance against mastitis of an indigenous Greek sheep breed, as compared to an improved high-production breed, was attributed to more efficient local defense mechanisms in the teat of ewes of the indigenous breed (Fragkou et al., 2007b). A genetic background to increased susceptibility in mastitis in dairy ewes has also been reported (Barillet et al., 2001; Rupp et al., 2009; Bramis et al., 2014). These authors have suggested that, in selection of ewes for resistance to mastitis, use of reduced log-transformed somatic cell score may be employed as an indirect trait. Recently, Bonnefont et al. (2011) and Toufeer et al. (2011) have used a 15 K ovine-specific microarray for transcriptional profiling to study the biological pathways and genes determining the underlying susceptibility and resistance against staphylococcal mammary infections, which would help identification of biological pathways and genes in mastitis resistance.

## 5. Pathogenetic processes

The pathogenesis of mastitis is dependent on both microbial (bacterial virulence and antibiotic resistance) and host factors. In general, bacterial virulence is determined by the nature of the existing or produced virulent factors, the adherence ability of the bacteria and their ability to form biofilms. Virulence factors can include bacterial cell antigens, excreted toxins or both of them.

The thermostable enterotoxins of *S. aureus* may be of some importance in the pathogenesis of mastitis (Zadoks et al., 2011), although their significance is greater as a concern for public health (Kadariya et al., 2014). Other toxins of importance produced by *S. aureus* are leucotoxins, as these destroy neutrophils and monocytes, limiting the defense abilities of animals against intra mammary infections (Contreras et al., 2007). Bacterial adherence refers to the ability of the pathogens to remain attached to the epithelial tissues, whereas biofilm formation refers to the ability of bacterial cells to adhere within a matrix of self-produced extracellular polymeric substance (slime), allowing efficient multiplication of bacteria under less favorable conditions (Melchior et al., 2006). The key role of these factors in the virulence of *S. aureus* strains in cases of ovine mastitis has been described (Vautor et al., 2007). This study characterised 46 isolates of *S. aureus* from dairy ewes, according to *agr* group, adherence, slime production and antibiotic resistance, reporting that about 70% of the isolates belonged to *agr* group 3, 39% and 26% were adherent and slime producers, respectively, whereas, two isolates were susceptible to oxacillin. Similarly, interaction between mammary epithelial cells and bacterial slime was found to play a critical role in the development of intramammary infections (Aguilar et al., 2001). Bacterial slime facilitates initial attachment of bacteria on the epithelium and participates in formation of micro-colonies (Cucarella et al., 2004), playing an indirect role in antibiotic resistance through decreased antibiotic concentration within the colony and the increased resistance of dormant bacteria located at the center of the colony (Vautor et al., 2007). In later stages, biofilm formation promotes multiplication and enhances survival of invading bacteria, due to a protective action against antibacterial substances (Varhimo et al., 2011), considered crucial in determining bacterial virulence through the genetic and physiological changes associated with biofilm formation.

Although several virulence factors of *M. haemolytica* have been recognised, their role in the pathogenesis of mastitis is not yet fully understood (Omaleki et al., 2011). Among these, leucotoxin produced by *M. haemolytica* at its logarithmic phase of growth (Zecchinon et al., 2005) has been characterised in great detail. Less studied virulence factors include sialoglycerase, neuraminidase, immunoglobulin peptidase, lipopolysaccharides capsule and membrane proteins (Omaleki et al., 2011). The ruminant-specific pathogenetic action of this organism could be attributed to the selective cytotoxic action of leucotoxin against ruminant leucocytes (Zecchinon et al., 2005), causing their apoptosis (Atapattu and Czuprynski, 2005). The bacterial capsule also plays a critical role in the adherence and invasion of these bacteria (Zecchinon et al., 2005), in fact occurring within 10 min following infection (Vilela et al., 2004), whilst membrane proteins decrease immune responses of affected hosts and neuraminidase reduces the viscosity of mucus supporting bacterial apposition closer to the cell surface.

Despite extensive research on pathogenetic processes following intra-mammary infections, the sequence of events stimulating the overall immunological response has not yet been fully elucidated (Bonnefont et al., 2011). It is generally accepted that occurrence of mastitis is favored in cases of impairment of the various defense mechanisms of the mammary gland, reducing protective actions against bacterial intra-mammary infections. These mechanisms can be distinguished into innate immunity and specific immunity (Sordillo, 2005).

The results of a detailed investigation of the early stage of bacterial invasion have been published recently. Bacterial entrance into the teat duct can take place during naturally occurring events; for example, *M. haemolytica* is transmitted during sucking by lambs (Fragkou et al., 2011) and staphylococci are transferred by from hands of milkers or from clusters of the milking machine (Marco Melero, 1994). Deposition of bacteria 6 mm deep into clinically healthy teats always resulted in inflammation, whilst deposition 2 mm deep did not (Mavrogianni et al., 2005), thus indicating that mechanical factors, such as pushing of bacteria by the tongue of lambs or dilatation of the teat after milking (Gougoulis et al., 2008b) can promote the disease. Lymphoid hyperplasia identified in the lamina propria at the junction between the teat duct and teat cistern is considered to have a clear protective role to that structure (Mavrogianni et al., 2005). These focal lymphoid accumulations were later characterised and found to contain $CD79^+$, $CD3^+$, $\gamma d$ T cells, $CD68^+$ and MHC-$II^+$ cells (Mavrogianni et al., 2007; Fragkou et al., 2010). However, in cases of *Orf Virus* infection in the teats, these structures could not be observed (Mavrogianni et al., 2006a), as was also the case in chapped teats (Fragkou et al., 2007a). In these cases, bacterial deposition even at the tip of the teat resulted in clinical mastitis, confirming the protective role of these structures. There appeared to be a genetic background in the development of these lymphoid accumulations, as differences have been identified between breeds of dairy sheep in their presence and function (Fragkou et al., 2007b). It has been suggested that these lympho-epithelial structures develop following bacterial invasion and result from recruitment and expansion of antigen-specific lymphocytes in situ (Mavrogianni et al., 2005; Fragkou et al., 2010).

Within the mammary parenchyma, defense responses to bacteria that have overcome the defenses of the teat, are mediated by macrophages and neutrophils. Pathogens that have passed through the teat duct, activate macrophages, producing chemo-attractants as inflammatory mediators (Bonnefont et al., 2011). These provoke migration of neutrophils from the blood stream into the intra-mammary infection site, commencing their phagocytic-bactericidal activity. The migration of the neutrophils occurs either through gaps in the epithelial lining due to the degeneration of alveolar cells, or through more extensive sloughing of parts of epithelial lining (Akers and Nickerson, 2011). Inflammation and the migration of neutrophils are associated with an overall damage and sloughing of the ductal and alveolar epithelial cells, transforming the later cells into poorly-differentiated, non-secretory cells (Akers and Nickerson, 2011).

Macrophages can facilitate innate and specific immune responses in the mammary parenchyma. Similar to neutrophils, macrophages exhibit phagocytosis and destroy bacteria with proteases and reactive oxygen species (Sordillo, 2005). However, in cases of mastitis both their number and their Fc receptors are decreased compared to neutrophils, which renders them more useful for the non-specific defenses of the mammary gland, exhibited mainly by means of production of prostaglandins, leukotrienes and cytokines which support inflammation processes, rather than for phagocytosis (Sordillo, 2005). Macrophages also stimulate the specific immune responses through processing and presentation of the bacterial antigens to T and B lymphocytes. $CD4^+$ lymphocytes are responsible for activation of lymphocytes and macrophages through secretion of cytokines, whereas $CD8^+$ have a cytotoxic or suppressor function against host cells expressing bacterial antigens (Sordillo, 2005). Albenzio et al. (2012) concluded that intramammary pathogens induced activation of $CD4^+$ and $CD8^+$ and were related to higher levels of both tumor-necrosis factor-$\alpha$ and IL-12. On the other hand, B lymphocytes are responsible for production of antibodies (immunoglobulins) against specific bacterial pathogens. Among immunoglobulins, $IgG_1$, $IgG_2$ and IgM facilitate phagocytosis by neutrophils and macrophages acting as opsonins, whereas, IgA cause the agglutination of pathogens preventing their spread within the mammary gland (Sordillo, 2005).

Potentially, the recently described defenses in the teat of ewes can be exploited toward improving control of the disease. The findings suggest that these organised lymphoid structures are inducible and contribute to the defense of the teat and there may be merit in vaccine strategies that induce local immune responses in the teat.

## 6. Diagnosis

Diagnosis of clinical or subclinical mastitis has recently been reviewed in detail (Fragkou et al., 2014). Diagnostic procedures used in mastitis include clinical examination, bacteriological tests, cytological examination of milk (direct by using fluoro-optoelectronic counters and microscopic cell counting, indirect by using the California Mastitis test or the Whiteside test), measurement of milk electrical conductivity and imaging techniques (ultrasonography, endoscopy, infrared thermography) (Fragkou et al., 2014). Appropriate samples for the relevant diagnostic techniques include udder or teat skin swabs, teat duct material, milk, mammary tissue and blood samples.

Diagnosis of clinical mastitis is usually straightforward, based on findings of the clinical examination (swollen and painful udder, abnormal milk, high rectal temperature, lameness on the side of the affected gland). Further diagnostic tests (especially microbiological examinations) will support aetiological diagnosis of mastitis, which is important for effective treatment (Mavrogianni et al., 2011).

In contrast, diagnosis of subclinical mastitis requires application of specific tests. Increased somatic cell counts in ewes' milk coupled with bacterial isolation remains the 'gold standard' for diagnosis of subclinical mastitis. Different threshold values for somatic cell counts have been proposed. An interesting approach regarding thresholds of somatic cell counts has been proposed by Berthelot et al. (2005) for prediction of subclinical mastitis, with somatic cell counting used initially, followed by bacteriological examination in the diagnosis of the problem. The authors have proposed that somatic cell counts $<0.5 \times 10^6$ or $>1.0 \times 10^6$ cells $mL^{-1}$ indicate absence or presence of subclinical mastitis, respectively; when cell counts are within this range, a bacteriological examination of milk would be required for confirmation of subclinical mastitis.

In the future, biomics analyses are expected to be used in the diagnosis of the disease. Initial studies have already been published about the proteome of ewes' milk (Pisanu et al., 2011; Anagnostopoulos et al., 2014). More recently, biomarkers potentially suitable for early diagnosis of intramammary infections caused by *S. chromogenes* in ewes have been reported (Chiaradia et al., 2013); increased levels of proteins (SPA, SPB, C3, Ig1γ, IgμC, IgLλ, ALB, TTR) were observed in the secretion of infected mammary glands. The identification of molecules suitable for the early and accurate diagnosis of intramammary infections can be a fruitful area of future research.

Moreover, methods for identifying bacterial nucleic acids directly in milk samples (e.g., 'real-time' PCR assays) will further improve accuracy of aetiological diagnosis of mastitis and will reduce time required for that. Although there are already available such assays for *S. aureus* (Viguier et al., 2009), considerable efforts will be required to creating a rapid and financially viable test for routine use in samples of sheep origin. The problem arises because these assays use cow-based models, in which animal species *M. haemolytica* is not a significant mammary pathogen, in contrast to its significance in ewes.

## 7. Control

Control of intramammary infections in ewes needs to consider the numerous causative organisms, as well as the diversity of the risk factors. In the design and implementation of a control program, there are factors that should be addressed in all situations and factors of relevance to specific farms. Importantly, the welfare of animals should be considered with relevant standards maintained.

### *7.1. Effective treatment of mastitis cases during lactation*

Details of mastitis treatment have been recently reviewed (Mavrogianni et al., 2011). In small ruminants, no detailed protocols for treatment of mastitis are available, this being a limiting factor for effective treatment of the disease. Moreover, very few veterinary pharmaceutical products are licenced for specific use in sheep in many countries of the world, hence therapy must be accompanied by careful consideration of appropriate withdrawal periods for milk from animals under treatment.

Nevertheless, there is one established rule for effective treatment of mastitis: the combination of speed and efficacy (Mavrogianni et al., 2011). Treatment should start immediately after detection of the first signs of the disease and should be carried out using effective antimicrobial agents (Erskine et al., 2003; Fragkou and Fthenakis, 2014). Early instigation of treatment is necessary in minimising mammary lesions and restoring the health of affected ewes. However, often although clinical cure takes place, bacteriological cure cannot be achieved. Subsequently, bacteria present in the mammary gland may cause decreased production, develop mammary abscesses or cause a recrudescence of clinical disease. Effective treatment is important in control programs, as it minimises bacterial dissemination in the environment, thus reducing chances of infection of other animals in the flock.

Use of non-steroidal anti-inflammatory agents in mastitis has been advocated for alleviation of the clinical signs of the disease and improvement of the welfare of animals (McKellar, 2006). Flunixin meglumine can contribute to improvement of clinical signs, particularly of the mammary gland, and to returning body temperatures to normal (Fthenakis, 2000). In severe cases of the disease, intravenous dextrose administration in combination with electrolytes has been advocated and should be continued until full recovery of the animal occurs (Fragkou and Fthenakis, 2014).

### *7.2. General husbandry measures*

Control of mastitis requires improvement of the general hygiene standards in flocks. Frequent removal of litter and disinfection of the shed supports control of intra-mammary infections caused by environmental pathogens. Attention to an appropriate stocking density, sufficient bedding material, and increased ventilation, provides increased hygiene and reduced bacterial concentrations, through reduction of humidity and improvement of bedding hygiene. These measures are more significant in intensive production systems (Bergonier et al., 2003).

Balanced nutrition with high-quality feedstuffs will contribute to enhanced health status of ewes and will support efficient mammary gland function. Specifically, vitamin E and selenium status, which have been found to have an effect in the leucocytic activity (Morgante et al., 1999; Rooke et al., 2004) need to be investigated, as sheep are often deficient in these two nutrients (Govasmark et al., 2005; White and Rewell, 2006). The possibility for injectable administration of selenium to ewes during the last month of pregnancy should be considered; this will additionally prevent cases of deficiency in newborn lambs (Rock et al., 2001; Rooke et al., 2004).

As large numbers of lambs and a prolonged suckling period have been associated with increased risk to mastitis development, early weaning of lambs in large litters is recommended. The potential of implementing artificial rearing should also be considered, especially in intensive dairy-type flocks.

In meat production systems, cessation of a lactation period, and consequent mammary involution, is abrupt, taking place when lambs are removed from their dam. In dairy-type production systems, cessation of lactation can be effected either progressively (i.e., milking frequency can be gradually decreased over a period of several days or weeks) or abruptly (i.e., milking ceases). No differences in the incidence risk of intramammary infections and mastitis associated with the procedure of cessation of a lactation period, i.e., abrupt or progressive, have been reported (Petridis et al., 2012, 2013).

Culling of ewes at the end of a lactation period will contribute to reducing incidence risk of mastitis in a flock. Based on the results of the clinical examination of the udder and the ancillary tests performed (e.g., cytological examination) the following categories of ewes should be considered for culling: (i) animals with at least one mammary gland permanently damaged, (ii) animals chronically affected, (iii) animals with incidents of relapsing mastitis (iv) animals with no full response to mastitis treatment during the preceding lactation period. The benefits of culling such animals include: (i) decrease of veterinary expenses for mastitis control in the flock, (ii) elimination of sources of potential infection for other animals in the flock and (iii) decrease of flock bulk somatic cell counts in the subsequent lactation period (Mavrogianni et al., 2011; Petridis and Fthenakis, 2014).

In general, an overall high health status of ewes will support efficient control of mastitis by preserving an effective immune system. As a general rule, ewes with long-standing or recurrent diseases, especially with mammary abnormalities, need to be removed from the flock. This will additionally help to minimise dissemination of pathogens in a flock.

### *7.3. Milking practices and routine*

Milking practices need to be revised and proper operation of milking equipment must be checked periodically (Contreras et al., 2007). A well-designed standard operating protocol has to be followed by trained staff during milking. Implementing milking order, with healthy ewes milked first has been reported to lead to a decrease in incidence risk of the disease (Bergonier et al., 2003).

Rough handling during milking and abrupt removal of clusters should be avoided. If necessary, appropriate milk-stripping should be performed for removal of residual milk, but over-milking must be avoided. Operating parametres of milking machines that need to be considered include vacuum level, pulsation rate and ratio and system cleaning procedures. Vacuum fluctuations and over-used liners should be avoided. Milking system disinfection with water and disinfectant is necessary for preventing the accumulation of dirt that can increase risk of intramammary infections (Bergonier et al., 2003).

Post-milking disinfection of teats is effective for prevention of new intramammary infections by contagious bacteria (Bergonier and Berthelot, 2003; Contreras et al., 2007), but is of lesser significance in preventing infections by environmental pathogens (Klinglmair et al., 2005). For this reason, Bergonier and Berthelot (2003) have suggested a targeted implementation of post-milking teat disinfection specifically during periods with increased prevalence of clinical mastitis and/or teat problems (i.e., orf), which usually coincide with the start of milking period, immediately after lambs are removed from their dams. Quality of the disinfectant is crucial, as transfected teat dip solutions have been implicated in leading to intramammary infections (Tzora and Fthenakis, 1998), particularly in the case of recirculating teat dip cups. In order to avoid contamination, teat spraying can be applied, which is a faster procedure, albeit it requires a greater quantity of disinfectant. A significant disadvantage of the method is that teat coverage is not complete, hence teats are exposed to infections (Biggadike et al., 2003). Disinfectants often used in teat disinfection include chlorexidine, dodecyl benzene sulfonic acid, fatty acid-based products, glycerol monolaurate, hydrogen peroxide, iodophors, nisin, quaternary ammonium products and sodium chlorite. It is noteworthy, that resistance of staphylococcal strains against some of the above has now been reported (Bjorland et al., 2005).

### 7.4. Breeding for mastitis resistance

Breeding for mastitis resistance presents a sustainable means for control of mastitis. Programs focused on breeding for resistance against mastitis have used various traits (Psifidi et al., 2014), including indirect predictor traits, e.g., somatic cell scores. For example, the last few years, a breeding program applied in animals of Lacaune breed in France has been using somatic cell counting to select animals for improved resistance to mastitis. Recently, Rupp et al. (2009) reported that using decreased somatic cell counts for selection purposes in these animals could be linked to subsequent reduction of incidence risk of intramammary infections. A relationship between toll-like receptor gene polymorphism and bacterial intramammary infections was also suggested for use in breeding programs (Swiderek et al., 2006). Moreover, improvement of milkability can also be of assistance if included into genetic selection programs, by using udder traits associated with udder morphology, teat size and placement (Gelasakis et al., 2012); the rationale of this approach is that efficiency of machine milking will subsequently lead to reduced incidence risk of intramammary infections at flock level. Recently, mapping of a quantitative trait locus for mastitis resistance on OAR3 in Lacaune dairy sheep has been reported (Rupp et al., 2013), whilst a genome-wide association analysis of resistance to mastitis in dairy sheep has also been published (Sechi et al., 2013).

### 7.5. Strategic use of antibiotics at the beginning of the dry-period

Strategic use of antibiotics is effective for control of intramammary infections and should be implemented at the beginning of each dry period ('dry-period' treatment). The principle of antibiotic administration at the end of a lactation period involves the intramammary infusion of a pharmaceutical preparation to both mammary glands of ewes in the flock. Antibiotic administration at that period is an integral part of udder health management, which aims to (i) cure infections that have occurred during the preceding lactation period and (ii) prevent development of new intramammary infections during the forthcoming dry-period (Fthenakis et al., 2012). The benefits of administration of antibiotics at the end of each lactation period have been recently reviewed in detail by Petridis and Fthenakis (2014). The procedure can lead to subsequent reduction of incidence risk of mastitis during the dry-period in flocks (e.g., Gonzalo et al., 2004; Linage and Gonzalo, 2008; Schwimmer et al., 2008), reduced bulk milk somatic cell counts (Schwimmer et al., 2008; Gonzalo et al., 2009) and increased milk yield (Schwimmer et al., 2008) in the subsequent lactation period.

In recent years, as the result of concerns regarding (i) potential antibiotic residues in the food chain and (ii) increased incidence of antibiotic-resistant bacterial strains in animals, 'selective' administration of antibiotics to ewes at drying-off has been advocated. This involves administration of antimicrobial agents only to animals considered to be infected, based on results of clinical examination of the udder and cytological examination of milk samples, to correctly identify ewes in need of antibiotic administration (Petridis and Fthenakis, 2014). The approach is effective in curing mammary infections of animals prevalent at the end of a lactation period, but does not afford protection to untreated ewes in the flock against new intramammary infections during the dry-period (Berry and Hillerton, 2002; Bergonier and Berthelot, 2003), especially during the stage of active involution when there is an increased risk of mastitis (Saratsis et al., 1998).

### 7.6. On-farm monitoring and recording

On-farm monitoring (e.g., by regular examination of milk samples by using the California Mastitis Test) provides surveillance of the mammary health of animals and needs to be included in the general strategy for control of mastitis in a flock. Finally, maintenance of accurate records of the health status of ewes will also contribute to the success of every mastitis control program.

### 7.7. Vaccination

Vaccines licenced against ovine mastitis aim to protect animals against staphylococcal mastitis. In general, these are of older technology and suboptimal efficacy, benefiting vaccinated animals primarily by reducing severity of clinical signs, with several publications appearing occasionally and describing modifications to existing licenced staphylococcal vaccines (e.g., Tollersrud et al., 2002; Hadimli et al., 2005). Vaccines active against other pathogens, e.g. the successful J5 vaccine against *E. coli* mastitis or experimental vaccines against *P. aeruginosa* (Leitner and Krifucks, 2007) or *Streptococcus uberis* (Gilchrist et al., 2013) are of little significance in sheep, as these organisms are only minor mammary pathogens.

More recently, biofilm matrix polysaccharides were used for development of protective immune response against *S. aureus* mastitis in ewes (Perez et al., 2009). This approach employs cell-free surface polysaccharide in various vehicles, bacterial unbound cells or bacterial cells embedded in their biofilm matrix in various adjuvants. Vaccination with whole bacterial cells surrounded by matrix with polysaccharide conferred protection against *S. aureus* mammary infection and mastitis, which appeared to be related to the level of antibodies to *S. aureus* polysaccharide and to the degree of biofilm formation (Perez et al., 2009). Subsequently to this approach a vaccine (Vimco®) has been licenced for use in ewes for protection against mastitis, with a recommended administration six to four weeks before the expected lambing, followed by a booster dose two weeks later; annual repeats of the scheme at the end of every subsequent pregnancy should be subsequently carried out.

## 8. Concluding remarks

In recent years, a large amount of literature has supported increase of knowledge about mastitis in sheep. Nevertheless, there are still facets of the disease that require further studies for elucidation, and there is still a long way to fully understand the problem. The present paper reviews the recently published literature about the disease. Recent publications have highlighted the significance and virulence factors of the causative agents, especially *S. aureus* and *M. haemolytica*, the primary causes of the disease. Research has also contributed to highlighting risk factors for the disease, including genetic susceptibility of animals to infections, which can support in the future means for sustainable control of the problem. Pathogenetic mechanisms, with special reference to the role of the local defenses in the teat, have also been elucidated in recent years and can be exploited in formulating strategies that induce local immune responses in the teat of ewes, as a means for protecting animals. Further to the well-established, bacteriological tests and somatic cell counting, advanced diagnostic methodologies (e.g., proteomics technologies) will contribute to rapid and accurate diagnosis of the problem, which in turn will enhance effective treatment efforts. Although control methods of the disease have been improved, in the future more sustainable approaches will be necessary.

## Conflict of interest

The authors have nothing to disclose.

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