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Symposium review: Intramammary infections—Major pathogens and strain-associated complexity*

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ABSTRACT

Intramammary infection (IMI) is one of the most costly diseases to the dairy industry. It is primarily due to bacterial infection and the major intramammary pathogens include *Escherichia coli*, *Streptococcus uberis*, and *Staphylococcus aureus*. The severity and outcome of IMI is dependent on several host factors including innate host resistance, energy balance, immune status, parity, and stage of lactation. Additionally, the infecting organism can influence the host immune response and progression of disease. It is increasingly recognized that not only the infecting pathogen species, but also the strain, can affect the transmission, severity, and outcome of IMI. For each of 3 major IMI-associated pathogens, *S. aureus*, *Strep. uberis*, and *E. coli*, specific strains have been identified that are adapted to the intramammary environment. Strain-dependent variation in the host immune response to infection has also been reported. The diversity of strains associated with IMI must be considered if vaccines effective against the full repertoire of mammary pathogenic strains are to be developed. Although important advances have been made recently in understanding the molecular mechanism underpinning strain-specific virulence, further research is required to fully elucidate the cellular and molecular pathogenesis of mammary adapted strains and the role of the strain in influencing the pathophysiology of infection. Improved understanding of molecular pathogenesis of strains associated with bovine IMI will contribute to the development of new control strategies, therapies, and vaccines. The development of enabling technologies such as pathogenomics, transcriptomics, and proteomics can facilitate system-level studies of strain-specific molecular pathogenesis and the identification of key mediators of host-pathogen interactions.

Key words: intramammary infection, strain, mastitis pathogens

INTRODUCTION

Bovine mastitis, inflammation of the mammary gland, is a disease of substantial economic importance. The major cause of mastitis is IMI by a bacterium, and although a wide variety of species have been associated with IMI, several pathogens, including *Staphylococcus aureus*, *Streptococcus uberis*, *Escherichia coli*, and CNS, account for the majority of cases (Bradley et al., 2007; Petrovski et al., 2011; Keane et al., 2013). The prevalence of these major IMI-associated pathogens varies from region to region. In the United States and United Kingdom, *E. coli* and *Strep. uberis* have been reported as the species most commonly associated with clinical IMI (Bradley et al., 2007; Hertl et al., 2014), whereas in the pasture-based production systems of Ireland and New Zealand, *S. aureus* and *Strep. uberis* predominate (Petrovski et al., 2011; Keane et al., 2013).

The costs associated with mastitis result from reduced milk yield and quality, discarded milk, veterinary and treatment costs, culling of chronically infected animals, and increased labor. It is difficult to accurately estimate the cost of bovine mastitis globally, but it has been reported that in Ireland an increase in the bulk milk SCC from <100,000 cells/mL to 300,000–400,000 cells/mL decreased net farm profit from €0.059/kg to €0.033/kg (Geary et al., 2012). In the United States it was reported that the average cost of a mastitis case was $325.75 for first lactation heifers and $426.50 in later lactations (Liang et al., 2017), whereas in the Netherlands the total economic losses associated with clinical and subclinical mastitis were estimated to be €114 to €182 per cow per year (Huijps et al., 2008). In addition to the economic impact, mastitis can have serious negative consequences for animal health and welfare.

Intramammary infections can present as clinical or subclinical. Clinical mastitis is associated with visible changes in the milk or udder (flakes or clots in the milk, redness, swelling, or hardness in the udder) whereas the

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more common subclinical mastitis is characterized by changes in some parameter of the milk; a rise in SCC above 200,000 cells/mL or an increase in the activity of *N*-acetyl-β-D-glucosaminidase can indicate that an infection has occurred (Bradley, 2002; Hovinen et al., 2016). The major mastitis-causing pathogen species are commonly associated with different courses of infection, although the relationship is not absolute. *Escherichia coli* is often associated with rapid onset, acute mastitis with severe symptoms. In many cases the infection is self-resolving without the need for veterinary intervention; however, it can occasionally lead to systemic infection and death (Zadoks and Fitzpatrick, 2009). In contrast, infection with *S. aureus* is usually less severe but frequently results in a chronic infection that is difficult to eradicate. It may also present with occasional clinical flare-ups (Wellnitz and Bruckmaier, 2012). *Streptococcus uberis* is regularly associated with subclinical IMI, which in some instances can persist in the intramammary environment; however, clinical *Strep. uberis* infection also occurs (Zadoks et al., 2003; Bannerman et al., 2004a).

Mastitis pathogens have historically been classified as contagious or environmental based on their primary reservoir and mode of transmission (Bradley, 2002). The prevalence of IMI in a herd is dependent on the rate at which new infections arise and the length of those infections (Wilson et al., 1997). These in turn are influenced by a variety of factors such as parity, stage of lactation, the nature of the infectious agent, and the nutrition and environment of the cow (Smith et al., 1984; Hertl et al., 2011; Elghafghuf et al., 2014; Moyes, 2015). Although contagious pathogens are transmitted from cow to cow, usually at milking, environmental pathogens, with a reservoir in the cow's environment, often infect opportunistically. The major contagious pathogens were traditionally considered to be *Streptococcus agalactiae* and *S. aureus* with *E. coli* and *Strep. uberis* considered environmental pathogens (Bradley, 2002). More recently it has been recognized that some IMI pathogens may be capable of both contagious and environmental transmission and that the mode of transmission may be associated with the strain rather than species of pathogen (Zadoks et al., 2000; Budd et al., 2015; Davies et al., 2016). Prevention and management of contagious mastitis relies on the implementation of several well-characterized infection control strategies such as those outlined in the National Mastitis Council's "5-point" plan (NMC, 2011), later extended to a "10-point" plan. Control programs have been quite successful in reducing infectious mastitis, and in particular in decreasing the prevalence of *Strep. agalactiae*. However, their success in controlling *S. aureus* mastitis has been more variable (Ruegg, 2017). The precise reason for this is unknown, but *S. aureus* can be irregularly shed in milk, can form walled-off infections, and has a low cure rate after anti-microbial therapy (Sears et al., 1990; Sol et al., 1997). The ability of *S. aureus* to act as a contagious or environmental pathogen with bovine and environmental reservoirs may also contribute to difficulties in controlling this pathogen (Sommerhauser et al., 2003; Klaas and Zadoks, 2018).

Mastitis control in dairy herds has traditionally involved the treatment of clinical cases with antimicrobial agents, with blanket use of antibiotic dry cow therapy at the end of lactation also commonly practiced (Francoz et al., 2016). Given societal concern regarding the use of antimicrobials in veterinary medicine, alternative strategies to prevent and manage bovine IMI are urgently required (Thanner et al., 2016). The purpose of this review is to highlight advances in our knowledge and understanding of pathogen and strain-specific IMI for the major pathogens *S. aureus*, *Strep uberis*, and *E. coli* and to share recent insights on the role of the pathogen strain in influencing the host response and disease progression.

PATHOGEN-ASSOCIATED HOST IMMUNE RESPONSE

The outcome of any IMI is dependent on several host factors including nutritional status, immune status, genetic makeup, parity, and stage of lactation (Rupp and Boichard, 2003; Hertl et al., 2011; Elghafghuf et al., 2014). Despite the extensive influence of the host on the progression and outcome of IMI, pathogen-specific factors also play an important role in the pathophysiology of infection. The immune response can vary depending on both the species of the infecting bacterium and the strain of the infecting agent. The immune response to the major IMI pathogen species *S. aureus*, *Strep uberis*, and *E. coli* has recently been reviewed (Schukken et al., 2011; Petzl et al., 2018), whereas strain-associated differences are detailed below. Innate immunity is a key component in the recognition and initiation of an immune response to IMI. Pathogen-associated molecular patterns (**PAMP**), conserved bacterial structures, are recognized by their cognate pattern recognition receptors expressed on the mammalian cell surface or within host cells. This instigates a signaling cascade that ultimately results in the activation of the transcription factor complex NF-κB and the induction of pro-inflammatory cytokines and chemokines (Akira and Takeda, 2004), resulting in the influx and activation of immune cells in the mammary gland. Resident mammary epithelial cells (**MEC**) are proposed to be key primary actors in the initiation of a species-associated response (Wellnitz and Bruckmaier,

2012; Bauer et al., 2015; Gunther et al., 2016b). It has been reported that experimental infection of first lactation cows with *E. coli* resulted in a large increase in mammary gland expression of *TNFα*, *IL8*, *IL10*, and *IL12* by 24 h postinfection, whereas no such increase was evident in *S. aureus* infected glands (Yang et al., 2008). Infection of primary bovine MEC (**pbMEC**) in vitro with heat-killed *E. coli* results in activation of TLR4, subsequent NF-κB activation, and a large increase in expression of *TNFα* and the chemokine *IL8*. In contrast, infection of pbMEC with heat-killed *S. aureus* fails to activate NF-κB and results in induction of pro-inflammatory cytokines to a much lower level than that induced by *E. coli* (Yang et al., 2008; Gunther et al., 2016b). The major PAMP of *E. coli*, LPS, is a potent stimulator of TLR4, and interaction of LPS with TLR4 and its co-receptor CD14 at the surface of MEC and milk somatic cells, particularly macrophages, alerts the host immune system to the presence of an infecting microbe (Wang et al., 2002). The chemokine IL-8 promotes the recruitment of neutrophils, potent phagocytic leukocytes, to the udder, and protection from infection is dependent on the rapid recruitment of neutrophils and subsequent phagocytosis of invading microorganisms (Paape et al., 2002). However, it has been demonstrated that *S. aureus*, *Strep. uberis*, and *E. coli* each have the ability to invade and persist within bMEC, representing a niche where the organisms may evade phagocytosis (Matthews et al., 1994; Hebert et al., 2000; Dopfer et al., 2001). Differences between strains in ability to resist phagocytosis by bovine PMN in vitro have also been demonstrated, which may facilitate persistence of the organism in the mammary gland (Leigh et al., 1990; Leigh and Field, 1991; Aarestrup et al., 1994; Mullarky et al., 2001; Blum et al., 2008; Roussel et al., 2017). Pathogen-associated variation in the intramammary immune response is evident in the first hours after infection as *E. coli* immediately activates expression of pro-inflammatory genes, whereas *S. aureus* infection results in a slower response of lower magnitude (Yang et al., 2008; Petzl et al., 2016). It has been hypothesized that failure of *S. aureus* to activate NF-κB in MEC contributes to the diminished immune response observed in cows challenged with *S. aureus* compared with those challenged with *E. coli* (Riollet et al., 2000; Bannerman et al., 2004b; Lee et al., 2006; Yang et al., 2008). Although much initial work on the species-associated immune response focused on *S. aureus* and *E. coli*, it was subsequently shown that infection of isolated pbMEC with *Strep. uberis* also fails to activate NF-κB and subsequent induction of pro-inflammatory cytokines (Gunther et al., 2016a). Studies using a macrophage cell line have suggested that this cell type responds to all 3 major IMI pathogen species by activation of NF-κB and strong induction of immune gene expression (Gunther et al., 2016b). Much of the research to date has focused on the in vitro response of the different cell types to challenge with the major IMI pathogens. The differential induction of host immunity and the local and systemic responses observed postinfection are likely due to the combined response of both resident and recruited cells and the cross-talk between them. Future research should focus on understanding the interaction between the major cell types important in generating an immune response in vivo.

GENOMICS OF IMI PATHOGENS

Within each of the major bovine IMI pathogen species, a diversity of strains and lineages (see Table 1) exists, each with a potentially unique repertoire of virulence factors and immune-stimulatory antigens (Budd et al., 2015; Hossain et al., 2015; Keane, 2016). Strain typing of *E. coli*, *Strep. uberis*, and *S. aureus* isolates recovered from IMI has relied on several well-characterized methods including pulsed-field gel electrophoresis (**PFGE**), ribotyping, and multilocus sequence typing (**MLST**) to provide insights into their molecular epidemiology. Each method has its advantages and disadvantages; PFGE, which compares banding patterns after restriction digestion of bacterial genomic DNA, has a high discriminatory power but is difficult to standardize across laboratories making comparisons across studies difficult. Multilocus sequence typing is a reproducible typing method based on the sequence of multiple house-keeping loci, with each unique sequence an allele. As MLST is based on allele rather than sequence comparison, it takes account of the fact that allele substitution by horizontal gene transfer will introduce many more polymorphisms than point mutation (Maiden et al., 2013). Multilocus sequence typing allows inference about the population structure and evolutionary history of the isolates, although it is less discriminatory than PFGE (Grundmann et al., 2002; Ikawaty et al., 2009; Adkins et al., 2016). The availability of online MLST databases for several microbial pathogens, including *S. aureus*, *Strep uberis*, and *E. coli*, facilitates data sharing. The association between the various genotyping methods ranges between good agreement to poor agreement (Boss et al., 2016), and the choice of typing method may depend on the population structure of the pathogen in question. *Staphylococcus aureus* has a predominantly clonal population structure with infrequent recombination among strains, whereas recombination is more common in *E. coli* and particularly in *Strep. uberis* (Zadoks et al., 2005a; Coffey et al., 2006; Wirth et al., 2006; Smyth et al., 2009). Specific bovine-adapted lineages of *Strep. uberis* and

Table 1. Definitions of terms

Term	Definition
Isolate	A pure culture of bacteria derived from a single organism.
Strain	Similar isolates that can be distinguished from other isolates of the same species by phenotypic or genotypic methods.
Lineage	A group of genetically related strains derived from a recent common ancestor. The rate of diversification depends on the extent of recombination.
Host-adapted	Infects, persists, and transmits within a specific host species.

E. coli have not been identified although an association between bacterial lineage and disease status has been reported for *Strep. uberis*, with CC5 associated with clinical disease, ST143 with subclinical disease, and ST86 with latent infection (Tomita et al., 2008). In contrast, for *S. aureus* distinct lineages are associated with bovine infection, although the prevalence of the different lineages may vary geographically (Smyth et al., 2009; Budd et al., 2015). Each lineage has evolved relatively independently with only infrequent horizontal gene transfer between lineages (Waldron and Lindsay, 2006). The genome of *S. aureus* consists of core genes, found in every isolate, and variable genes that can be present or absent depending on the strain. Many of the *S. aureus* virulence factors are encoded by variable genes, and carriage of these genes is often conserved within isolates of a given lineage (Lindsay, 2010; Budd et al., 2015). Therefore identifying the lineage of the infecting strain of *S. aureus* can also characterize the strain with respect to virulence factors.

Traditional strain-typing methods are fast being replaced by typing by whole-genome sequencing (**WGS**; Schurch et al., 2018). Next-generation sequencing greatly increases discriminatory power, thereby allowing closely related strains to be distinguished. Importantly, it also enables rapid identification of emerging strains. Whole-genome MLST increases the number of alleles typed while maintaining back-compatibility with traditional MLST (Maiden et al., 2013). It further allows identification of antimicrobial resistance and virulence genes. Identification of the most prevalent IMI strains, using a technique such as WGS or whole-genome MLST, which is unambiguous and easily comparable across laboratories, will facilitate the identification of globally distributed strains or lineages. It will also allow systematic review and meta-analysis of studies examining the association between strain or lineage and infection characteristics such as infection severity, infection duration, transmission, and cure rate after antimicrobial therapy. Comparative genomics with strains isolated from humans or other animals may also lead to the identification of loci important for pathogenicity in each specific host, an approach that has already shown much promise (Lowder et al., 2009; Guinane et al., 2010; Blum et al., 2018). The cost and labor requirements of WGS means this technique is likely to remain a research tool in the short term. However, comparative genomics of large numbers of IMI-associated strains could identify common genomic features of the predominant strains and lineages facilitating the development of fast, cheap, and accurate strain-typing methods that could be used in a clinical diagnostic setting.

BACTERIAL STRAIN INFLUENCES THE HOST IMMUNE RESPONSE

E. coli

Within each of the major mastitis-associated pathogen species, a variety of strains have been associated with disease or with different courses of disease. Although many *E. coli* IMI are transient infections of short duration, persistent *E. coli* IMI, characterized by repeated episodes of clinical mastitis interspersed by periods of subclinical infection, can also be observed (Dopfer et al., 1999; Bradley and Green, 2001). The existence of genetically distinct strains of *E. coli* responsible for persistent and transient mastitis has been postulated (Dogan et al., 2012; Kerro Dego et al., 2012). Evidence for phenotypic differences between strains isolated from persistent and transient mastitis was provided when it was shown that strains of *E. coli* associated with persistent infection invaded, survived, and replicated in bMEC more highly than those associated with transient infection (Dogan et al., 2006). It was additionally shown that persistent IMI-associated and transient IMI-associated strains used different pathways for internalizing within bMEC (Passey et al., 2008; Almeida et al., 2011). Despite these phenotypic differences, isolates from persistent and transient IMI are genetically diverse with no individual gene found to be more prevalent in either group, suggesting they evolved on multiple occasions from various commensal ancestors (Dogan et al., 2012; Richards et al., 2015).

For many years it was believed that the host immune response to *E. coli* IMI was driven by the conserved PAMP LPS and the infecting strain of *E. coli* had little or no influence on the host immune response to IMI (Burvenich et al., 2003). This view was supported by

studies that demonstrated that signs and symptoms of *E. coli* IMI were more homogeneous than those observed after *S. aureus* challenge (Petzl et al., 2008). However, although infection of pbMEC with strains of *E. coli* associated with either persistent or transient mastitis was reported to result in the upregulation of a large number of common genes, each strain type also had a unique gene expression signature. Infection with a strain isolated from a case of acute mastitis resulted in upregulation of immune response genes compared with infection with a strain isolated from a case of persistent mastitis, reflecting the higher level of inflammation in the acute case (Kerro Dego et al., 2012). More recently it has also been shown that the bovine immune response to intramammary challenge with 4 distinct strains of *E. coli* varied according to the infecting strain (Blum et al., 2017). The strains were isolated from various sources (acute mastitis, persistent mastitis, or the cow environment). Infection with strains VL2874 (acute mastitis) and VL2732 (persistent mastitis) resulted in the greatest increase in SCC and decrease in milk yield, whereas infection with strain P4 (acute mastitis) also resulted in increased SCC and decreased milk yield but the infection was resolved in 7 d. In comparison, *E. coli* strain K71 (cow environment) failed to establish an infection in the mammary gland and did not elicit any detectable immune or inflammatory response. Significant differences were also present between the strains in their ability to induce expression of several secreted cytokines in milk (Blum et al., 2017).

Several studies have examined *E. coli* isolates for evidence of a specific subset of mammary-pathogenic *E. coli* (**MPEC**) with sometimes conflicting results. A variety of *E. coli* phylogroups have been associated with IMI and in some studies particular phylogroups or phylogenetic lineages were more commonly associated with mastitis, whereas in others no association was found (Blum et al., 2008; Blum and Leitner, 2013; Keane, 2016; Leimbach et al., 2017). However, limitations of the strain-typing methods used may have contributed to the failure to detect MPEC as a recent whole-genome comparison of 66 phylogroup A mammary-derived *E. coli* with phylogroup A dairy farm-derived *E. coli* found that mammary isolates had a reduction in phylogenetic diversity and a larger core genome but smaller pangenome than dairy farm isolates (Goldstone et al., 2016). Nineteen genes clustered in 3 loci were identified that were proposed to be essential for MPEC including the *fecIRABCDE* genes encoding the iron dicitrate utilization pathway. The genes *fecABCDE* form an operon composed of structural genes encoding for the outer membrane siderophore receptor (FecA) and a transport system (FecBCDE), whereas *fecI* and *fecR* encode ferric-citrate transport regulator proteins. Genes belonging to this locus were found in 100% of the MPEC isolates but only 68% of the other phylogroup A genomes (Goldstone et al., 2016). The protein products of this locus allow the bacteria to capture iron from ferric citrate and were subsequently demonstrated to facilitate growth of *E. coli* in milk. They were additionally shown to be required for *E. coli* to cause IMI as deletion of the *fec* genes rendered the MPEC strain P4 nonpathogenic, whereas their introduction into the nonpathogenic strain K71 rendered this strain capable of causing IMI (Blum et al., 2018).

Strep. uberis

Streptococcus uberis is an IMI pathogen with reservoirs in the cow's environment such as soil, bedding material, bovine body sites, and fecal matter (Zadoks et al., 2005b). Initial studies on the epidemiology of *Strep. uberis* IMI demonstrated a wide variety of genotypes (Jayarao et al., 1993; Oliver et al., 1998; Phuektes et al., 2001). It was further reported that similar strains were recovered from environmental sources as from IMI, consistent with an environmental mode of transmission (Zadoks et al., 2005b). However, despite the importance of environmental sources in the transmission of infection, it has also been demonstrated that, in some instances, within-cow or cow-to-cow transmission of *Strep. uberis* may occur (Phuektes et al., 2001; Zadoks et al., 2001; Tomita et al., 2008). Persistent *Strep. uberis* infection has additionally been documented, with the same strain recovered from the mammary gland of some cows from one lactation to the next (Oliver et al., 1998).

Early evidence for strain-associated virulence of *Strep. uberis* was provided by Hill (1988) who demonstrated that strain 0140J reliably induced clinical mastitis after intramammary infusion and was resistant to killing in vitro by PMN, whereas strain EF20 only rarely induced clinical mastitis after intramammary infusion and was killed by PMN in vitro (Hill, 1988). However, the association between ability to induce clinical mastitis and resisting killing by PMN was not replicated in a separate study using different strains (Tassi et al., 2015). Although the same strains of *Strep. uberis* are frequently associated with both clinical and subclinical mastitis, there have been reports of particular strains of *Strep. uberis* more commonly associated with clinical mastitis, further suggesting strain-specific virulence (Jayarao et al., 1993; Phuektes et al., 2001; Zadoks et al., 2003). The duration of *Strep. uberis* IMI has also been reported to be strain-dependent, with some strains causing infections of significantly longer duration than others (Zadoks et al., 2003). *Streptococcus uberis* has been reported to persist for extended periods

of time within bMEC without loss of host cell viability and strains also appear to vary in their invasiveness (Matthews et al., 1994; Tamilselvam et al., 2006).

The host immune response to *Strep. uberis* IMI has been proposed to vary according to the infecting strain. Infection of pbMEC with 2 different strains of *Strep. uberis* had different effects on the induction of immune response genes as determined by quantitative reverse-transcription PCR (Swanson et al., 2009). Infection of pbMEC with strain 233 had no effect on immune gene expression, whereas infection with strain 266, a hyaluronic acid encapsulated strain isolated from a case of clinical mastitis, induced expression of *C3*, *IL1β*, and *SAA3*. An immune response particular to the infecting *Strep. uberis* strain was further demonstrated by Wellnitz et al. (2012) who examined in vitro the response of pbMEC and milk somatic cells to a strain of *Strep. uberis* associated with persistent mastitis compared with a strain associated with acute mastitis. The strain isolated from acute mastitis caused a greater increase in expression of certain pro-inflammatory cytokines (*IL8*, *IL1*β) potentially reflecting the more severe inflammation associated with this strain (Wellnitz et al., 2012). Contrary to these reports, Gunther et al. (2016a) reported no difference between heat-killed preparations of 7 strains of *Strep. uberis* in their ability to induce immune gene expression in pbMEC, and this cell type was found to fail to activate TLR signaling or NF-κB activation after *Strep. uberis* challenge (Gunther et al., 2016a). In contrast they reported that RAW267.4 cells, a murine macrophage cell line, upregulated immune gene expression in response to *Strep. uberis* infection, albeit in a strain-independent manner (Gunther et al., 2016a). However, they used only heat-killed preparations of *Strep. uberis* when investigating the immune response, which may not have fully reflected the variability between strains.

The in vivo host response to a strain of *Strep. uberis* associated with persistent infection was compared with the response to a strain associated with transient infection. Intramammary infusion of the persistent IMI-associated strain led to clinical mastitis, decreased milk yield, elevated SCC, and increased expression of pro-inflammatory cytokines in milk. In contrast, infusion of the strain associated with transient mastitis failed to induce any clinical signs and bacteria were eliminated from the mammary gland by 3 d postinfection (Tassi et al., 2013). The strain from a case of persistent mastitis was more resistant to killing by macrophages but less resistant to killing by PMN than the strain from transient mastitis. The persistent IMI-associated strain also formed a weaker biofilm and was better able to adhere to bMEC, although it did not internalize more efficiently. The authors therefore suggested that ability to adhere to bMEC and to resist killing by milk macrophages may be key virulence factors for *Strep. uberis* (Tassi et al., 2015).

The molecular mechanism underpinning the relationship between the infecting strain and the pathophysiology of *Strep. uberis* IMI has proven difficult to elucidate. Despite the differences between strain 0140J and EF20 in ability to induce IMI, whole-genome analysis of these strains, along with other strains associated with clinical and subclinical infection, failed to illuminate an obvious genetic reason for this difference (Hossain et al., 2015). Further research is required to reveal the virulence factors necessary for *Strep. uberis* to cause bovine IMI and the basis of the observed strain-specific virulence.

S. aureus

Staphylococcus aureus has a highly clonal population structure and it is well known that particular lineages have adapted to infect a bovine host (Smyth et al., 2009; Budd et al., 2015). These lineages include the pandemic bovine-adapted lineages CC97 and CC151 (also known as CC705) as well as CC8, CC479, and CC133. Each lineage carries a particular variable genome characteristic of that lineage, and differences between lineages in geographical distribution and virulence attributes have been reported (Zbinden et al., 2014; Budd et al., 2016; Cosandey et al., 2016). Despite the limited number of lineages that predominate among bovine-adapted *S. aureus,* there is nonetheless considerable strain diversity. Early studies on the molecular epidemiology of *S. aureus* IMI differed on whether the strain influenced disease presentation. Whereas some studies found that the infecting *S. aureus* strain had no bearing on IMI indicators such as SCC and *N*-acetyl-β-D-glucosaminidase activity (Middleton et al., 2002), others reported that the severity of disease was related to the strain (Zadoks et al., 2000; Haveri et al., 2005). Additionally, particular genotypes, most notably genotype B (which predominantly consists of strains belonging to CC8), have been reported to be associated with contagious mastitis with a high within-herd prevalence (Graber et al., 2009). It has also been reported that the inoculum required to induce IMI varies depending on the *S. aureus* strain. Whereas 10,000 cfu is required to reliably induce IMI with strain 1027 (CC133; Petzl et al., 2008), an inoculum of <100 cfu is sufficient for strain Newbould 305 (CC97; Bannerman et al., 2004b; Kauf et al., 2007; Bannerman et al., 2008).

Considerable evidence also points to differences between strains and lineages in virulence traits. Adherence to epithelial cells has been proposed to play a role in the pathogenesis of *S. aureus* and to prevent the removal of the bacteria by milk flow. Subsequent

epithelial cell invasion by receptor-mediated endocytosis may contribute to the persistence of *S. aureus* IMI and the failure of antibiotic therapy to clear *S. aureus* from the infected mammary gland. Differences between strains of *S. aureus* in their ability to adhere to and invade pbMEC have been reported (Hensen et al., 2000). Strains belonging to CC151 lack several well-characterized *S. aureus* surface proteins known to mediate interaction with host cells, either directly or via extracellular matrix proteins (Herron-Olson et al., 2007; Budd et al., 2015) and strains from this lineage show reduced adherence to bMEC compared with strains from other lineages (Zbinden et al., 2014). Persistent infection has also been proposed to be related to the ability of *S. aureus* to form small colony variants (**SCV**) that can survive within host cells with minimal deleterious effects (Atalla et al., 2008). Challenging cows with a parental strain and its SCV demonstrated that the SCV elicited a milder form of mastitis characterized by a lower SCC and a delay in mounting an SCC response. The authors ascribed the ability of the SCV to induce a mild IMI to the reduced growth rate of the bacterium in combination with reduced production of α-toxin (Atalla et al., 2009). Antimicrobial susceptibility has also been reported to be associated with *S. aureus* lineage (Sakwinska et al., 2011; Moser et al., 2013; Budd et al., 2015).

The ability of the infecting strain of *S. aureus* to modulate the host immune response was reported by Zecconi et al. (2005) who found that infection with isolates of particular genotypes was associated with a lower SCC response (Zecconi et al., 2005). A potential molecular mechanism underpinning this strain-dependent effect on SCC was provided by Lahouassa et al. (2007) who infected pbMEC with 3 strains of *S. aureus* and demonstrated differences between the strains in their ability to induce host cells to upregulate transcription of the pro-inflammatory cytokines *IL1*β, *TNF*α, and *IL8* and to produce chemoattractant factors capable of polarizing neutrophils (Lahouassa et al., 2007). However, the strains used in this study were not typed. More recently, the ability of strains of defined lineages to induce a pro-inflammatory response from bMEC has been characterized. It was found that infection of bMEC with strains of *S. aureus* belonging to CC151 resulted in lower pro-inflammatory gene and protein expression in addition to a longer lag time before gene expression was induced compared with strains from other bovine-adapted lineages (Budd et al., 2016). The CC151 strains were further demonstrated to fail to induce the production of chemoattractants from bMEC. Conditioned media from bMEC infected with strains belonging to CC97 strongly attracted bovine neutrophils, whereas conditioned media from bMEC infected with strains belonging to CC151 failed to result in neutrophil chemotaxis (unpublished data). The failure of strains belonging to CC151 to stimulate bMEC was also observed by Zbinden et al., who reported that infection of bMEC with strains of CC151 induced lower levels of pro-inflammatory cytokine production than strains of other bovine-adapted lineages (CC8, CC97, CC20; Zbinden et al., 2014). They hypothesized that this would result in strains belonging to CC151 being less virulent than other bovine-adapted strains. However, a prompt response to an infecting pathogen has been shown to be crucial for controlling IMI and preventing invasive disease (Rainard and Riollet, 2003). Therefore, the failure of CC151 strains to elicit a robust immune response and production of chemotactic factors from bMEC may lead to a later or weaker attraction (or both) of leukocytes to the mammary gland, allowing strains from this lineage time to be established in the mammary gland. Interestingly, experimental infection of cows with CC151 *S. aureus* strains has demonstrated the propensity for strains from this lineage to cause clinical mastitis (Keane et al., 2018; Wilson et al., 2018).

Attempts to identify a set of genes necessary for *S. aureus* to cause IMI have so far not been successful and few genes that are unique to bovine-adapted strains have been identified (Kozytska et al., 2010). The ruminant-specific leukotoxin *lukM*/*lukF′* has been hypothesized to be a major virulence factor as it confers on *S. aureus* the ability to lyse migrating neutrophil effector cells at a distance (Monecke et al., 2007; Hata et al., 2010; Vrieling et al., 2015). The *lukMF′* genes are highly prevalent among strains belonging to CC151, CC479, and CC133 but uncommon among other bovine-adapted lineages (Schlotter et al., 2012; Budd et al., 2015). Among strains that encode *lukM*/*lukF*, production of functional leukotoxin also varies. Deletion of *lukMF′* has been shown to result in a failure of *S. aureus* to kill bovine neutrophils (Vrieling et al., 2015). Intramammary challenge of cows with *S. aureus* strain S1444, which produces high levels of LukM*F′*, resulted in more severe clinical signs than challenge with strains S1449 and S1463, which produce intermediate levels of LukM*F′*, although SCC was similar between the groups (Vrieling et al., 2016). However, S1444 belongs to CC479 (Hoekstra et al., 2018), whereas the lineage to which S1449 and S1463 belong was not reported. Therefore, the strains may vary in more than just production of LukM*F′* and challenge of cows with strain S1444 lacking *lukMF′* would confirm the role of this toxin in virulence of bovine-adapted *S. aureus*.

Although cow factors are undoubtedly important in determining the response to IMI, species and strain-dependent variation in immune response, mastitis indi-

cators such as SCC, and infection severity and outcome has been demonstrated for *S. aureus*, *Strep. uberis*, and *E. coli* (de Haas et al., 2004; Yang et al., 2008; Atalla et al., 2009; Tassi et al., 2013; Blum et al., 2017; Keane et al., 2018). Several virulence traits have been hypothesized to underpin this variation including differences in ability to invade and survive within epithelial cells and professional phagocytes and survive neutrophil killing (Dogan et al., 2006; Tassi et al., 2015; Budd et al., 2016). However, much of the evidence supporting these hypotheses comes from in vitro studies. Bacterial gene expression is tightly regulated in vivo and further work is required to determine the importance of these traits in the intramammary environment. Deep transcriptome and proteome analysis of mammary gland tissue and different immune cell types will enable the unbiased identification of bovine and bacterial genes and proteins expressed in vivo, even those expressed at low levels. Interaction proteomics can be applied to identify the range of bacterial adhesins capable of interacting with bovine cells, along with their cognate receptors, contributing to the identification of novel diagnostic markers and vaccine and therapeutic targets.

VACCINES

Of the major IMI pathogen species, vaccines developed against *E. coli* have arguably been the most successful and are generally based on the bacterins of strains such as J5. Such vaccines do not provide complete protection against *E. coli* IMI but rather result in increased levels of specific IgG1 and IgG2 antibodies and a reduction in the severity of clinical signs and in milk loss. Importantly, protection against heterologous strains of *E. coli* is also observed (Hogan et al., 1992a,b, 1995). More recent studies have focused on intramammary vaccination against *E. coli* and demonstrated that local immunization resulted in reduced inflammation and conferred superior protection to intramuscular vaccination and that this was due to a modification of the local cytokine profile rather than an improved humoral adaptive immune response (Herry et al., 2017).

Vaccination using repeated intramammary inoculation of whole killed *Strep. uberis* has been reported to protect against infection with the homologous strain, whereas subcutaneous vaccination resulted in partial protection. Vaccination resulted in an increase in specific IgG1, IgG2, and IgM, but there was no evidence for enhanced opsonic activity leading to increased phagocytic uptake (Finch et al., 1994). Subcutaneous vaccination of cows with live *Strep. uberis* combined with intramammary infusion of bacterial surface extract also afforded protection against the homologous strain; however, vaccination did not provide adequate protection against a heterologous strain (Finch et al., 1997). The *Strep. uberis* proteins plasminogen activator PauA, the cell surface associated protein GapC, and *Strep. uberis* adhesion molecule SUAM have all been examined for their ability to provide protection against *Strep. uberis* mastitis (Leigh et al., 1999; Prado et al., 2011; Song et al., 2017). However, it must be noted that while the prevalence of the *gapC*, *sua*, and *pauA* genes among bovine *Strep. uberis* isolates is high, heterogeneity in gene carriage and gene sequence has also been observed (Gilchrist et al., 2013; Perrig et al., 2015). Therefore, any vaccine based on these single proteins may only provide strain-specific protection and may confer a selective advantage to *Strep. uberis* strains that are not covered by the vaccine. A commercial subunit vaccine for *S. uberis*, UBAC, has been recently launched on the market. The precise composition is not described but it consists of a preparation of biofilm adhesion component and includes lipoteichoic acid. Challenge of vaccinated cows with a heterologous strain of *Strep. uberis* showed that vaccination did not prevent infection but reduced clinical mastitis signs, bacterial count in milk, and milk yield (Collado et al., 2018). This vaccine requires the administration of 3 doses yet does not induce long-lasting immunity (https://www.ema.europa.eu/documents/product-information/ubac-epar-product-information_en.pdf). Further studies are required to assess the efficacy of this vaccine on commercial dairy farms under a variety of management systems and against a range of *Strep. uberis* strains.

The development of an effective vaccine against *S. aureus* has long been a research priority in both human and veterinary medicine. Societal concern regarding the use of antimicrobials in veterinary medicine and the potential spread of antimicrobial resistance determinants to human-associated *S. aureus* has acted as a new impetus to vaccine research. However, infection with *S. aureus* does not result in the development of immunity to re-infection and the role of the adaptive immune system is poorly understood.

A wide variety of *S. aureus* virulence factors and surface proteins have been investigated for their ability to act as antigens, which could induce a protective immune response against subsequent *S. aureus* challenge. Protein A, encoded by the *spa* gene, was one of the first cell wall anchored proteins of *S. aureus* to be characterized and was an early target of vaccine research (Pankey et al., 1985). Well known for its ability to bind the Fc fragment of mammalian IgG (Moks et al., 1986), SpA has subsequently been found to bind other ligands including the Fab domain of V_H3 immunoglobulins (Hillson et al., 1993), von Willebrand factor (Hartleib et al., 2000), and tumor necrosis factor receptor-1 (Gomez et al., 2004). Noted for its polymorphism, *spa*

is also commonly exploited in epidemiological studies of *S. aureus* (Zecconi et al., 2005). By binding immunoglobulins in the incorrect orientation, SpA inhibits opsonization and subsequent phagocytosis and is a well characterized virulence factor of *S. aureus* (Haraldsson and Jonsson, 1984; Patel et al., 1987). However, results of vaccine trials with SpA have been equivocal. Vaccination of first lactation cows with SpA was reported to increase the spontaneous cure rate and decrease SCC after *S. aureus* challenge; however, it did not reduce the incidence of IMI (Pankey et al., 1985). In a mouse IMI model, vaccination with SpA reduced the bacterial load in the mammary gland but did not protect against tissue damage after *S. aureus* challenge (Gogoi-Tiwari et al., 2016). The suitability of SpA as a vaccine target was further called into question by the discovery that strains belonging to the globally distributed bovine-adapted lineage CC151 do not express this protein on their cell surface (Stutz et al., 2011). In CC151 the *spa* gene is truncated, resulting in a protein product lacking the C-terminal LPxTG motif, required for Sortase A-mediated anchoring of SpA to the cell wall (Herron-Olson et al., 2007; Budd et al., 2016). Therefore, any vaccine based on protein A alone would not be expected to protect against mastitis caused by strains belonging to CC151.

The development of a vaccine based on surface expressed adhesins of *S. aureus* has also been widely pursued (Brouillette et al., 2002; Castagliuolo et al., 2006; Pujato et al., 2018). Major vaccine targets have included MSCRAMM proteins (microbial surface components recognizing adhesive matrix molecules) such as fibronectin-binding proteins FnBPA and FnBPB as well as clumping factor A (**ClfA**), and DNA-protein vaccination against these adhesins has been demonstrated to provide some protection against bovine IMI (Shkreta et al., 2004). However, it has also been shown that while a DNA vaccine directed against 3 different adhesins, fibrinogen-binding protein (Ebf), FnBPA, and ClfA induced significant levels of antigen-specific IgG and IgM in mice (Castagliuolo et al., 2006), the ability of the antibodies to recognize *S. aureus* was strain-dependent with several strains only poorly recognized (Scarpa et al., 2010). The carriage and expression of adhesins among bovine-adapted *S. aureus* is predominantly conserved within a lineage, although variation among strains of a lineage has also been reported (McCarthy and Lindsay, 2010; Budd et al., 2015). Of note, CC151 lacks several well-characterized adhesins such as *fnbB*, *cna*, *sasG*, and *sasK* and does not appear to express FnBPA. Additionally, CC71 lacks *sasD* whereas CC97 lacks *cna*. Therefore, any vaccine based on *S. aureus* adhesins would need to account for such variability and polymorphisms among bovine-adapted strains.

Currently 2 vaccines are marketed for the control of *S. aureus* IMI. These vaccines, Startvac and Lysigin, both contain whole inactivated *S. aureus* and require multiple administrations of the vaccine to confer short-term protection. As both products fail to induce long-term protective immunity, regular re-administration is required. Startvac, currently available in Europe and Canada, includes a bacterin based on *S. aureus* SP140, a strain which expresses the slime associated antigenic complex (**SAAC**) in addition to inactivated *E. coli* J5 (Prenafeta et al., 2010). The critical parameters for evaluating this vaccine depend on whether the aim is to control contagious or environmental IMI. For a contagious pathogen, limiting transmission is the goal and so a reduction in new infections or in the duration of infection is desirable. For environmental pathogens, a reduction in the severity of disease is the objective. There have been conflicting reports on the efficacy of this vaccine. Schukken et al. (2014) found that vaccination reduced the incidence of new *S. aureus* IMI in addition to reducing the duration, whereas Landin et al. (2015) reported that vaccination offered no protection against new *S. aureus* IMI and did not have any effect on SCC or milk yield. Bradley et al. (2015) found no difference in the proportion of cows with subclinical mastitis or in the incidence of IMI between vaccinated and unvaccinated cows, albeit they primarily evaluated the *E. coli* component of the vaccine as the study was carried out in herds where clinical IMI was predominantly due to *E. coli*. They did however report that vaccination resulted in increased milk yield and a reduction in the severity of clinical IMI (Bradley et al., 2015). It has been proposed that SAAC is the major *S. aureus* protective antigen of Startvac (Prenafeta et al., 2010). The SAAC primarily consists of the surface polysaccharide poly-*N*-acetyl β-1,6 glucosamine (**PNAG**), the product of the intercellular adhesion (*ica*) operon. It must be noted, however, that production of biofilm/SAAC is highly variable among *S. aureus* strains and not all strains encode the *ica* operon necessary for the production of PNAG. Strains belonging to CC71 do not encode this operon due to a large genomic rearrangement near the origin of replication in this lineage (Budd et al., 2015) and so Startvac may not provide any protection against infection with strains from this lineage.

Despite extensive research the development of an effective *S. aureus* vaccine that confers long-term immunity is still elusive, and may be unrealistic given that long-term immunity does not arise after natural infections. Most staphylococcal vaccine research has focused on antigens that drive the development of an adaptive humoral immune response (Brouillette et al., 2002; Shkreta et al., 2004; Prenafeta et al., 2010; Pujato et al., 2018), although *S. aureus* possesses a variety of

virulence factors that interfere with antibody function. A vaccine that drives both cell-mediated and humoral immune responses may confer additional protection. An improved understanding of the cell-mediated immune response and in particular the role of the various T cell subsets in regulating phagocyte effector functions and bacterial clearance would aid the development of the next generation of vaccines against *S. aureus* IMI. In the future the antigenic repertoire of the range of *S. aureus* lineages associated with bovine IMI should be considered, with the goal of developing a vaccine that protects against the major bovine-adapted *S. aureus* strains. Multiple variants of the antigenic targets may need to be included in any vaccine cocktail and vaccine trials should test for efficacy against the common *S. aureus* lineages.

CONCLUSIONS

An increased demand for milk and milk products has precipitated an expansion in dairy production across the world. In tandem the need to reduce our reliance on nonselective antibiotic use has brought a renewed focus on dairy production systems. Therefore, the impetus to develop new and sustainable strategies to manage and control infectious diseases such as IMI has never been greater. Intramammary infection is a multi-factorial disease influenced by a variety of factors related to the host, pathogen, and environment. Strategies to manage and control this disease will benefit from an improved understanding of these factors and how they interact. The host immune response, as determined by the extent of leukocyte recruitment to the mammary gland, is a key trait used for mastitis diagnosis and breeding for mastitis resistance. Understanding inter-strain variation in ability to elicit a host response will therefore facilitate tailored diagnosis and breeding strategies based on the species and strain of infecting pathogen. Additionally, the activation of an immune response is a key precursor to subsequent bacterial clearance from the mammary gland. Strains that cause chronic or persistent infection have developed means to evade immune detection, such as internalization into host cells. Identification of key mechanisms by which bacteria evade immune system detection will likely yield novel therapeutic and vaccine targets. This will require knowledge of the repertoire of bacterial lineages and strains that cause bovine IMI, their major virulence factors, and molecular mechanisms of pathogenesis. Characterization of the diversity of strains associated with bovine IMI and how different strains interact with the host will contribute to the development of new therapies and vaccines in addition to furthering our understanding of the factors required for the development of a protective immune response. Recent advances in the areas of pathogenomics, transcriptomics, and proteomics provide us with the tools to deepen our understanding of host-pathogen interactions and strain-specific interactions for IMI and to identify key mediators of these interactions.

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