Invited review: A critical appraisal of mastitis vaccines for dairy cows

Pascal Rainard, Florence Gilbert, Pierre Germon, Gilles Foucras

A critical appraisal of mastitis vaccines for dairy cows

Pascal Rainard[1]*, Florence B. Gilbert[1], Pierre Germon[1], and Gilles Foucras[2]

[1]ISP, INRAE, Université de Tours, UMR1282, Nouzilly, France

[2]IHAP, INRAE, ENVT, Université de Toulouse, Toulouse, France

* Corresponding author Pascal Rainard

ABSTRACT

Infections of the mammary gland remain a frequent disease of dairy ruminants that negatively impact animal welfare, milk quality, farmer serenity, farming profitability, and increases use of antimicrobials. There is a need for efficacious vaccines to alleviate the burden of mastitis in dairy farming, but despite decades of research this need has not been satisfactorily fulfilled. A careful appraisal of past and current research on mastitis vaccines reveals the peculiarities but also the commonalities among mammary gland infections associated with the major mastitis pathogens, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis*, *S. agalactiae* or *S. dysgalactiae*. A major pitfall is that the immune mechanisms of effective protection have not been fully identified. Until now, vaccine development has been directed towards the generation of antibodies. In this review, we drew up an inventory of the main approaches used to design vaccines aiming at the major pathogens for the mammary gland, and critically appraised the current and tentative vaccines. In particular, we sought to relate efficacy to vaccine-induced defense mechanisms, in order to shed light on some possible reasons for current vaccine shortcomings. Based on the lessons learned from past attempts and the recent results of current research, the design of effective vaccines may take a new turn in the years to come.

Keywords: cattle; mammary gland; mastitis; adaptive immunity; vaccine

INTRODUCTION

Mastitis is one of the most costly diseases in the dairy industry, costing US dairy industry up to $2 billions per year and from €17 to €198 per cow at the farm level in Europe (Hogeveen et al., 2011). In addition to its economic impact, mastitis is also of concern regarding animal welfare and farmer serenity. Mastitis, even subclinical, affects milk quality and can be a source of foodborne pathogens, such as livestock- associated Methicillin Resistant *S. aureus* (LA-MRSA) (Goerge et al., 2017, Garcia et al., 2019). Mastitis is the most common reason for antimicrobials use in dairy farms, and a prudent use of antibiotics is recommended (Ruegg, 2017). As the widespread prophylactic use of antibiotics is no longer sustainable, and considering its importance as a mastitis control practice, an additional or replacement procedure is needed. This is only partly achieved by internal teat sealants along with selective dry cow therapy (Bradley et al., 2010). Efficacious vaccines, by reducing the incidence of new infections and the occurrence of clinical cases, would be an appropriate and convenient way to fulfill this need. Most mastitis cases are caused by the so-called major pathogens, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Escherichia coli,* and *Klebsiella pneumoniae*. Documented reviews on *S. aureus* vaccines are available that present in detail the results obtained in field experiments, their merits and limitations (Middleton et al., 2009, Scali et al., 2015, Côté-Gravel et al., 2019). Vaccines directed at other major pathogens are less well covered (Keane, 2019). Vaccine development faces a number of hurdles. Some relate to the return on investment, which is conditioned by the size of the market and by the acceptability of the vaccine, which largely depends on its proven effectiveness.

These uncertainties certainly dampen the enthusiasm or will of developers, which in turn reduces investment in research and therefore progress. These issues are not dealt with in this review. The purpose of this review on mastitis vaccines is to assess current knowledge, including newly released vaccines, with a particular attention to the supposed defense mechanisms put to effect by vaccination. This approach will help to analyze the possible reasons for the disconnect between expectations and reality, and to devise alternative or complementary means for the development of mastitis vaccines.

COLIFORM MASTITIS VACCINES

Escherichia coli J5 Mastitis Vaccines

Intramammary infections (IMI) by Gram-negative bacteria ("coliforms") have long been the major cause of clinical mastitis of dairy cows worldwide, and are still a major challenge for mastitis research (Ruegg, 2017). In the late 1970s, mastitis control programs based on teat dipping and dry cow therapy were found to be effective in controlling staphylococcal and streptococcal mastitis, but had little effect on coliform infections. Only tedious control methods (improved hygiene of bedding and walking corridors by frequent removal of manure or flushing) had effectively reduced coliform mastitis under field conditions (NRC et al., 1979). In the late 1980s, evidence began to emerge that vaccination with bacterins made up of killed rough *E. coli* and adjuvant had some efficacy against clinical coliform mastitis in cows (Wilson and Gonzalez, 2003). The rationale behind the use of rough *E. coli* was that they were purported to elicit antibodies cross-reacting with mastitis strains of different serotypes and protective against these strains in models of infection or endotoxemia by passive transfer of immune serum (Ziegler et al., 1973, Ziegler et al., 1982, Sakulramrung and Domingue, 1985). The most studied rough mutant was the J5 mutant derived from an O111:B4 *E. coli* strain. This mutant lacks oligosaccharide side chains of the lipopolysaccharide (LPS), so that the core LPS, which is

nearly identical to that of most other Gram-negative bacteria, is exposed at the surface of bacteria. The immunization with normal pathogenic smooth strains of Gram-negative bacteria induces the formation of serotype-specific antibodies directed at the O side chains, but usually little if any antibodies to the core region or to membrane-associated proteins (Baumgartner and Glauser, 1993). It is likely that O antigens are immunodominant when compared to core antigens and that they prevent the induction of antibodies to core epitopes. The drawback is that there are about 180 *E. coli* different O-serotypes and that none is prevalent among mastitis-associated strains (Linton et al., 1979, Linton and Robinson, 1984, Stenutz et al., 2006). Several studies have established that immunization with smooth wild-type strains of Gram-negative bacteria elicits protection against a challenge with the homologous strain, contrary to immunization with rough mutants, reviewed in (Greisman and Johnston, 1997). The protection afforded by core-antigen antibodies is thus a controversial topic.

Nevertheless, vaccines based on J5 mutant strains attracted the interest of mastitis researchers and were used as a component of the control of clinical coliform mastitis in parturient and early lactation cows. The observation that dairy cows with low ELISA titers of IgG_1 to *E. coli* J5 were associated with five times the rate of clinical coliform mastitis compared to animals with higher titers (Tyler et al., 1988) elicited interest in immunization with rough mutants of Gram-negative bacteria. Several field studies were carried out, mainly with the mutant strain J5 but also with a rough mutant of *Salmonella enterica* Typhimurium Re-17 strain, as reviewed by (Wilson and Gonzalez, 2003). In the late 1980s, evidence emerged that vaccination of cows with J5 bacterins had some efficacy against clinical coliform mastitis. The bacterins were administered by the subcutaneous route on several occasions, often at drying-off, with a booster before calving and another after calving, with significant reductions in the incidence of clinical mastitis in vaccinated compared to unvaccinated cows (Gonzalez et al., 1989, Cullor, 1991, Hogan et al., 1992a). A vaccine based on the use of killed *Salmonella* Re-

17 produced a comparable reduction in clinical cases of coliform mastitis and mortality rate (McClure et al., 1994), raising interrogation about the underlying mechanisms other than the developement of cross-reactive antibody immunity, as discussed below.

An early study of the financial return on the use of the J5 vaccine indicated that when more than 1% of cow lactations were affected by clinical coliform mastitis, the vaccination would be profitable (DeGraves and Fetrow, 1991). The efficiency of a recently licensed vaccine against coliform and staphylococcal mastitis (Startvac®, Hipra SA) has been investigated in a field study: there was no decrease in the incidence rate of clinical mastitis, but mastitis severity was significantly reduced, with lessened milk losses resulting in a return on investment of 2.57 to 1 (Bradley et al., 2015). It has been proposed that through the reduction in milk production losses and replacement of culled or dead cow, J5 vaccination is profitable in herds that experience cases of clinical mastitis by Gram-negative bacteria beyond a threshold of more than 4% of lactating cows per month (Wilson and Gonzalez, 2003). An overview of the main coliform vaccine trials is given in Table 1.

Supposed Mechanisms of Protection by J5 Vaccines

Remarkably, the relative success of rough Gram-negative bacteria vaccines remains unexplained in terms of their underlying mechanisms. The initial rationale for these vaccines was that they could elicit antibodies capable of neutralizing the toxic activity of the lipid A moiety of LPS and opsonizing mastitis-associated coliform bacteria. Early studies in mice and humans indicated that passive transfer of antibodies induced by J5 immunization protected against endotoxic shock (Ziegler et al., 1982). However, the neutralization of endotoxic activity is controversial. Several attempts of passive transfer of antibodies to J5 were unsuccessful, possibly because of insufficient antibody concentration, so that the use of J5 antiserum to prevent or treat endotoxic shock is not of standard medical practice (Cross, 2014). As lipid A

126 is responsible for the toxicity of LPS, monoclonal antibodies to lipid A have been developed
127 for the therapy of Gram-negative sepsis. However, their efficiency has been doubted as they
128 did not consistently show neutralizing activity (Warren et al., 1993). The treatment of calves
129 with J5 antiserum did not protect against shock induced by intravenous LPS injection (Morris
130 et al., 1986). Antibodies to the core part of LPS have also been tested for protective activity.
131 Coupling the core glycolipid to a carrier protein improved the immunogenicity and resulted in
132 higher antibody titers than those elicited by a J5 bacterin vaccine (Cross et al., 2014). However,
133 there are five different *E. coli* core antigens (K-12, R1, R2, R3 and R4) that can induce core-
134 specific antibodies. A mixture of those core antigens conjugated to a protein carrier was used
135 to vaccinate cows. Compared to control cows, SCC and *E. coli* shedding in milk did not differ
136 in vaccinated animals after intramammary challenge, although high titers of antibodies to the
137 core type of the challenge strain had been elicited (Brade et al., 2013). It is not even certain that
138 the J-5 vaccines induce antibodies to the core LPS, as one study suggested that the raised
139 antibodies were not directed to rough LPS but to outer membrane proteins such as OmpA
140 (Chaiyotwittayakun et al., 2004).

141 Several studies have investigated the effect of J5 vaccines on the opsonic activity of
142 serum and milk of vaccinated cows. The focus has been on the induction of antibodies that are
143 opsonic for neutrophils of dairy ruminants, i.e. IgG_2 and IgM antibodies, as these antibodies
144 play a major role in the opsonization of *E. coli* (Williams and Hill, 1982, Hill et al., 1983a).
145 Most natural opsonic antibodies to *E. coli* are of the IgM isotype and usually there is little IgG_2
146 activity in the serum and milk of unimmunized cows (Williams and Hill, 1982). Natural
147 infection of the mammary gland (MG) elicits opsonic antibodies, but they are serotype specific
148 (Hill et al., 1983a). Immunization with smooth bacteria can induce opsonic antibodies in serum
149 and milk (Rainard, 1983, Herry et al., 2017), but these antibodies are mainly serotype-specific,
150 which make them useless given the multiplicity of serotypes among mastitis-causing coliform

bacteria (Linton et al., 1979, Sanchez-Carlo et al., 1984, Lipman et al., 1995). To obviate the narrow specificity of opsonic antibodies, J5 bacterins were used to induce cross-reactive opsonic antibodies. It appeared that eliciting antibodies to J5 bacteria in the IgG_2 sub-isotype was difficult, requiring more than three administrations to reach significantly higher than initial titers (Chaiyotwittayakun et al., 2004). Increased IgG_2 antibodies to J-5 obtained by hyperimmunization correlate with a decreased occurrence of clinical mastitis (Erskine et al., 2007). However, antibody titers tend to dwindle in a few months (Erskine, 2012).

The opsonic activity of antibodies raised with J5 bacterins is a matter of controversy. Studies showed either some increase in opsonic activity following vaccination (Hogan et al., 1992b) or not (Vreede et al., 1986). The antibodies bound to their antigen target have to be accessible to the phagocytes, and specifically to their immunoglobulin receptors, for ingestion to be triggered. First, the antigen targets must be accessible to antibodies. The accessibility of lipid A, core antigens and outer membrane proteins has been doubted, owing to the shielding effect of the side chains of LPS (O-antigen) of smooth strains (Greisman and Johnston, 1997). This shielding has been confirmed with mastitis strains: immunization of cows with J5 induced cross-reactive antibodies to rough strains but these antibodies, except those directed at fimbriae, reacted very poorly with smooth strains and did not improve on the opsonic activity of pre-existing natural antibodies (Figure 1) (Rainard et al., 2021). Absorption of J5 immune serum with smooth strains did not reduce the antibody reactivity with rough strains, demonstrating the efficient shielding by the O-antigen polysaccharide layer toward outer membrane antigens. In accordance, immunization of rabbits with *E. coli* J5 did not augment the opsonization titer to smooth strains (van Dijk et al., 1981). Nevertheless, J5 bacterins induce cross-reactive antibodies, even though their specificity has not been characterized (Ziegler et al., 1973), except for the outer membrane protein OmpA and type 1 fimbriae (Rainard et al., 2021). The role played by these cross-reacting antibodies in protection against infection has been evaluated in

vaccination and challenge experiments. Several studies did not find noticeable protective effect following systemic or intramammary J5 vaccination (Hill, 1991, Smith et al., 1999, Tomita et al., 2000, Steele et al., 2019, Tashakkori et al., 2020, Vangroenweghe et al., 2020). One study found that the J5 vaccine was associated with faster clearance of *E. coli* in milk and less reduction in milk yield following an intramammary challenge with a strain that did not induce clinical mastitis (Wilson et al., 2007). Of note, the IgM and IgG_2 milk titers were not different from titers of control cows just before challenge, and higher IgM antibody titers against J5 at 12 h post-challenge tended to be associated with higher milk production losses, suggesting that the vaccine effect was probably independent of opsonic antibodies.

The vaccine formulation is likely to account for part of the discrepant results of experimental challenges. The comparison of two J5 vaccines using two different adjuvants yielded different outcomes, one showing some protection and the other none compared to control cows (Hogan et al., 2005). The route of administration could also influence the efficacy of the vaccine. The common way of J5 vaccine administration is subcutaneous injection in the neck. A study compared this route to the area drained by the supramammary lymph node, without much difference in efficacy, with a comparable increase in antibody titers to J5 (Tomita et al., 1998). In another vaccine trial, the two J5 vaccines tested failed to improve serum and mammary secretion IgM antibody titers but increased IgG_1 and IgG_2 titers, with no change in clinical status of challenged cows among treatment groups (Tomita et al., 2000). The *E. coli* strains used for the challenge are also likely to affect the results of vaccine trials. In all of these experiments, the role of anti-J5 antibodies was not established. One study showed a slight augmentation of phagocytosis of a smooth *E. coli* strain after vaccination with J5 that was attributed to a slight augmentation of IgM antibodies to the smooth strain in the serum of vaccinated cows, although there was no increase of titers to J5 (Hogan et al., 1992b). The specificity of the opsonic antibodies was not determined. It

appeared that J5 vaccines elicit antibodies to molecules protruding from the O-antigen shield, such as type 1 fimbriae, which can bind to smooth strains (Rainard et al., 2021). Since a proportion of mastitis *E. coli* isolates have the genetic equipment to produce these structures (Lipman et al., 1995), and as fimbriae are supposed to contribute to adhesion to mammary epithelial cells (MECs), antibodies to fimbriae could play a role in vaccine-induced protection. However, their opsonic activity has not been evidenced (Rainard et al., 2021). Another type of fimbriae, the long polar fimbriae, can be produced by mastitis strains and could be associated with the ability to invade MECs (Dogan et al., 2012). Their role in mastitis pathogenesis and the possible contribution of antibodies to these fimbriae to mammary defense is worth investigating.

All things considered, the importance of opsonic antibodies as a defense mechanism induced by J5 vaccines is questionable. The observation that early lactation pooled whey (5–10 days post-partum) was opsonic for all mastitis strains of *E. coli* tested suggests that since early lactation milk contains sufficient opsonins, severe *E. coli* mastitis at this stage of lactation is not due to opsonic deficiency (Hill et al., 1983b). In other words, opsonic antibodies are not a limiting factor in defense against *E. coli* mastitis even in early lactation when coliform mastitis tends to be severe. This conclusion is supported by the absence of reinforcement of milk neutrophils phagocytic activity at the onset of infection in the quarters of vaccinated cows challenged with the homologous strain compared to control cows, although antibody titers to this strain were markedly augmented in the IgG_2 and IgM isotypes (Herry et al., 2017). Another factor contributing to reducing the potential benefit of vaccine-induced antibodies is the difficulty in maintaining elevated concentrations because of dilution in milk. It can be concluded that the role of J5 vaccine-induced opsonic antibodies remains dubious and that the experimental challenge trials displayed limited protection and did not yield many clues as to the mechanism behind the activity of the J5 vaccine observed in field experiments.

As an alternative to the role of the antibody response, it has been proposed that J5 vaccines promote a T cell-mediated immunity polarized towards a T helper type 1 (Th1) response (Dosogne et al., 2002). Vaccination by the systemic route would induce memory helper T cells, some of which would home into the mammary tissue (Taylor et al., 1994). There, these lymphocytes would increase the recruitment and activation of neutrophils through the secretion of cytokines on presentation of *E. coli* antigens at the onset of infection, finally allowing the cow to cope more effectively with the bacteria. The benefit of this response might be most appropriate around parturition, a time when the immune response of cows seems to be biased towards Th2 type immune responses (Shafer-Weaver et al., 1999). Indeed, a systemic (subcutaneous) immunization of cows with a model antigen such as ovalbumin can elicit an antigen-specific pathway in the MG (De Cueninck, 1979). A hallmark of this cell-mediated immune response is the accelerated or amplified influx of neutrophils at the onset of infection, as exemplified by the reinforced recruitment of leukocytes upon challenge of cows immunized intramammarily with killed *E. coli* (Rainard, 1983). Experimental infections of cows vaccinated with J5 bacterins do not support this cell-mediated hypothesis. In one study, the authors specify that "the SCC did not differ between treatments in speed or magnitude of response" (Hogan et al., 1995). No amplified neutrophil recruitment was reported in other experimental challenges (Hill, 1991, Hogan et al., 1992c, Smith et al., 1999, Tomita et al., 2000). There is no evidence either supporting the improvement of the neutrophil phagocytic or bactericidal activity following J5 vaccination. Remarkably, the induction of T cell-mediated immune response by J5 vaccines has not been investigated.

The mechanisms of protection observed in field studies with J5 vaccination remain unclear (Baumgartner et al., 1991). It has been suggested that J5 vaccines may reduce the severity of mastitis by acting more on the systemic immune response than on the local mammary defenses (Erskine, 2012). The mechanism is elusive. We have seen that the

hypothesis of endotoxin activity neutralization by antibodies has not been validated. Antibodies to lipid A or core antigens (passive transfer of immune plasma or IVIG or mAb) have been mainly considered to alleviate sepsis, not to combat infection limited to an infected organ, as it occurs in coliform mastitis. The induction of opsonic antibodies is at best marginal with the licensed protocols of vaccination, and their contribution to protection can be questioned. The experimental challenge experiments did not confirm the hypothesis of cell-mediated immunity manifesting itself by an amplified influx of neutrophils at the onset of infection. The downside stemming from our ignorance of protection mechanisms is that we are unable to optimize the J5 vaccine on a rational basis.

Sub-unit Vaccines against Coliforms

Another approach to vaccination against coliform mastitis has been explored. Blocking the growth of coliform bacteria in mammary secretions has the potential to prevent IMI rather than reducing only its severity. Iron is an essential element for the survival and multiplication of coliform bacteria. Most iron in mammary secretions is bound to lactoferrin during the dry period and to citrate during lactation (Reiter, 1978). Coliform bacteria can acquire iron in bodily fluids by utilizing high-affinity iron acquisition systems (Garenaux et al., 2011). Two of those systems have been targeted to develop a mastitis vaccine. The enterobactin iron acquisition system is common in coliforms isolated from mastitis. The ferric enterobactin receptor FepA, which is an iron-regulated outer membrane protein, binds ferric enterobactin, an efficient iron chelator (siderophore). Cows were immunized with purified FepA, and the serum IgG fraction tested for bacteriostatic activity (Lin et al., 1999): the growth of all the *E. coli* and *Klebsiella pneumoniae* isolates tested was inhibited by 4 mg/mL of the IgG preparation in the presence à 0.5 mg/mL apolactoferrin. Of note, purified IgG from cows immunized with *E. coli* J5 had little inhibitory effect on the growth of *E. coli* or *K. pneumoniae* mastitis isolates (Lin et al., 1999).

The authors suggested that antibodies to FepA could help the MG to deal with *E. coli* during the dry period when lactoferrin concentrations are high in mammary secretion. Another inducible siderophore receptor, the ferric citrate receptor FecA (Braun, 1997), is likely to play an important role for coliform mastitis isolates as citrate concentration is high (7–11 mM) in milk (Gaucheron, 2005), the *fecABCD* operon is present in most, if not all, mastitis *E. coli* strains (Goldstone et al., 2016), and loss of expression causes a loss of pathogenicity for the MG (Blum et al., 2018). Cows were immunized twice by the subcutaneous and once by the intramammary routes with either purified FecA or J5 vaccine, with a control unimmunized group (Takemura et al., 2002). There was no difference between groups in bacterial counts after challenge, duration of infection, milk somatic cell counts or milk production, showing that the FecA and J5 vaccination were ineffective, despite an increase in antibody titers to FecA in the serum of the FecA immunized cows. Purified IgG from FecA-immunized cows had little effect on the growth of *E. coli* in vitro under Fe-restricted conditions (Takemura et al., 2004). In the conclusion of their in vitro and in vivo trials on blocking the growth of *E. coli* by immunizing cows with bacterial ferric iron receptors, the authors deemed that this vaccination approach may not be feasible because the required effective antibody concentrations could not be induced and maintained in milk (Wolf et al., 2004).

In the same line of thought, a *K. pneumoniae* bacterial extract was used as the active principle of a vaccine for *Klebsiella* mastitis in dairy cattle (KLEBVax™ SRP, Epitopix, Willmar, Minnesota) recently licensed by the USDA. This vaccine is based on the proprietary patented SRP® (Siderophore Receptor and Porin) technology, which consists in extracting bacterial surface proteins of a representative mastitis *K. pneumoniae* isolate, used as antigens for active immunization. A field trial was carried out in a university dairy farm in which *Klebsiella* mastitis caused 14% of all clinical cases, 19% of which led to the death of the cows (Gorden et al., 2018). All cows were vaccinated with a commercially available J5 vaccine. The

Klebsiella SRP® extract was emulsified in oil-in-water adjuvant and two subcutaneous doses administered three weeks apart to half the cows, the other half receiving a placebo (adjuvant only). In all, 229 pairs of cows were analyzed. There was no significant difference in culling because of *Klebsiella pneumoniae* mastitis between groups (10 vs 19 in vaccinated vs control cows) or coliform mastitis other than *Klebsiella* (6 vs 5) or in clinical cases of *Klebsiella* mastitis (31 vs 38) or of coliform mastitis (65 vs 81). However, cows vaccinated before calving had less SCC and milk losses than control cows. The concurrent administration of a J5 vaccine and the different schedules of administration of the *Klebsiella* vaccine complicated the study, as a positive effect on the number of clinical cases and milk production occurred only when the first injection took place before calving. The SRP antigen induced antibodies, but their protective activity was not established. Another study involving more than 3000 cows did not show any effect of the KLEBVax™ vaccine, either on the incidence of clinical mastitis or the postcalving risk of death or culling in relation to *Klebsiella* spp. Mastitis (Tomazi et al., 2021). In the latter study, a new vaccine based on the recombinant protein YidR, highly conserved between mastitis *Klebsiella pneumoniae* isolates but also with *Escherichia coli* strains, was evaluated in a study involving more than 3000 cows. It is impossible to judge the efficacy against *E. coli* mastitis because the cows also received a J-5 vaccine, even in the control placebo group (aluminum hydroxide adjuvant only). The main positive result of the two injection of the vaccine, one at drying-off, the other before calving, was a reduction (about 37%, $p < 0.05$) of the adjusted incidence of clinical mastitis (Tomazi et al., 2021). The mechanism associated with this effect remains elusive. In particular, the ELISAs used to measure the antibody response showed a high baseline level against the purified recombinant protein or whole bacteria, little increase elicited by the vaccine wuth the recombiantn protein and no significant increase with whole *Klebsiella*.

Intramammary immunization during the dry period with a bacterial extract from a smooth mastitis strain (P4) has been carried out to test the influence of the route of immunization and the possibility to protect at least against the homologous challenge strain (Herry et al., 2017). Compared to a protocol involving two subcutaneous injections, the protocol including a local (intramammary) booster immunization was more successful, by improving bacterial clearance while limiting inflammation. Of note, the efficiency of opsonisation and neutrophil-dependent bactericidal activity in milk was not improved by the vaccination, despite a sizable increase in antibody titers to the O-antigen of the challenge strain. It is thus possible that the improved MG defenses were not linked to strain-specific antigens, but rather to the route of immunization. Indeed, a further analysis of data from CD4 T cell gene expression indicated that IL-17 and type 3 immunity-related T cells had been elicited in the mammary tissue of locally immunized glands (Cebron et al., 2020).

STREPTOCOCCAL VACCINES

Streptococcus uberis Mastitis Vaccines

Streptococcus uberis tends to be the most common cause of clinical mastitis in pasture-based dairy herds in regions and countries with a temperate humid climate such as New Zealand, the UK, Ireland and France (Denis et al., 2009, Petrovski et al., 2011, Green and Bradley, 2013, Keane et al., 2013, Poutrel et al., 2018). Current strategies aiming at reducing the incidence of IMI by *S. uberis* are not very effective so that the control of these infections remains problematic, a situation that makes efficient vaccines a highly desirable but so far elusive goal despite multiple attempts (Denis et al., 2009, Klaas and Zadoks, 2018). An early attempt to vaccinate against *S. uberis* mastitis used live bacteria of a virulent strain (0140J) administered by the subcutaneous route and a bacterial surface extract intramammarily seven days after drying-off (Hill et al., 1994). Vaccinated cows challenged with the vaccine strain shed 10^5 times

lower bacterial concentration and more than 10 times lower leukocyte concentrations in milk than unvaccinated animals. Interestingly, although specific antibacterial antibodies were elicited in the IgG_2 and IgM isotypes, the opsonic activity of serum or milk was not improved, despite a modest initial phagocytic activity (50% bacterial survival). Contrary to vaccination with a surface extract of the challenge strain, which did not induce protection, the live vaccine induced a proliferative response of blood lymphocytes to *S. uberis* antigen. A further study showed that vaccination with live bacteria protected against the homologous strain but was less effective against a heterologous strain (Finch et al., 1997). Another attempt indicated that local vaccination (intramammary) with killed bacteria without adjuvant administered six times one week apart during the dry period protected the gland against homologous challenge by preventing bacterial growth without inflammation (Finch et al., 1994). Again, the opsonic activity of milk or serum of vaccinated cows was not improved. Furthermore, in experimental MG infections with virulent strains of *S. uberis*, intense recruitment of neutrophils into mammary tissue and milk occurred concomitantly with high concentrations of bacteria, which were not frequently observed associated with neutrophils (Hill et al., 1994, Thomas et al., 1994). Those findings were surprising. Indeed, the phagocytic killing of bacteria by neutrophils is considered an essential defense of the MG, a position supported by cogent arguments with regard to mastitis-associated *E. coli* and *S. aureus* (Paape et al., 2002). Accordingly, the prompt and intense recruitment of activated neutrophils at the onset of infection is assumed to have a positive effect on the outcome of infection (Craven and Williams, 1985, Rainard and Riollet, 2003). These views may not apply to *S. uberis* mastitis, an oddity that prompted investigations on the interactions of *S. uberis* with phagocytic cells.

The investigation of a small panel of five *S. uberis* clinical isolates exposed to bovine neutrophils in the presence of skim milk displayed three phenotypes: bacteria that resisted phagocytosis when cultured in laboratory medium (strain C197C), bacteria that became

375 resistant when cultured in medium supplemented with casein hydrolysate (strains 0140J and
376 ST10), and bacteria susceptible to phagocytosis under both conditions (strains EF20 and CC21)
377 (Leigh and Field, 1991). A previous study, involving the most susceptible strain (EF20) and a
378 resistant strain (0140J), had shown that both could produce a hyaluronic capsule and that the
379 susceptibility to the bactericidal activity of neutrophils was not dictated by the capsule (Leigh
380 et al., 1990). In effect, strain EF20, which loses its capsule at the stationary phase of culture,
381 was equally susceptible to phagocytosis at the exponential and stationary phases, and the
382 decapsulation of strain 0140J with hyaluronidase did not render it susceptible to phagocytosis.
383 Those two strains had shown different pathogenicity for the lactating MG, as the strain EF20
384 failed to induce mastitis most of the time, whereas the strain 0140J was usually successful (Hill,
385 1988). Surprisingly, the strain EF20 was eliminated from inoculated glands in the absence of
386 neutrophil influx, thus challenging the presupposed view that the difference in pathogenicity of
387 the two strains was linked to their differing resistance to phagocytosis. It should be noted that
388 the two strains were equally efficient at inducing mastitis in dry MG. Further studies with
389 strain 0140J showed that the presence of the capsule appears to contribute to resistance to
390 phagocytosis as neutrophils killed 0140J acapsular mutants (Ward et al., 2001). Nevertheless,
391 other studies have indicated that although the hyaluronic acid capsule contributes to resistance
392 to phagocytosis, it was not required for strain 0140J to induce clinical mastitis (Field et al.,
393 2003). Intriguingly, the authors explained the inefficiency of the MG phagocytic defense by a
394 potent antiphagocytic activity exerted by the strain 0140J culture supernate. This inefficiency
395 is consistent with in vivo data that has shown that bacterial numbers in milk remain high despite
396 a massive influx of neutrophils (Field et al., 2003). The identity of this putative inhibitor and
397 its mode of action have not been established. Among antiphagocytic streptococcal factors,
398 hyaluronic acid has been reported to prevent attachment of streptococci to macrophages
399 (Whitnack et al., 1981). It is worth noting that not all *S. uberis* isolates are able to produce a

400 capsule even at their primary in vitro culture (Matthews et al., 1994b), but that most of the
401 strains isolated from mastitis possess the genes (*hasABC*) required for capsule synthesis (Field
402 et al., 2003).

403 Contrary to neutrophils, macrophages obtained from dried MGs were able to
404 phagocytose a strain resistant to phagocytosis by neutrophils, in the presence of IgG_1 or IgG_2
405 antibodies from normal serum, independently of complement (Grant and Finch, 1996). This
406 observation is in keeping with the experimental infection of the lactating MG with *S. uberis*
407 showing that bacteria were seen within macrophages but not within neutrophils (Thomas et al.,
408 1994). Mammary gland macrophages were able to take up capsulated or unencapsulated
409 mastitis isolates of *S. uberis*, but the capsule reduced phagocytosis and intracellular killing
410 (Almeida and Oliver, 1993). In these studies, the bactericidal efficacy of MG macrophages
411 appeared to be limited even in the presence of serum, consistent the idea that MG macrophages
412 have depressed phagocytic and pro-inflammatory functions, especially milk macrophages
413 during lactation (Denis et al., 2006).

414 All these results suggest that phagocytosis by neutrophils and macrophages (at least
415 during lactation) is not an effective defense of the MG against *S. uberis* (Leigh, 1999).
416 Consequently, several other avenues have been followed to develop effective vaccines. One of
417 them stemmed from the observation that effective vaccine trials showed protection of the gland
418 without inflammation and prevented bacterial growth in vivo (Hill et al., 1994). *S. uberis* are
419 fastidious bacteria that are auxotrophic for at least eight amino-acids (Leigh, 2000). One way
420 streptococci acquire their nutrients in the MG is by activating milk plasminogen and binding
421 the activated plasmin to their surface, thus being able to use peptides from hydrolyzed milk
422 proteins for their growth and multiplication (Leigh and Lincoln, 1997). The plasminogen
423 activator PauA is produced by most mastitis isolates of *S. uberis* and is highly conserved across
424 strains (Johnsen et al., 1999). A small-scale vaccine experiment with PauA showed that

neutralizing antibodies were induced that coincided with reduced severity of mastitis, low bacterial shedding, and low milk leukocytosis compared to unvaccinated control cows (Leigh et al., 1999). A remarkable aspect of this protective effect is that it was obtained with a reduction rather than an increase in inflammation as usual with other vaccines. Moreover, inflammation could well promote the growth of *S. uberis* in the MG, possibly by the activation of inflammasome and the production of IL-1β by macrophages (Archer et al., 2020).

However, PauA would not be a requirement for *S. uberis* to grow in milk (Ward et al., 2003), which casts some doubt on the effectiveness of a vaccine based exclusively on this antigen. It has been shown that the glyceraldehyde-3-phosphate dehydrogenase GapC contributes to the binding of plasmin at the bacterial surface (Cunningham, 2000). Vaccination of cows with recombinant *S. uberis* GapC or CAMP factor was reported to induce protection after homologous challenge (Fontaine et al., 2002), but peculiarities of the challenge model (low virulence of the *S. uberis* strain, size or viability of the inoculum) made this experiment unconvincing (Leigh, 2002). Field studies with PauA, GapC, or CAMP subunit vaccines are awaited.

Another avenue of research for vaccine design derived from the observation that various *S. uberis* strains have been shown to adhere in vitro to a mammary epithelial cell line (MAC-T cells) and to be actively internalized (Matthews et al., 1994a). In MAC-T cells, *S. uberis* can survive for several days (Tamilselvam et al., 2006). By using primary cultures of mammary epithelial cells, adhesion occurred readily only to cells that had lost their microvilli (Ditcham et al., 1996). However, adherence and invasion of intact mammary epithelial cells have not been confirmed using mammary tissue explants (Thomas et al., 1992), and examination of mammary tissue after experimental infection with *S. uberis* revealed bacteria adhering only to damaged epithelium (Thomas et al., 1994). There are two conflicting views on the adherence and invasion of MECs by *S.uberis*, one which posits that adhesion and invasion is a central

mechanism in the pathogenesis of *S. uberis* mastitis (Almeida et al., 2015), another considering that adhesion is not required and cannot play a major role in the initiation of infection (Leigh, 1999). Adherence and invasion of MAC-T cells depend on the presence of the surface *S. uberis* adhesion molecule (SUAM) (Almeida et al., 2006). Antibodies obtained by immunizing cows with recombinant SUAM reduced the adherence and internalization of *S. uberis* by MAC-T cells (Prado et al., 2011). Infection of the MG with a mastitis isolate and its isogenic mutant devoid of SUAM showed that the defective mutant induced mastitis of lesser intensity with fewer bacterial shedding and milk leukocytosis (Almeida et al., 2015). However, bacterial shedding and milk leukocytosis were still sizable, indicating that SUAM was not the only virulence factor at play during MG infection. In an experimental *S. uberis* intramammary challenge involving 40 cows half of which were vaccinated with SUAM and adjuvant, all the cows developed mastitis (Siebert et al., 2017). These results suggest that SUAM alone would not suffice to design an efficient vaccine, and would need to be associated with other antigen targets.

A possible way *S. uberis* could resist MG immune defenses and antimicrobial treatments is the formation of biofilm. Biofilms are dense aggregates of surface-adherent microorganisms embedded in a complex matrix mainly comprising exopolysaccharides. Most if not all *S. uberis* mastitis isolates can grow in biofilm under suitable culture conditions (Crowley et al., 2011, Dieser et al., 2017). In particular, casein proteolytic peptides contribute to increased biofilm formation, conditions likely to occur during MG infection (Varhimo et al., 2011). A recently licensed vaccine produced by Laboratorios Hipra S. A. (UBAC®) is based on a slime preparation as antigen (Collado et al., 2018). The Biofilm Adhesion Component (BAC) comprises an extract of biofilm produced by a mastitis isolate cultivated on a solid surface and extracted by autoclaving. This complex antigen contains lipoteichoic acid (LTA). The antigen preparation is adjuvanted with monophosphoryl lipid A (MPLA), and emulsioned water-in-oil

475 in Montanide ISA (mineral oil; Seppic, France). The vaccine is administered by the
476 intramuscular route on three occasions, two months before calving, about 40 days later, finally
477 about 15 days after calving. A trial involving experimental infection with a strain different from
478 the vaccine strain has been conducted with 13 vaccinated and 12 control cows. The initial
479 response to the challenge was similar in the two groups, but the clinical score was lower in the
480 vaccinated group 3 days after challenge, and bacterial shedding after six days. Milk production
481 losses were reduced in the vaccinated group. Nevertheless, milk leukocytosis was similar and
482 high (more than 10^6 cells/mL during the first 8 days) in the challenged quarters in both groups.
483 Antibodies to LTA increased in the vaccinated group, and immune serum was somewhat active
484 in reducing biofilm formation. Since control cows only received PBS injection, a potential non-
485 specific immunostimulation due to the presence of MPLA and adjuvant in vaccinated cows
486 could have contributed to the results obtained. The results of a field study were presented at the
487 National Mastitis Council Conference in Milan (Puig et al., 2018). Involving 401 vaccinated
488 and 380 control cows in six herds, the study showed a reduction in clinical mastitis cases in
489 vaccinated animals compared to controls (6.1% vs 13.5%) through reduction in infection
490 severity, but the impact on the milk yield was small (36.8 vs 36.4 L/day). The effect of
491 vaccination on phagocytosis by neutrophils or adherence to MECs was not reported.

492 In all the studies mentioned above, the contribution of vaccine-induced antibodies to
493 MG defense was either doubtful (opsonic antibodies) or not unquestionably established
494 (prevention of adherence and invasion). Most attempts to improve the immune response to *S.*
495 *uberis* have focused on humoral immunity. However, a few observations point to a role of cell-
496 mediated immunity in the control of *S. uberis* MG infection. Blood lymphocytes of cows
497 protected after vaccination with live *S. uberis* proliferated in vitro when exposed to killed
498 vaccine bacteria (Hill et al., 1994). Spontaneous resolution of infection coincided with a
499 measurable interleukin IL-17A response in the milk of cows experimentally infected with *S.*

500 *uberis*, suggesting the involvement of IL-17A-producing lymphocytes (Tassi et al., 2013).
501 Gamma Interferon (IFN-γ), another cytokine mainly produced by T lymphocytes, is found in
502 the milk of *S. uberis*-infected quarters (Bannerman et al., 2004). Memory lymphocytes of CD8+
503 CD45RO+ phenotype proliferating when exposed to *S. uberis* crude antigen extract in the
504 presence of antigen-presenting cells were found in the blood and mammary secretion (dry
505 glands) of most cows, particularly from infected quarters (Denis et al., 2011): CD8 T cell lines
506 derived by exposure to *S. uberis* antigen released IFN-γ and had substantial killing activity
507 towards *S. uberis* in vitro. Immunization of cows by the subcutaneous route with an *S. uberis*
508 crude extract (twice before drying-off and once before calving) elicited an antibody response,
509 a moderate IL-17A and IFN-γ release in blood upon stimulation with antigen, and cells that
510 could exert a cytotoxic activity towards monocytes that had phagocytosed *S. uberis* (Wedlock
511 et al., 2014). The protective effect of these humoral and cell-mediated immune responses was
512 not determined.

513 Important questions regarding the immune mechanisms that are efficient in controlling
514 *S. uberis* mastitis remain unanswered. Is phagocytosis by neutrophils required, and what is the
515 cause of the apparent ineffective opsonophagocytic killing? Is inflammation a beneficial or
516 detrimental response to *S. uberis* infection of the MG? What cell-mediated immune response is
517 best suited to allow the MG to get rid of *S. uberis* infections? The interaction of *S. uberis* with
518 the MG remains intriguing in many respects. These uncertainties hamper the development of
519 an effective vaccine.

520

521 ## *Streptococcus agalactiae* Mastitis Vaccines

522 Clinical and subclinical mastitis due to *Streptococcus agalactiae* are frequent in
523 countries that develop their dairy industry, such as Brazil, China or Portugal (Almeida et al.,
524 2016, Bi et al., 2016, Carvalho-Castro et al., 2017, Pang et al., 2017), and are reemerging in

525 countries where dairy farming is developed but farming conditions change as in Northern
526 Europe (Mweu et al., 2012, Lyhs et al., 2016). This situation could rekindle interest in vaccine
527 design after a long period of neglect owing to the effectiveness of conventional control
528 measures in ridding herds of *S. agalactiae* MG infection and keeping them free from infection
529 (Keefe, 1997, Erskine, 2012).

530 The development of vaccines to prevent life-threatening infections of human neonates
531 or adults with underlying medical conditions is an active field of medical research (Song et al.,
532 2018), although, currently, no licensed *S. agalactiae* medical vaccine is available on the market
533 (Lin et al., 2018). Owing to the importance of *S. agalactiae* capsules in the pathogenesis of
534 infections, attempts have primarily targeted capsular polysaccharides to elicit opsonic
535 antibodies by conjugating purified polysaccharides to a protein carrier to enhance their
536 immunogenicity (Baker and Edwards, 2003). Complications are the multiplicity of capsules
537 serotypes (ten are considered in the serotype classification), which are susceptible to change
538 under vaccine pressure, and the cost and complexity of vaccine formulation. Bacterial surface
539 proteins are also used as antigens to elicit a humoral response aiming at improving phagocytosis
540 (Lindahl et al., 2005) and several clinical trials are ongoing (Song et al., 2018). With those
541 antigens, problems come from gene diversity and allelic variability of surface and secreted
542 proteins, mostly related to genomic islands and other mobile genetic elements (Brochet et al.,
543 2006). Besides, the accessibility of surface antigen differs from strain to strain (Maione et al.,
544 2005).

545 Early attempts to develop efficient vaccines against *S. agalactiae* mastitis relied on
546 bacterins, with variable results. An encouraging outcome was obtained with an autogenous
547 vaccine in combination with antibiotic therapy (Johnson and Norcross, 1971). Another study
548 showed that vaccination or natural infections were not protective despite the induction of
549 circulating strain-specific antibodies (Mackie et al., 1983). More recently, subunit vaccines

based on purified bacterial components have been assessed. The group B polysaccharide coupled to a protein carrier was shown to elicit opsonic antibodies in immunized cows, but the protective effect was not tested (Rainard, 1992). Streptococcal surface proteins have been the target of several trials, as they are shared by sizable proportions of mastitis isolates. Immunization of cows with the protein X elicited opsonic antibodies and increased the recruitment and bactericidal activity of neutrophils in milk at the onset of infection (Rainard et al., 1991). Another surface protein shared by most mastitis isolates is the CAMP factor, which induces some protection as a vaccine antigen in a mouse mastitis model (Liu et al., 2017).

These encouraging preliminary studies await their translation to the field. One possible pitfall is the capacity of *S. agalactiae* strains to modify the expression of their surface antigens, with possible eclipse or loss of capsular and protein production (Jensen, 1980, Rosini et al., 2015). It will probably be necessary to combine several of these antigens to circumvent that issue.

Streptococcus dysgalactiae Mastitis Vaccines

S. dysgalactiae subspecies *dysgalactiae* ranks highly among bacteria responsible for clinical and subclinical mastitis (Sampimon et al., 2009, Lundberg et al., 2014, Zhang et al., 2018). The dearth in research on the pathogenesis and immune response to this pathogen, relatively to the extensive research on pathogens of similar importance such as *S. uberis* or *S. aureus*, is surprising (Klaas and Zadoks, 2018). Consequently, we know little about the MG defenses specific to this pathogen. In an early vaccine trial, formalin-killed bacteria were administered by the subcutaneous route in the region of the supramammary lymph node (Stark and Norcross, 1970). Repeated, frequent immunizations were necessary to get partial protection which was attributed to the elicited antibodies. Recently, several groups used the dehydrogenase and protein receptor GapC as vaccine antigen. In a dry cow model, vaccination with GapC

575 reduced the challenge-induced inflammation as assessed by the concentrations of cells in the
576 MG secretion (Bolton et al., 2004). This preliminary study was not continued. A chimeric
577 protein comprising *S. dysgalactiae* GapC and two *S. aureus* proteins (IsdB and TRAP) elicited
578 protection in a mouse lethal challenge after two vaccine injections, the first in complete Freund
579 adjuvant, the second in incomplete Freund adjuvant (Yu et al., 2014). One asset of GapC is that
580 this protein shows a high sequence identity across several bacterial species so that cross-
581 protection can be expected. Indeed, cross-protection was obtained by immunizing mice with a
582 genetically modified *E. coli* expressing *S. dysgalactiae* GapC (11–150 sequence) in a lethal
583 mouse challenge with either *S. dysgalactiae*, *S. uberis* or *S. agalactiae* (1.5 to 2 x 10^9 CFU by
584 intraperitoneal injection) (Song et al., 2017). Translation to the cow will need challenges less
585 distant from natural infection of the MG. GapC-induced cross-protection is not systematic, as
586 immunization of cows with recombinant *S. uberis* GapC did not protect against challenges with
587 *S. dysgalactiae* (Fontaine et al., 2002). Another potential asset of GapC is that it elicits specific
588 CD4+ T cell immune responses preferentially towards Th1/Th17 polarization (Yao et al., 2016).
589 This line of research is worth pursuing.
590

591 ## *Staphylococcus aureus* VACCINES

592 ### Whole-bacterial Cell Vaccines

593 Despite the implementation of the standard mastitis prevention program, *S. aureus* mastitis
594 remains endemic in most countries and is the most frequent cause of subclinical mastitis of
595 dairy cows in many regions (Omore et al., 1996, Petzer et al., 2009, Kalmus et al., 2011, Mistry
596 et al., 2016, Poutrel et al., 2018). Mastitis by *S. aureus* remains a major problem to the dairy
597 industry owing to its contagiousness, persistence in infected glands, resistance to treatment and
598 threat to public health (Rainard et al., 2018). The development of an efficacious vaccine has
599 long been a research target. Some success has been achieved with bacterins combined with

600 detoxified staphylococcal toxins (formalin-treated culture supernate) to prevent the most severe
601 forms of mastitis of small ruminants (Derbyshire and Smith, 1969). This result can be
602 considered acceptable as the prevalence of *S. aureus* in small ruminant herds is usually limited
603 and the main issue is with severe, often gangrenous mastitis (Bergonier and Berthelot, 2003,
604 Contreras et al., 2003, Gelasakis et al., 2015). In cows, *S. aureus* mastitis is usually subclinical,
605 so what is expected of an efficient vaccine is to reduce the incidence of new infections and limit
606 their spread within herds (Middleton, 2008). As most infections persist over long periods,
607 another indication would be the elimination of chronic infections. The results obtained with
608 bacterin plus toxoids have been inconsistent (Slanetz et al., 1965, Brock et al., 1975). The
609 addition of toxoids to killed bacteria was necessary to obtain the best protection (Derbyshire,
610 1960). In this regard, several studies have suggested that the alpha and beta toxins are involved
611 in the pathogenesis of mastitis (Bramley and Neave, 1975, Yancey, 1993). An improvement
612 over this first approach has been sought by taking into consideration the production of bacterial
613 antigens during infection. Live vaccines confer better protection than do killed vaccines, an
614 edge that was attributed to the production of "in vivo antigens" and to the elicitation of
615 antibodies in the IgG2 isotype (Watson, 1981). In addition, the live vaccine boosted the
616 recruitment of neutrophils into the MG during the initiation phase of the inflammatory response
617 (Colditz and Watson, 1982). A preparation of bacteria grown under suitable in vitro conditions
618 stimulating the production of a pseudocapsule or slime, administered by the systemic
619 (subcutaneous) route with an appropriate adjuvant favoring the production of antibodies in the
620 IgG_2 isotype (dextran sulfate), was proposed as a strategy to get protection (Watson, 1992a).
621 The live vaccine lead has recently been refined by engineering a defective *VraG* mutant in a
622 genetically stable small colony variant (SCV) *S. aureus* (Côté-Gravel et al., 2016). SCVs have
623 altered metabolism and slow growth, associated with reduced production of toxins but increased
624 capacity to invade, survive and hide within epithelial cells compared to their parent strains

(Atalla et al., 2011). It has been proposed that SCVs are associated with chronic infections of the MG, and shown that they induce in cows immune responses different from those induced by parent strains (Atalla et al., 2010). Strongly expressed by *S. aureus* during mastitis, *VraG* encodes a putative ABC transporter that could be required for cationic antimicrobial peptide sensing and resistance (Allard et al., 2013). The resulting virulence-attenuated mutant induced a marked antibody response cross-reacting with a variety of mastitis *S. aureus* isolates in mice when administered as a live vaccine by the subcutaneous route (Côté-Gravel et al., 2016). Besides antibodies, the live-attenuated vaccine elicited a cell-mediated response characterized by the induction of splenocytes secreting IFN-γ and IL-17, contrary to a bacterin combined or not with inactivated *E. coli* J5 (Côté-Gravel et al., 2019). These studies in mice prompt further research and translation to ruminants. The SCV formation provides the possibility of delivering antigens in the host cell cytoplasmic compartment, which could modify the orientation of the cell-mediated immune response (Côté-Gravel et al., 2019).

The "in vivo antigen" approach was patented (Watson, 1992a) but has not find a way into widespread commercialization. Another patented bacterin vaccine preparation, comprising three *S. aureus* strains grown in a medium favoring the production of exopolysaccharides, showed encouraging results in a small-scale experiment, but without published follow-up (Leitner et al., 2003). More recently, the "slime track" has been followed and developed (Pérez et al., 2009). The production of exopolysaccharides by *S. aureus* mastitis isolates is inducible by growth in special laboratory media rich in salt and sugars or in milk whey (Baselga et al., 1994). Among these exopolysaccharides there are true capsular polysaccharides, which form thin and discontinuous microcapsules of mainly two serotypes, CP5 and CP8 (Sutra et al., 1990a, Poutrel and Sutra, 1993), with a variable expression between strains and within bacterial populations of most mastitis isolates (Poutrel et al., 1997). Specific antibodies can be elicited by immunizing cows with capsular polysaccharides conjugated to a protein carrier (Gilbert et

al., 1994, Lee et al., 2005). The anti-capsular antibodies are opsonic (Kampen et al., 2005) but are not required to opsonize mastitis isolates. Moreover, a number of isolates from chronic infections do not produce capsules because they repress their production or have mutated in the capsule operon (Tuchscherr et al., 2005). Those nontypable variants or mutants are supposed to have an increased capacity to adhere to and invade epithelial cells than their capsulated counterparts (Tuchscherr et al., 2010). On the other hand, the growth of mastitis isolates in exopolysaccharide-promoting media interferes with phagocytosis by neutrophils (Sutra et al., 1990b). Apart from capsular polysaccharides, the contribution of inducible slime is likely. Bacterins comprising strong slime-producing *S. aureus* induce antibodies against poly-N-acetyl-β-glucosamine (PNAG), a major component of slime, and have conferred protection of lactating ewes against infection and mastitis (Pérez et al., 2009). Bacterins were better inducers of antibodies to PNAG than was purified slime, even when slime was incorporated into liposomes. After an intramammary challenge with a live heterologous *S. aureus* strain, the shedding of bacteria was reduced by two orders of magnitude in the groups vaccinated with the high slime-producing bacteria compared to controls and animals in the groups vaccinated with the low slime-producing bacteria. Surprisingly, bacterial concentrations in the milk of ewes immunized with crude or purified PNAG exceeded concentrations in milk of control ewes. Clinical data and mammary tissue lesions tended to correlate negatively with the antibody titers and bacterial shedding reduction in milk (Pérez et al., 2009). Accordingly, antibodies induced by the deacylated form of PNAG (dPNAG) arre opsonic (Maira-Litran et al., 2005). The study suggests that antibodies to slime, including PNAG, are induced when associated with bacterial bodies, and that they protect against, or correlate with protection to, severe mastitis in ewes. This protection manifested itself by a marked reduction in the severity of the symptoms. It should be noted, however, that not all mastitis isolates are high producers of slime (Oliveira et al., 2006, Vautor et al., 2008, Dhanawade et al., 2010). The slime vaccine approach was pursued

675 in a trial involving cows that were vaccinated with *S. aureus* bacterins with either high or low
676 slime content (Prenafeta et al., 2010). Two doses of bacterins were administered 45 days apart
677 to primiparous gestating cows before calving, followed by an intramammary challenge with a
678 heterologous strain of *S. aureus*. Compared to unvaccinated heifers, only those vaccinated with
679 the high slime-producing bacterin shed less colony-forming units (CFU) of bacteria in their
680 milk on day 1 post-challenge, but there was no difference in clinical signs between groups.
681 Antibodies to slime were associated with the reduction in milk CFU numbers. This line of
682 research led to the release of a licensed vaccine in Europe in 2009 (Startvac®. Hipra SA).

683 A field study was conducted in two large dairy herds by using the StartVac® vaccine
684 according to the label directions (Schukken et al., 2014). Cows received three doses of the
685 vaccine, at 45 days before the expected parturition date, 35 days later, and at 52 days in milk.
686 The vaccine consists of killed *E. coli J5* bacteria and killed *S. aureus* slime-producing
687 strain SP140 emulsified in liquid paraffin. It is administered by the intramuscular route. Over
688 the 21-month observation period, vaccination resulted in a moderate reduction in the incidence
689 of new staphylococcal IMI, and complex modeling of the IMI dynamics yielded a reduction of
690 the "basic reproduction ratio" of 45% for *S. aureus* and 35% for coagulase-negative
691 staphylococci. In a field study involving seven farms, the vaccine administered following the
692 label regimen did not reduce the incidence or prevalence of clinical or subclinical mastitis but
693 was associated with a significant reduction in the severity of clinical cases and milk losses, with
694 a return on investment of 2.57 to 1 (Bradley et al., 2015). In that study, most clinical cases
695 (25%) were due to *E. coli*, clinical cases caused by *S. aureus* or coagulase-negative
696 staphylococci CoNS accounting for only 2.5 and 5.6% of cases, respectively. Another field
697 study conducted in two large farms in which *S. aureus* mastitis prevalence was high did not
698 show any beneficial effect on udder health, milk production or culling rate (Landin et al., 2015).
699 Another trial carried out in a herd in which *S. aureus* was the predominant pathogen led the

700 authors to conclude that the vaccine was not an appropriate tool to manage the *S. aureus*
701 problem (Freick et al., 2016).

702 These discrepant results can be compared to the efficacy assessment of another vaccine
703 licensed in Northern America, the Lysigin® vaccine (Boehringer Ingelheim Vetmedica, Inc.).
704 The vaccine is composed of a lysate of one serotype 5, two serotype 8 and two nontypable
705 ("serotype 336", not a capsular polysaccharide but probably cell wall teichoic acid (Verdier et
706 al., 2007)) strains (Ma et al., 2004), administered by the subcutaneous route with an alum-based
707 proprietary adjuvant. Lysigin vaccine efficacy has been evaluated in several studies, with
708 variable results showing a decreased clinical severity of mastitis, lower milk SCC, sometimes
709 a reduction in the incidence of IMI (reviewed in (Middleton, 2008)). However, unfavorable
710 results were also obtained, as in a study with heifers vaccinated twice in late gestation, in which
711 the only positive effect of the vaccine was a reduction in the duration of clinical mastitis after
712 challenge (Middleton et al., 2006). A subsequent field study by the same group did not show a
713 reduction in the prevalence or incidence of *S. aureus* or CoNS IMI by the Lysigin® vaccine
714 (Middleton et al., 2009).

715

716 **Subunit Vaccines**

717 Overall, the commercial vaccines against *S. aureus* mastitis tend to reduce the severity
718 of clinical mastitis but do not solve the issue of chronic subclinical mastitis. The current bacterin
719 vaccines are not very different from the vaccine approach advocated by Dennis Watson
720 (Watson, 1992a). Many other attempts to improve on these vaccines have been based on the
721 use of bacterial components supposed to play a role in the pathogenesis or to be the target of
722 efficient immune defenses of the host (Scali et al., 2015). An early attempt was the evaluation
723 of staphylococcal protein A to protect cows from repeated exposure to *S. aureus* by dipping the
724 teats in a bacterial suspension immediately after milking (Pankey et al., 1985). Vaccinated and

control cows had a similar incidence of *S. aureus* mastitis and milk production, but the vaccine improved the spontaneous cure rate of IMI. However, the definition of infection was based on only one positive diagnostic, which is not sufficient to define an established infection, even more so if we consider that transient colonization of the teat canal by *S. aureus* is likely to have occurred bearing in mind the challenge procedure.

Staphylococcus aureus is well equipped to adhere to epithelial cells, thanks to the regulated expression of a number of redundant adhesins (Foster and Hook, 1998). Among the adhesins that are likely to contribute to adhesion and invasion, Fibronectin-binding proteins (FnBPA and FnBPB) are important contributors (Brouillette et al., 2004). Immunization with FnBP, collagen-binding protein, or clumping factor usually decreased the severity of mastitis induced by intramammary challenges (Mamo et al., 1995, Mamo et al., 2000, Hu et al., 2010). These studies were conducted in murine mastitis models, and their translation to the cow remains to be performed. A DNA vaccine based on a plasmid encoding the bovine granulocyte-macrophage—colony-stimulating factor (GM-CSF) and the *S. aureus FnBP* and clumping factor A (*ClfA*) genes was used to vaccinate heifers twice before a final booster injection of the two recombinant *S. aureus* proteins (Shkreta et al., 2004). The intramammary challenge with *S. aureus* Newbould 305 was carried out in three quarters of vaccinated (n= 4) and control (n = 4) cows three weeks after calving. The vaccine elicited antibodies and lymphoproliferative responses, with partial protection illustrated by a reduced bacterial shedding and an increased cure rate in the vaccinated animals (Shkreta et al., 2004). These encouraging results await confirmation on a larger scale.

The capacity to acquire iron or growth in milk is an important attribute of mastitis-associated bacteria. The expression of several *S. aureus* iron-regulated genes is upregulated during growth in vivo (Allard et al., 2006). Among these genes, those of the iron-regulated surface determinants (Isd) system (*IsdABSDEFGHI*) are specialized in the acquisition of iron

750 from the heme proteins (Skaar and Schneewind, 2004). Cattle immunization with IsdB and
751 IsdH induced strong antibody responses and proliferation of CD4+ but not CD8+ cells in a
752 PBMC stimulation assay (Ster et al., 2010). Protection in cattle induced by these antigens has
753 not been established.

754 Other shared antigens have been identified as vaccine antigen candidates. Two proteins
755 with homology to glyceraldehyde-3-phosphate dehydrogenase, GapB and GapC, have highly
756 conserved sequences and are shared by mastitis-associated *S. aureus* (Goji et al., 2004). DNA
757 vaccination with plasmids encoding the GapC and GapB proteins boosted with recombinant
758 proteins induced humoral and cellular immune responses in mice, but protection was not tested
759 (Kerro-Dego et al., 2006). Research is ongoing to optimize the production of neutralizing
760 antibodies in the MG to *S. aureus* immune evasion proteins such as adhesins or leukotoxins by
761 combining adjuvants and selecting the most appropriate site of injection (Boerhout et al., 2015,
762 Boerhout et al., 2018, Misra et al., 2018). An overview of the main *S. aureus* vaccine trials is
763 given in Table 2.

764 The general picture emerging from these numerous *S. aureu*s vaccine trials, and still
765 more numerous not cited here due to redundancy and space constraints, is one of encouraging
766 perspectives followed by aborted development. The few vaccines that reached licensing and
767 field use so far are based on killed whole cell bacteria and have had mixed results. All
768 researchers agreed that there is ample room for improvement.

769

770 **CONCLUSIONS**

771 This review aims at giving an objective account of the present state of mastitis vaccines and
772 current research. It is clear that the achievements are not up to expectations, whatever the
773 bacteria involved. There are several obstacles to the development of efficacious vaccines
774 against mammary pathogens. Some result from the MG physiology, particularly in lactation, as

775 dairy cows have been selected to secrete large amounts of milk which blunts and waters down
776 immune defenses. Others pertain to the pathogens, such as their diversity and their adaptation
777 to the MG niche. The complexities of the host-pathogen interaction within the cow's MG
778 represent another major obstacle to the development of efficacious mastitis vaccines of the host-
779 pathogen interaction within the cow's mammary gland represent a major obstacle to the
780 development of efficacious mastitis vaccines. These difficulties may be confounded by a
781 possible misdirection of most of past research, biased towards antibody-dependent defenses
782 (Figure 2). There is a need for an in-depth analysis of the reasons that could explain the lack of
783 success of past attempts, with a view to proposing new ways of getting out of the mastitis
784 vaccine predicament. One way is to explore how cell-mediated immunity could strengthen MG
785 defenses, and how this immunity could be harnessed by vaccination. Eliciting type 3 immunity
786 in the MG is an attractive option (Rainard et al., 2020). It will be useful to select adjuvants that
787 orient the immune response towards protection and to validate reliable correlates of protection.
788 The route of administration is also likely to be of major importance. In addition, lessons learned
789 from successes and failures of vaccines directed at other diseases could help identify the
790 peculiarities of the MG niche and the different ways bacteria cause mastitis, hence helping to
791 focus on relevant vaccine targets.

792 There have been a number of attempts to follow new research leads that have already
793 yielded some promising results, but much attention has been paid to the choice of antigens
794 and adjuvant (vaccinology) and little to the induced immune response (immunology). Doubts
795 about vaccination have been expressed due to the numerous failures and misleading claims of
796 success. In our opinion, there is no unsurmontable obstacle to the development of efficacious
797 mastitis vaccines. Nonetheless, lessons must be learned from past attempts, a frank appraisal
798 of current achievements made and new approaches boldly adopted. Then a much greater
799 chance of success will arise.

Acknowledgments

The authors are grateful to many colleagues for the fruitful discussions and exchanges over the past years. We apologize to all the researchers whose studies we could not mention due to space limitations. We also had to make choices for the sake of clarity, as there have been so many studies in the field covered by our review. We hope that our blindspots have not hampered the pertinence of our findings and conclusions. The authors have not stated any conflict of interests.

REFERENCES

Allard, M., H. Moisan, E. Brouillette, A. L. Gervais, M. Jacques, P. Lacasse, M. S. Diarra, and F. Malouin. 2006. Transcriptional modulation of some *Staphylococcus aureus* iron-regulated genes during growth in vitro and in a tissue cage model in vivo. Microbes Infect. 8(7):1679-1690.

Allard, M., C. Ster, C. L. Jacob, D. Scholl, M. S. Diarra, P. Lacasse, and F. Malouin. 2013. The expression of a putative exotoxin and an ABC transporter during bovine intramammary infection contributes to the virulence of *Staphylococcus aureus*. Vet. Microbiol. 162(2-4):761-770.

Almeida, A., C. Alves-Barroco, E. Sauvage, R. Bexiga, P. Albuquerque, F. Tavares, I. Santos-Sanches, and P. Glaser. 2016. Persistence of a dominant bovine lineage of group B *Streptococcus* reveals genomic signatures of host adaptation. Environ. Microbiol. 18(11):4216-4229.

Almeida, R. A., O. K. Dego, S. I. Headrick, M. J. Lewis, and S. P. Oliver. 2015. Role of *Streptococcus uberis* adhesion molecule in the pathogenesis of *Streptococcus uberis* mastitis. Vet. Microbiol. 179(3-4):332-335.

Almeida, R. A., D. A. Luther, H. M. Park, and S. P. Oliver. 2006. Identification, isolation, and partial characterization of a novel *Streptococcus uberis* adhesion molecule (SUAM). Vet. Microbiol. 115(1-3):183-191.

828 Almeida, R. A. and S. P. Oliver. 1993. Antiphagocytic Effect of the Capsule of *Streptococcus*
829 *uberis*. J. Vet. Med. Series B 40(9-10):707-714.
830 Archer, N., S. A. Egan, T. J. Coffey, R. D. Emes, M. F. Addis, P. N. Ward, A. M. Blanchard, and J.
831 A. Leigh. 2020. A Paradox in Bacterial Pathogenesis: Activation of the Local Macrophage
832 Inflammasome Is Required for Virulence of *Streptococcus uberis*. Pathogens 9(12):997.
833 Atalla, H., C. Gyles, and B. Mallard. 2011. *Staphylococcus aureus* small colony variants (SCVs)
834 and their role in disease. Anim Health Res. Rev. 12(1):33-45.
835 Atalla, H., B. Wilkie, C. Gyles, K. Leslie, L. Mutharia, and B. Mallard. 2010. Antibody and cell-
836 mediated immune responses to *Staphylococcus aureus* small colony variants and their parental
837 strains associated with bovine mastitis. Dev. Comp. Immunol. 34(12):1283-1290.
838 Baker, C. J. and M. S. Edwards. 2003. Group B streptococcal conjugate vaccines. Arch. Dis.
839 Child. 88(5):375-378.
840 Bannerman, D. D., M. J. Paape, J. P. Goff, K. Kimura, J. D. Lippolis, and J. C. Hope. 2004. Innate
841 immune response to intramammary infection with *Serratia marcescens* and *Streptococcus uberis*.
842 Vet. Res. 35(6):681-700.
843 Baselga, R., I. Albizu, and B. Amorena. 1994. *Staphylococcus aureus* capsule and slime as
844 virulence factors in ruminant mastitis. A review. Vet. Microbiol. 39(3-4):195-204.
845 Baumgartner, J. D. and M. P. Glauser. 1993. Immunotherapy of Endotoxemia and Septicemia.
846 Immunobiol. 187(3-5):464-477.
847 Baumgartner, J. D., D. Heumann, T. Calandra, and M. P. Glauser. 1991. Antibodies to
848 lipopolysaccharides after immunization of humans with the rough mutant *Escherichia coli* J5. J.
849 Infect. Dis. 163(4):769-772.
850 Bergonier, D. and X. Berthelot. 2003. New advances in epizootiology and control of ewe
851 mastitis. Livestock Prod. Sci. 79(1):1-16.
852 Bi, Y., Y. J. Wang, Y. Qin, R. Guix Vallverdu, J. Maldonado Garcia, W. Sun, S. Li, and Z. Cao. 2016.
853 Prevalence of Bovine Mastitis Pathogens in Bulk Tank Milk in China. PLoS ONE 11(5):e0155621.
854 Blum, S. E., R. J. Goldstone, J. P. R. Connolly, M. Reperant-Ferter, P. Germon, N. F. Inglis, O.
855 Krifucks, S. Mathur, E. Manson, K. McLean, P. Rainard, A. J. Roe, G. Leitner, and D. G. E. Smith. 2018.
856 Postgenomics Characterization of an Essential Genetic Determinant of Mammary Pathogenic
857 *Escherichia coli*. MBio 9(2).
858 Boerhout, E., M. Vrieling, L. Benedictus, I. Daemen, L. Ravesloot, V. Rutten, P. Nuijten, J. van
859 Strijp, A. Koets, and S. Eisenberg. 2015. Immunization routes in cattle impact the levels and
860 neutralizing capacity of antibodies induced against *S. aureus* immune evasion proteins. Vet. Res.
861 46(1):115.

862 Boerhout, E. M., A. P. Koets, T. G. T. Mols-Vorstermans, P. J. M. Nuijten, M. J. H. Hoeijmakers,
863 V. Rutten, and J. J. E. Bijlsma. 2018. The antibody response in the bovine mammary gland is
864 influenced by the adjuvant and the site of subcutaneous vaccination. Vet. Res. 49(1):25.
865 Bolton, A., X. M. Song, P. Willson, M. C. Fontaine, A. A. Potter, and J. Perez-Casal. 2004. Use of
866 the surface proteins GapC and Mig of *Streptococcus dysgalactiae* as potential protective antigens
867 against bovine mastitis. Can. J. Microbiol. 50(6):423-432.
868 Brade, L., S. Hensen, and H. Brade. 2013. Evaluation of a LPS-based glycoconjugate vaccine
869 against bovine *Escherichia coli* mastitis: Formation of LPS Abs in cows after immunization with *E. coli*
870 core oligosaccharides conjugated to hemocyanine. Innate immunity 19(4):368-377.
871 Bradley, A. J., J. E. Breen, B. Payne, V. White, and M. J. Green. 2015. An investigation of the
872 efficacy of a polyvalent mastitis vaccine using different vaccination regimens under field conditions in
873 the United Kingdom. J. Dairy Sci. 98(3):1706-1720.
874 Bradley, A. J., J. E. Breen, B. Payne, P. Williams, and M. J. Green. 2010. The use of a
875 cephalonium containing dry cow therapy and an internal teat sealant, both alone and in
876 combination. J. Dairy Sci. 93(4):1566-1577.
877 Bramley, A. J. and F. K. Neave. 1975. Studies on the control of coliform mastitis in dairy cows.
878 Br. Vet. J. 131(2):160-169.
879 Braun, V. 1997. Surface signaling: Novel transcription initiation mechanism starting from the
880 cell surface. Arch. Microbiol. 167(6):325-331.
881 Brochet, M., E. Couve, M. Zouine, T. Vallaeys, C. Rusniok, M. C. Lamy, C. Buchrieser, P. Trieu-
882 Cuot, F. Kunst, C. Poyart, and P. Glaser. 2006. Genomic diversity and evolution within the species
883 *Streptococcus agalactiae*. Microbes Infect. 8(5):1227-1243.
884 Brock, J. H., E. D. Steel, and B. Reiter. 1975. The effect of intramuscular and intramammary
885 vaccination of cows on antibody levels and resistance to intramammary infection by *Staphylococcus*
886 *aureus*. Res. Vet. Sci. 19(2):152-158.
887 Brouillette, E., G. Grondin, C. Lefebvre, B. G. Talbot, and F. Malouin. 2004. Mouse mastitis
888 model of infection for antimicrobial compound efficacy studies against intracellular and extracellular
889 forms of *Staphylococcus aureus*. Vet. Microbiol. 101(4):253-262.
890 Carvalho-Castro, G. A., J. R. Silva, L. V. Paiva, D. A. C. Custodioa, R. O. Moreira, G. F. Mian, I. A.
891 Prado, A. Chalfun, and G. M. Costa. 2017. Molecular epidemiology of *Streptococcus agalactiae*
892 isolated from mastitis in Brazilian dairy herds. Braz. J. Microbiol. 48(3):551-559.
893 Cebron, N., S. Maman, S. Walachowski, B. Gausseres, P. Cunha, P. Rainard, and G. Foucras.
894 2020. Mammary Th17-related immunity, but not high systemic Th1 response is associated with
895 protection against *E. coli* mastitis. NPJ Vaccines 5(1):108.

Chaiyotwittayakun, A., J. L. Burton, P. S. Weber, K. Kizilkaya, F. F. Cardoso, and R. J. Erskine. 2004. Hyperimmunization of steers with J5 *Escherichia coli* bacterin: effects on isotype-specific serum antibody responses and cross reactivity with heterogeneous Gram-negative bacteria. J. Dairy Sci. 87(10):3375-3385.

Colditz, I. G. and D. L. Watson. 1982. Effect of immunisation on the early influx of neutrophils during staphylococcal mastitis in ewes. Res. Vet. Sci. 33(2):146-151.

Collado, R., C. Montbrau, M. Sitja, and A. Prenafeta. 2018. Study of the efficacy of a *Streptococcus uberis* mastitis vaccine against an experimental intramammary infection with a heterologous strain in dairy cows. J. Dairy Sci. 101(11):10290-10302.

Contreras, A., C. Luengo, A. Sanchez, and J. C. Corrales. 2003. The role of intramammary pathogens in dairy goats. Livestock Product.Sci. 79:273-283.

Côté-Gravel, J., E. Brouillette, and F. Malouin. 2019. Vaccination with a live-attenuated small-colony variant improves the humoral and cell-mediated responses against *Staphylococcus aureus*. PLoS ONE 14(12):e0227109.

Côté-Gravel, J., E. Brouillette, N. Obradovic, C. Ster, B. G. Talbot, and F. Malouin. 2016. Characterization of a vraG Mutant in a Genetically Stable *Staphylococcus aureus* Small-Colony Variant and Preliminary Assessment for Use as a Live-Attenuated Vaccine against Intrammamary Infections. PLoS ONE 11(11):e0166621.

Craven, N. and M. R. Williams. 1985. Defences of the bovine mammary gland against infection and prospects for their enhancement. Vet. Immunol. Immunopathol. 10(1):71-127.

Cross, A. S. 2014. Anti-endotoxin vaccines: back to the future. Virulence 5(1):219-225.

Cross, A. S., H. J. Karreman, L. Zhang, Z. Rosenberg, S. M. Opal, and A. Lees. 2014. Immunization of cows with novel core glycolipid vaccine induces anti-endotoxin antibodies in bovine colostrum. Vaccine 32(46):6107-6114.

Crowley, R. C., J. A. Leigh, P. N. Ward, H. M. Lappin-Scott, and L. D. Bowler. 2011. Differential Protein Expression in *Streptococcus uberis* under Planktonic and Biofilm Growth Conditions. Appl. Environ. Microbiol. 77(1):382-384.

Cullor, J. S. 1991. The *Escherichia coli* J5 vaccine: investigating a new tool to combat coliform mastitis. Veterinary Medicine 86(8):836-844.

Cunningham, M. W. 2000. Pathogenesis of group A streptococcal infections. Clin. Microbiol. Rev. 13(3):470-511.

De Cueninck, B. J. 1979. Immune-mediated inflammation in the lumen of the bovine mammary gland. Int. Arch. Allergy Appl. Immunol. 59(4):394-402.

DeGraves, F. J. and J. Fetrow. 1991. Partial budget analysis of vaccinating dairy cattle against coliform mastitis with an Escherichia coli J5 vaccine. J. Am. Vet. Med. Assoc. 199(4):451-455.

931 Denis, M., S. J. Lacy-Hulbert, B. M. Buddle, J. H. Williamson, and D. N. Wedlock. 2011.
932 *Streptococcus uberis*-specific T cells are present in mammary gland secretions of cows and can be
933 activated to kill. Vet. Res. Commun. 35(3):145-156.
934 Denis, M., N. A. Parlane, S. J. Lacy-Hulbert, E. L. Summers, B. M. Buddle, and D. N. Wedlock.
935 2006. Bactericidal activity of macrophages against *Streptococcus uberis* is different in mammary
936 gland secretions of lactating and drying off cows. Vet. Immunol. Immunopathol. 114(1-2):111-120.
937 Denis, M., D. N. Wedlock, S. J. Lacy-Hulbert, J. E. Hillerton, and B. M. Buddle. 2009. Vaccines
938 against bovine mastitis in the New Zealand context: what is the best way forward? New Zealand Vet.
939 J. 57(3):132-140.
940 Derbyshire, J. B. 1960. Studies in immunity to experimental staphylococcal mastitis in the goat
941 and cow. J. Comp. Pathol. 70:222-231.
942 Derbyshire, J. B. and G. S. Smith. 1969. Immunization against experimental staphylococcal
943 mastitis in the goat by the intramammary infusion of cell-toxoid vaccine. Res. Vet. Sci. 10(6):559-564.
944 Dhanawade, N. B., D. R. Kalorey, R. Srinivasan, S. B. Barbuddhe, and N. V. Kurkure. 2010.
945 Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated
946 from bovine subclinical mastitis. Vet. Res. Commun. 34(1):81-89.
947 Dieser, S. A., A. S. Fessia, M. P. Ferrari, C. G. Raspanti, and L. M. Odierno. 2017. *Streptococcus*
948 *uberis*: In vitro biofilm production in response to carbohydrates and skim milk. Rev. Argent.
949 Microbiol. 49(4):305-310.
950 Ditcham, W. G., J. A. Leigh, A. P. Bland, and A. W. Hill. 1996. Adhesion of *Streptococcus uberis*
951 to monolayers of cultured epithelial cells derived from the bovine mammary gland. FEMS Immunol.
952 Med. Microbiol. 14(2-3):145-150.
953 Dogan, B., M. Rishniw, G. Bruant, J. Harel, Y. H. Schukken, and K. W. Simpson. 2012.
954 Phylogroup and lpfA influence epithelial invasion by mastitis associated *Escherichia coli*. Vet.
955 Microbiol. 159(1-2):163-170.
956 Dosogne, H., F. Vangroenweghe, and C. Burvenich. 2002. Potential mechanism of action of J5
957 vaccine in protection against severe bovine coliform mastitis. Vet. Res. 33(1):1-12.
958 Erskine, R. J. 2012. Vaccination strategies for mastitis. Vet. Clin. North Am. Food Anim. Pract.
959 28(2):257-270.
960 Erskine, R. J., E. J. VanDyk, P. C. Bartlett, J. L. Burton, and M. C. Boyle. 2007. Effect of
961 hyperimmunization with an *Escherichia coli* J5 bacterin in adult lactating dairy cows. J. Am. Vet. Med.
962 Assoc. 231(7):1092-1097.
963 Field, T. R., P. N. Ward, L. H. Pedersen, and J. A. Leigh. 2003. The hyaluronic acid capsule of
964 *Streptococcus uberis* is not required for the development of infection and clinical mastitis. Infect.
965 Immun. 71(1):132-139.

966 Finch, J. M., A. W. Hill, T. R. Field, and J. A. Leigh. 1994. Local vaccination with killed
967 *Streptococcus uberis* protects the bovine mammary gland against experimental intramammary
968 challenge with the homologous strain. Infect. Immun. 62(9):3599-3603.
969 Finch, J. M., A. Winter, A. W. Walton, and J. A. Leigh. 1997. Further studies on the efficacy of a
970 live vaccine against mastitis caused by *Streptococcus uberis*. Vaccine 15(10):1138-1143.
971 Fontaine, M. C., J. Perez-Casal, X. M. Song, J. Shelford, P. J. Willson, and A. A. Potter. 2002.
972 Immunisation of dairy cattle with recombinant *Streptococcus uberis* GapC or a chimeric CAMP
973 antigen confers protection against heterologous bacterial challenge. Vaccine 20(17-18):2278-2286.
974 Foster, T. J. and M. Hook. 1998. Surface protein adhesins of *Staphylococcus aureus*. Trends
975 Microbiol. 6(12):484-488.
976 Freick, M., Y. Frank, K. Steinert, A. Hamedy, O. Passarge, and A. Sobiraj. 2016. Mastitis
977 vaccination using a commercial polyvalent vaccine or a herd-specific *Staphylococcus aureus* vaccine
978 Results of a controlled field trial on a dairy farm. Tieraerztl Prax G N 44(4):219-229.
979 Garcia, S. N., B. I. Osburn, and J. S. Cullor. 2019. A one health perspective on dairy production
980 and dairy food safety. One Health 7:9.
981 Garenaux, A., M. Caza, and C. M. Dozois. 2011. The Ins and Outs of siderophore mediated iron
982 uptake by extra-intestinal pathogenic Escherichia coli. Vet. Microbiol. 153(1-2):89-98.
983 Gaucheron, F. 2005. The minerals of milk. Reprod. Nutr. Dev. 45(4):473-483.
984 Gelasakis, A. I., V. S. Mavrogianni, I. G. Petridis, N. G. C. Vasileiou, and G. C. Fthenakis. 2015.
985 Mastitis in sheep - The last 10 years and the future of research. Vet. Microbiol. 181(1-2):136-146.
986 Gilbert, F. B., B. Poutrel, and L. Sutra. 1994. Immunogenicity in cows of *Staphylococcus aureus*
987 type 5 capsular polysaccharide-ovalbumin conjugate. Vaccine 12(4):369-374.
988 Goerge, T., M. B. Lorenz, S. van Alen, N. O. Hubner, K. Becker, and R. Kock. 2017. MRSA
989 colonization and infection among persons with occupational livestock exposure in Europe:
990 Prevalence, preventive options and evidence. Vet. Microbiol. 200:6-12.
991 Goji, N., A. A. Potter, and J. Perez-Casal. 2004. Characterization of two proteins of
992 *Staphylococcus aureus* isolated from bovine clinical mastitis with homology to glyceraldehyde-3-
993 phosphate dehydrogenase. Vet. Microbiol. 99(3-4):269-279.
994 Goldstone, R. J., S. Harris, and D. G. Smith. 2016. Genomic content typifying a prevalent clade
995 of bovine mastitis-associated *Escherichia coli*. Sci. Rep. 6:30115.
996 Gonzalez, R. N., J. S. Cullor, D. E. Jasper, T. B. Farver, R. B. Bushnell, and M. N. Oliver. 1989.
997 Prevention of clinical coliform mastitis in dairy cows by a mutant *Escherichia coli* vaccine. Can. J. Vet.
998 Res. 53(3):301-305.
999 Gorden, P. J., M. D. Kleinhenz, J. A. Ydstie, T. A. Brick, L. M. Slinden, M. P. Peterson, D. E.
1000 Straub, and D. T. Burkhardt. 2018. Efficacy of vaccination with a *Klebsiella pneumoniae* siderophore

1001 receptor protein vaccine for reduction of Klebsiella mastitis in lactating cattle. J. Dairy Sci.
1002 101(11):10398-10408.
1003 Grant, R. G. and J. M. Finch. 1996. Phagocytosis of *Streptococcus uberis* by bovine mammary
1004 gland macrophages. Res.Vet.Sci. 62(1):74-78.
1005 Green, M. J. and A. J. Bradley. 2013. The changing face of mastitis control. Vet. Rec. 173:517-
1006 521.
1007 Greisman, S. E. and C. A. Johnston. 1997. Evidence against the hypothesis that antibodies to
1008 the inner core of lipopolysaccharides in antisera raised by immunization with enterobacterial deep-
1009 rough mutants confer broad-spectrum protection during Gram-negative bacterial sepsis. J. Endotoxin
1010 Res. 4(2):123-153.
1011 Herry, V., C. Gitton, G. Tabouret, M. Reperant, L. Forge, C. Tasca, F. B. Gilbert, E. Guitton, C.
1012 Barc, C. Staub, D. G. E. Smith, P. Germon, G. Foucras, and P. Rainard. 2017. Local immunization
1013 impacts the response of dairy cows to *Escherichia coli* mastitis. Sci. Rep. 7(1):3441.
1014 Hill, A. W. 1988. Pathogenicity of two strains of Streptococcus uberis infused into lactating and
1015 non-lactating bovine mammary glands. Res. Vet. Sci. 45(3):400-404.
1016 Hill, A. W. 1991. Vaccination of cows with rough *Escherichia coli* mutants fails to protect
1017 against experimental intramammary bacterial challenge. Vet.Res.Commun. 15:7-16.
1018 Hill, A. W., J. M. Finch, T. R. Field, and J. A. Leigh. 1994. Immune modification of the
1019 pathogenesis of *Streptococcus uberis* mastitis in the dairy cow. FEMS Immunol. Med. Microbiol.
1020 8(2):109-117.
1021 Hill, A. W., D. J. Heneghan, T. R. Field, and M. R. Williams. 1983a. Increase in specific opsonic
1022 activity in bovine milk following experimental *Escherichia coli* mastitis. Res.Vet.Sci. 35(2):222-226.
1023 Hill, A. W., D. J. Heneghan, and M. R. Williams. 1983b. The opsonic activity of bovine milk whey
1024 for the phagocytosis and killing by neutrophils of encapsulated and non-encapsulated *Escherichia*
1025 *coli*. Vet. Microbiol. 8(3):293-300.
1026 Hogan, J. S., V. B. Cannon, K. L. Smith, C. Rinehart, and S. Miller. 2005. Effects of adjuvants on
1027 safety and efficacy of an *Escherichia coli* J5 bacterin. J. Dairy Sci. 88(2):534-542.
1028 Hogan, J. S., K. L. Smith, D. A. Todhunter, and P. S. Schoenberger. 1992a. Field trial to
1029 determine efficacy of an *Escherichia coli* J5 mastitis vaccine. J. Dairy Sci. 75(1):78-84.
1030 Hogan, J. S., D. A. Todhunter, G. M. Tomita, K. L. Smith, and P. S. Schoenberger. 1992b. Opsonic
1031 activity of bovine serum and mammary secretion after *Escherichia coli* J5 vaccination. J. Dairy Sci.
1032 75(1):72-77.
1033 Hogan, J. S., W. P. Weiss, K. L. Smith, D. A. Todhunter, P. S. Schoenberger, and L. M. Sordillo.
1034 1995. Effects of an *Escherichia coli* J5 vaccine on mild clinical coliform mastitis. J. Dairy Sci. 78(2):285-
1035 290.

1036 Hogan, J. S., W. P. Weiss, D. A. Todhunter, K. L. Smith, and P. S. Schoenberger. 1992c. Efficacy
1037 of an *Escherichia coli* J5 mastitis vaccine in an experimental challenge trial. J. Dairy Sci. 75(2):415-422.
1038 Hogeveen, H., K. Huijps, and T. J. Lam. 2011. Economic aspects of mastitis: new developments.
1039 New Zealand Vet. J. 59(1):16-23.
1040 Hu, C. M., R. Gong, A. Z. Guo, and H. C. Chen. 2010. Protective effect of ligand-binding domain
1041 of fibronectin-binding protein on mastitis induced by *Staphylococcus aureus* in mice. Vaccine
1042 28(24):4038-4044.
1043 Jensen, N. E. 1980. Variation of type antigens of group-B streptococci. I. Variation of the X-
1044 antigen and of other type antigens in herds where the X-antigen occurs. Acta Vet.Scand. 21(3):367-
1045 374.
1046 Johnsen, L. B., K. Poulsen, M. Kilian, and T. E. Petersen. 1999. Purification and cloning of a
1047 streptokinase from *Streptococcus uberis*. Infect. Immun. 67(3):1072-1078.
1048 Johnson, S. D. and N. L. Norcross. 1971. An experimental method of vaccination for a
1049 *Streptococcus agalactiae* infected herd. Cornell Vet. 61(2):258-264.
1050 Kalmus, P., B. Aasmae, A. Karssin, T. Orro, and K. Kask. 2011. Udder pathogens and their
1051 resistance to antimicrobial agents in dairy cows in Estonia. Acta Vet. Scand. 53.
1052 Kampen, A. H., T. Tollersrud, and A. Lund. 2005. *Staphylococcus aureus* capsular polysaccharide
1053 types 5 and 8 reduce killing by bovine neutrophils in vitro. Infect. Immun. 73(3):1578-1583.
1054 Keane, O. M. 2019. Symposium review: Intramammary infections-Major pathogens and strain-
1055 associated complexity. J. Dairy Sci. 102(5):4713-4726.
1056 Keane, O. M., K. E. Budd, J. Flynn, and F. McCoy. 2013. Pathogen profile of clinical mastitis in
1057 Irish milk-recording herds reveals a complex aetiology. Vet. Rec. 173(1):17.
1058 Keefe, G. P. 1997. *Streptococcus agalactiae* mastitis: a review. Can. Vet. J. 38:429-437.
1059 Kerro-Dego, O., T. Prysliak, A. A. Potter, and J. Perez-Casal. 2006. DNA-protein immunization
1060 against the GapB and GapC proteins of a mastitis isolate of *Staphylococcus aureus*. Vet. Immunol.
1061 Immunopathol. 113(1-2):125-138.
1062 Klaas, I. C. and R. N. Zadoks. 2018. An update on environmental mastitis: Challenging
1063 perceptions. Transboundary and emerging diseases 65 Suppl 1:166-185.
1064 Landin, H., M. J. Mork, M. Larsson, and K. P. Waller. 2015. Vaccination against *Staphylococcus*
1065 *aureus* mastitis in two Swedish dairy herds. Acta Vet. Scand. 57:81.
1066 Lee, J. W., C. N. O'Brien, A. J. Guidry, M. J. Paape, K. A. Shafer-Weaver, and X. Zhao. 2005.
1067 Effect of a trivalent vaccine against Staphylococcus aureus mastitis lymphocyte subpopulations,
1068 antibody production, and neutrophil phagocytosis. Can. J. Vet. Res. 69(1):11-18.
1069 Leigh, J. A. 1999. *Streptococcus uberis*: a permanent barrier to the control of bovine mastitis?
1070 Vet. J. 157(3):225-238.

1071 Leigh, J. A. 2000. Vaccines against bovine mastitis due to *Streptococcus uberis* current status
1072 and future prospects. Adv. Exp. Med. Biol. 480:307-311.
1073 Leigh, J. A. 2002. Immunisation of dairy cattle with recombinant Streptococcus uberis GapC or
1074 a chimeric CAMP antigen confers protection against heterologous bacterial challenge M.C. Fontaine
1075 et al. Vaccine 20 (2002) 2278-2286. Vaccine 20(25/26):3047-3048.
1076 Leigh, J. A. and T. R. Field. 1991. Killing of *Streptococcus uberis* by bovine neutrophils following
1077 growth in chemically defined media. Vet. Res. Commun. 15(1):1-6.
1078 Leigh, J. A., T. R. Field, and M. R. Williams. 1990. Two strains of *Streptococcus uberis*, of
1079 differing ability to cause clinical mastitis, differ in their ability to resist some host defence factors.
1080 Res. Vet. Sci. 49(1):85-87.
1081 Leigh, J. A., J. M. Finch, T. R. Field, N. C. Real, A. Winter, A. W. Walton, and S. M. Hodgkinson.
1082 1999. Vaccination with the plasminogen activator from *Streptococcus uberis* induces an inhibitory
1083 response and protects against experimental infection in the dairy cow. Vaccine 17(7-8):851-857.
1084 Leigh, J. A. and R. A. Lincoln. 1997. *Streptococcus uberis* acquires plasmin activity following
1085 growth in the presence of bovine plasminogen through the action of its specific plasminogen
1086 activator. FEMS Microbiol. Lett. 154(1):123-129.
1087 Leitner, G., E. Lubashevsky, A. Glickman, M. Winkler, A. Saran, and Z. Trainin. 2003.
1088 Development of a *Staphylococcus aureus* vaccine against mastitis in dairy cows. I. Challenge trials.
1089 Vet. Immunol. Immunopathol. 93(1-2):31-38.
1090 Lin, J., J. S. Hogan, and K. L. Smith. 1999. Growth responses of coliform bacteria to purified
1091 immunoglobulin G from cows immunized with ferric enterobactin receptor FepA. J. Dairy Sci.
1092 82(1):86-92.
1093 Lin, S. M., Y. Zhi, K. B. Ahn, S. Lim, and H. S. Seo. 2018. Status of group B streptococcal vaccine
1094 development. Clin Exp Vaccine Res 7(1):76-81.
1095 Lindahl, G., M. Stalhammar-Carlemalm, and T. Areschoug. 2005. Surface proteins of
1096 *Streptococcus agalactiae* and related proteins in other bacterial pathogens. Clin. Microbiol. Rev.
1097 18(1):102-127.
1098 Linton, A. H., K. Howe, W. J. Sojka, and C. Wray. 1979. Range of *Escherichia-Coli* O-Serotypes
1099 Causing Clinical Bovine Mastitis and Their Antibiotic-Resistance Spectra. J. Appl. Bacteriol. 46(3):585-
1100 590.
1101 Linton, A. H. and T. C. Robinson. 1984. Studies on the association of *Escherichia coli* with
1102 bovine mastitis. Br. Vet. J. 140(4):368-373.
1103 Lipman, L. J., A. de Nijs, and W. Gaastra. 1995. Isolation and identification of fimbriae and toxin
1104 production by *Escherichia coli* strains from cows with clinical mastitis. Vet. Microbiol. 47(1-2):1-7.

1105 Liu, G., J. H. Yin, H. W. Barkema, L. B. Chen, M. Shahid, O. Szenci, J. De Buck, J. P. Kastelic, and
1106 B. Han. 2017. Development of a single-dose recombinant CAMP factor entrapping poly(lactide-co-
1107 glycolide) microspheres-based vaccine against *Streptococcus agalactiae*. Vaccine 35(9):1246-1253.
1108 Lundberg, Å., A. Nyman, H. E. Unnerstad, and K. P. Waller. 2014. Prevalence of bacterial
1109 genotypes and outcome of bovine clinical mastitis due to *Streptococcus dysgalactiae* and
1110 Streptococcus uberis. Acta Vet. Scand. 56(1):80.
1111 Lyhs, U., L. Kulkas, J. Katholm, K. P. Waller, K. Saha, R. J. Tomusk, and R. N. Zadoks. 2016.
1112 *Streptococcus agalactiae* Serotype IV in Humans and Cattle, Northern Europe(1). Emerg. Infect. Dis.
1113 22(12):2097-2103.
1114 Ma, J., J. Cocchiaro, and J. C. Lee. 2004. Evaluation of serotypes of *Staphylococcus aureus*
1115 strains used in the production of a bovine mastitis bacterin. J. Dairy Sci. 87(1):178-182.
1116 Mackie, D. P., D. A. Pollock, D. J. Meneely, and E. F. Logan. 1983. Clinical features of
1117 consecutive intramammary infections with *Streptococcus agalactiae* in vaccinated and non-
1118 vaccinated heifers. Vet. Rec. 112(20):472-476.
1119 Maione, D., I. Margarit, C. D. Rinaudo, V. Masignani, M. Mora, M. Scarselli, H. Tettelin, C.
1120 Brettoni, E. T. Iacobini, R. Rosini, N. D'Agostino, L. Miorin, S. Buccato, M. Mariani, G. Galli, R.
1121 Nogarotto, V. Nardi-Dei, F. Vegni, C. Fraser, G. Mancuso, G. Teti, L. C. Madoff, L. C. Paoletti, R.
1122 Rappuoli, D. L. Kasper, J. L. Telford, and G. Grandi. 2005. Identification of a universal Group B
1123 *streptococcus* vaccine by multiple genome screen. Science 309(5731):148-150.
1124 Maira-Litran, T., A. Kropec, D. A. Goldmann, and G. B. Pier. 2005. Comparative opsonic and
1125 protective activities of *Staphylococcus aureus* conjugate vaccines containing native or deacetylated
1126 Staphylococcal Poly-N-acetyl-beta-(1-6)-glucosamine. Infect. Immun. 73(10):6752-6762.
1127 Mamo, W., G. Froman, and H. P. Muller. 2000. Protection induced in mice vaccinated with
1128 recombinant collagen-binding protein (CnBP) and alpha-toxoid against intramammary infection with
1129 *Staphylococcus aureus*. Microbiol. Immunol. 44(5):381-384.
1130 Mamo, W., P. Jonsson, and H. P. Muller. 1995. Opsonization of *Staphylococcus aureus* with a
1131 fibronectin-binding protein antiserum induces protection in mice. Microb. Pathog. 19(1):49-55.
1132 Matthews, K. R., R. A. Almeida, and S. P. Oliver. 1994a. Bovine mammary epithelial cell
1133 invasion by *Streptococcus uberis*. Infect. Immun. 62(12):5641-5646.
1134 Matthews, K. R., B. M. Jayarao, A. J. Guidry, E. F. Erbe, W. P. Wergin, and S. P. Oliver. 1994b.
1135 Encapsulation of *Streptococcus-Uberis* - Influence of Storage and Cultural Conditions. Vet. Microbiol.
1136 39(3-4):361-367.
1137 McClure, A. M., E. E. Christopher, W. A. Wolff, W. H. Fales, G. F. Krause, and J. Miramonti.
1138 1994. Effect of Re-17 mutant *Salmonella typhimurium* bacterin toxoid on clinical coliform mastitis. J.
1139 Dairy Sci. 77(8):2272-2280.

1140 Middleton, J. R. 2008. *Staphylococcus aureus* antigens and challenges in vaccine development.
1141 Expert Rev. Vaccines 7(6):805-815.
1142 Middleton, J. R., C. D. Luby, and D. S. Adams. 2009. Efficacy of vaccination against
1143 staphylococcal mastitis: a review and new data. Vet. Microbiol. 134(1-2):192-198.
1144 Middleton, J. R., J. Ma, C. L. Rinehart, V. N. Taylor, C. D. Luby, and B. J. Steevens. 2006. Efficacy
1145 of different Lysigin formulations in the prevention of *Staphylococcus aureus* intramammary infection
1146 in dairy heifers. J. Dairy Res. 73(1):s10-19.
1147 Misra, N., T. F. Wines, C. L. Knopp, R. Hermann, L. Bond, B. Mitchell, M. A. McGuire, and J. K.
1148 Tinker. 2018. Immunogenicity of a *Staphylococcus aureus*-cholera toxin A(2)/B vaccine for bovine
1149 mastitis. Vaccine 36(24):3513-3521.
1150 Mistry, H., P. Sharma, S. Mahato, R. Saravanan, P. A. Kumar, and V. Bhandari. 2016. Prevalence
1151 and Characterization of Oxacillin Susceptible mecA-Positive Clinical Isolates of *Staphylococcus aureus*
1152 Causing Bovine Mastitis in India. PLoS ONE 11(9):e0162256.
1153 Morris, D. D., J. S. Cullor, R. H. Whitlock, M. Wickstrom, and L. B. Corbeil. 1986. Endotoxemia in
1154 neonatal calves given antiserum to a mutant *Escherichia coli* (J-5). Am. J. Vet. Res. 47(12):2554-2565.
1155 Mweu, M. M., S. S. Nielsen, T. Halasa, and N. Toft. 2012. Annual incidence, prevalence and
1156 transmission characteristics of *Streptococcus agalactiae* in Danish dairy herds. Prev. Vet. Med. 106(3-
1157 4):244-250.
1158 NRC, C. s. o. t. r. c. o. t. n. m. council, R. J. Eberhart, R. P. Natzke, F. H. S. Newbould, B.
1159 Nonnecke, and P. Thompson. 1979. Coliform mastitis - A review. J. Dairy Sci. 62:1-22.
1160 Oliveira, M., R. Bexiga, S. F. Nunes, C. Carneiro, L. M. Cavaco, F. Bernardo, and C. L. Vilela.
1161 2006. Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis*
1162 mastitis isolates. Vet. Microbiol. 118(1-2):133-140.
1163 Omore, A. O., J. J. McDermott, S. M. Arimi, M. N. Kyule, and D. Ouma. 1996. A longitudinal
1164 study of milk somatic cell counts and bacterial culture from cows on smallholder dairy farms in
1165 Kiambu district, Kenya. Prev. Vet. Med. 29(1):77-89.
1166 Paape, M., J. Mehrzad, X. Zhao, J. Detilleux, and C. Burvenich. 2002. Defense of the bovine
1167 mammary gland by polymorphonuclear neutrophil leukocytes. J Mammary Gland Biol. Neoplasia
1168 7(2):109-121.
1169 Pang, M. D., L. C. Sun, T. He, H. D. Bao, L. L. Zhang, Y. Zhou, H. Zhang, R. C. Wei, Y. J. Liu, and R.
1170 Wang. 2017. Molecular and virulence characterization of highly prevalent *Streptococcus agalactiae*
1171 circulated in bovine dairy herds. Vet. Res. 48.
1172 Pankey, J. W., N. T. Boddie, J. L. Watts, and S. C. Nickerson. 1985. Evaluation of protein A and a
1173 commercial bacterin as vaccines against *Staphylococcus aureus* mastitis by experimental challenge. J.
1174 Dairy Sci. 68(3):726-731.

Pérez, M. M., A. Prenafeta, J. Valle, J. Penadés, C. Rota, C. Solano, J. Marco, M. J. Grillo, I. Lasa, J. M. Irache, T. Maira-Litran, J. Jimenez-Barbero, L. Costa, G. B. Pier, D. de Andres, and B. Amorena. 2009. Protection from *Staphylococcus aureus* mastitis associated with poly-N-acetyl beta-1,6 glucosamine specific antibody production using biofilm-embedded bacteria. Vaccine 27(17):2379-2386.

Petrovski, K. R., N. B. Williamson, N. Lopez-Villalobos, T. J. Parkinson, and I. G. Tucker. 2011. Culture results from milk samples submitted to veterinary diagnostic laboratories from August 2003 to December 2006 in New Zealand. New Zealand Vet. J. 59(6):317-322.

Petzer, I. M., J. Karzis, J. C. Watermeyer, T. J. van der Schans, and R. van Reenen. 2009. Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. J. S. Afr. Vet. Assoc. 80(1):17-22.

Poutrel, B., S. Bareille, G. Lequeux, and F. Leboeuf. 2018. Prevalence of mastitis pathogens in France: antimicrobial susceptibility *of Staphylococcus aureus, Streptococcus uberis* and *Escherichia coli*. J Vet. Sci. Technol. 9:2.

P.outrel, B., P. Rainard, and P. Sarradin. 1997. Heterogeneity of cell-associated CP5 expression on *Staphylococcus aureus* strains demonstrated by flow cytometry. Clin. Diagn. Lab. Immunol. 4(3):275-278.

Poutrel, B. and L. Sutra. 1993. Type 5 and 8 capsular polysaccharides are expressed by *Staphylococcus aureus* isolates from rabbits, poultry, pigs, and horses. J.Clin.Microbiol. 31(2):467-469.

Prado, M. E., R. A. Almeida, C. Ozen, D. A. Luther, M. J. Lewis, S. J. Headrick, and S. P. Oliver. 2011. Vaccination of dairy cows with recombinant *Streptococcus uberis* adhesion molecule induces antibodies that reduce adherence to and internalization of S. uberis into bovine mammary epithelial cells. Vet. Immunol. Immunopathol. 141(3-4):201-208.

Prenafeta, A., R. March, A. Foix, I. Casals, and L. Costa. 2010. Study of the humoral immunological response after vaccination with a *Staphylococcus aureus* biofilm-embedded bacterin in dairy cows: Possible role of the exopolysaccharide specific antibody production in the protection from *Staphylococcus aureus* induced mastitis. Vet. Immunol. Immunopathol. 134(3-4):208-217.

Puig, A., E. Perozo, F. Roura, R. Armengol, D. Ponté, J. Mallo, J. M. Echevarria, C. Arrieta, D. Herrera, O. Franquesa, L. Urtado, I. Calm, R. Collado, A. Prenafeta, R. March, and D. Sabaté. 2018. Efficacy under field conditions of the new vaccine UBAC® in the reduction of *Streptococcus uberis* clinical mastitis in dairy cows during a whole lactation period. in Proc. NMC 2018. NMC 2018. The 2018 international bovine mastitis conference, Milan.

Rainard, P. 1983. Experimental mastitis with *Escherichia coli*: sequential response of leukocytes and opsonic activity in milk of immunised and unimmunised cows. Ann. Rech. Vet. 14(3):281-286.

1210 Rainard, P. 1992. Isotype antibody response in cows to *Streptococcus agalactiae* group B
1211 polysaccharide-ovalbumin conjugate. J.Clin.Microbiol. 30(7):1856-1862.
1212 Rainard, P., P. Cunha, R. P. Martins, F. B. Gilbert, P. Germon, and G. Foucras. 2020. Type 3
1213 immunity: a perspective for the defense of the mammary gland against infections. Vet. Res.
1214 51(1):129.
1215 Rainard, P., G. Foucras, J. R. Fitzgerald, J. L. Watts, G. Koop, and J. R. Middleton. 2018.
1216 Knowledge gaps and research priorities in *Staphylococcus aureus* mastitis control. Transbound.
1217 Emerging Dis. 65(Suppl. 1):149-165.
1218 Rainard, P., Y. Lautrou, P. Sarradin, A. Coulibaly, and B. Poutrel. 1991. The kinetics of
1219 inflammation and phagocytosis during bovine mastitis induced by *Streptococcus agalactiae* bearing
1220 the protein X. Vet. Res. Commun. 15(3):163-176.
1221 Rainard, P., M. Reperant-Ferter, C. Gitton, and P. Germon. 2021. Shielding Effect of *Escherichia*
1222 *coli* O-Antigen Polysaccharide on J5-Induced Cross-Reactive Antibodies. mSphere 6(1):e01227-20.
1223 Rainard, P. and C. Riollet. 2003. Mobilization of neutrophils and defense of the bovine
1224 mammary gland. Reprod. Nutr. Dev. 43(5):439-457.
1225 Reiter, B. 1978. Review of the progress of dairy science: antimicrobial systems in milk. J.Dairy
1226 Res. 45(1):131-147.
1227 Rosini, R., E. Campisi, M. De Chiara, H. Tettelin, D. Rinaudo, C. Toniolo, M. Metruccio, S.
1228 Guidotti, U. B. Sorensen, M. Kilian, M. Ramirez, R. Janulczyk, C. Donati, G. Grandi, and I. Margarit.
1229 2015. Genomic analysis reveals the molecular basis for capsule loss in the group B *streptococcus*
1230 population. PLoS ONE 10(5):e0125985.
1231 Ruegg, P. L. 2017. A 100-Year Review: Mastitis detection, management, and prevention. J.
1232 Dairy Sci. 100(12):10381-10397.
1233 Sakulramrung, R. and G. J. Domingue. 1985. Cross-Reactive Immunoprotective Antibodies to
1234 *Escherichia-coli* O111 Rough Mutant J5. J. Infect. Dis. 151(6):995-1004.
1235 Sampimon, O., H. W. Barkema, I. Berends, J. Sol, and T. Lam. 2009. Prevalence of
1236 intramammary infection in Dutch dairy herds. J. Dairy Res. 76(2):129-136.
1237 Sanchez-Carlo, V., R. A. Wilson, J. S. McDonald, and R. A. Packer. 1984. Biochemical and
1238 serologic properties of *Escherichia coli* isolated from cows with acute mastitis. Am. J. Vet. Res.
1239 45(9):1771-1774.
1240 Scali, F., C. Camussone, L. F. Calvinho, M. Cipolla, and A. Zecconi. 2015. Which are important
1241 targets in development of *S. aureus* mastitis vaccine? Res. Vet. Sci. 100:88-99.
1242 Schukken, Y. H., V. Bronzo, C. Locatelli, C. Pollera, N. Rota, A. Casula, F. Testa, L. Scaccabarozzi,
1243 R. March, D. Zalduendo, R. Guix, and P. Moroni. 2014. Efficacy of vaccination on *Staphylococcus*

aureus and coagulase-negative staphylococci intramammary infection dynamics in 2 dairy herds. J. Dairy Sci. 97(8):5250-5264.

Shafer-Weaver, K. A., C. M. Corl, and L. M. Sordillo. 1999. Shifts in bovine CD4+ subpopulations increase T-helper-2 compared with T-helper-1 effector cells during the postpartum period. J. Dairy Sci. 82(8):1696-1706.

Shkreta, L., B. G. Talbot, M. S. Diarra, and P. Lacasse. 2004. Immune responses to a DNA/protein vaccination strategy against *Staphylococcus aureus* induced mastitis in dairy cows. Vaccine 23(1):114-126.

Siebert, L., S. Headrick, M. Lewis, B. Gillespie, C. Young, L. Wojakiewicz, O. Kerro-Dego, M. E. Prado, R. Almeida, S. P. Oliver, and G. M. Pighetti. 2017. Genetic variation in CXCR1 haplotypes linked to severity of *Streptococcus uberis* infection in an experimental challenge model. Vet. Immunol. Immunopathol. 190:45-52.

Skaar, E. P. and O. Schneewind. 2004. Iron-regulated surface determinants (Isd) of *Staphylococcus aureus*: stealing iron from heme. Microbes Infect. 6(4):390-397.

Slanetz, L. W., C. H. Bartley, and F. E. Allen. 1965. Evaluation of Cell-Toxoid Vaccines for the Vaccination of Dairy Cattle against Staphylococcic Mastitis. Am. J. Vet. Res. 26:688-695.

Smith, J. L., J. S. Hogan, and K. L. Smith. 1999. Efficacy of intramammary immunization with an *Escherichia coli* J5 bacterin. J. Dairy Sci. 82(12):2582-2588.

Song, B. F., X. J. Yang, H. N. Sun, L. Q. Yu, J. Z. Ma, Z. J. Wu, and Y. D. Cui. 2017. Immunogenicity of amino acids 1-150 of Streptococcus GapC displayed on the surface of Escherichia coli. Microb. Pathog. 105:288-297.

Song, J. Y., J. H. Lim, S. Lim, Z. Yong, and H. S. Seo. 2018. Progress toward a group B streptococcal vaccine. Hum. Vacc. Immunother. 14(11):2669-2681.

Stark, D. M. and N. L. Norcross. 1970. Response of bovine to immunization against *Streptococcus dysgalactiae*. Cornell Vet. 60(4):604-612.

Steele, N. M., T. H. Swartz, K. M. Enger, H. Schramm, R. R. Cockrum, S. J. Lacy-Hulbert, R. R. White, J. Hogan, and C. S. Petersson-Wolfe. 2019. The effect of J5 bacterins on clinical, behavioral, and antibody response following an *Escherichia coli* intramammary challenge in dairy cows at peak lactation. J. Dairy Sci. 112(12):11233-11249.

Stenutz, R., A. Weintraub, and G. Widmalm. 2006. The structures of *Escherichia coli* O-polysaccharide antigens. FEMS Microbiol. Rev. 30(3):382-403.

Ster, C., F. Beaudoin, M. S. Diarra, M. Jacques, F. Malouin, and P. Lacasse. 2010. Evaluation of some *Staphylococcus aureus* iron-regulated proteins as vaccine targets. Vet. Immunol. Immunopathol. 136(3-4):311-318.

Sutra, L., C. Mendolia, P. Rainard, and B. Poutrel. 1990a. Encapsulation of *Staphylococcus aureus* isolates from mastitic milk: relationship between capsular polysaccharide types 5 and 8 and colony morphology in serum-soft agar, clumping factor, teichoic acid, and protein A. J.Clin.Microbiol. 28(3):447-451.

Sutra, L., P. Rainard, and B. Poutrel. 1990b. Phagocytosis of mastitis isolates of *Staphylococcus aureus* and expression of type 5 capsular polysaccharide are influenced by growth in the presence of milk. J.Clin.Microbiol. 28(10):2253-2258.

Takemura, K., J. S. Hogan, J. Lin, and K. L. Smith. 2002. Efficacy of immunization with ferric citrate receptor FecA from Escherichia coli on induced coliform mastitis. J. Dairy Sci. 85(4):774-781.

Takemura, K., J. S. Hogan, and K. L. Smith. 2004. Growth responses of *Escherichia coli* to immunoglobulin G from cows immunized with ferric citrate receptor, FecA. J. Dairy Sci. 87(2):316-320.

Tamilselvam, B., R. A. Almeida, J. R. Dunlap, and S. P. Oliver. 2006. *Streptococcus uberis* internalizes and persists in bovine mammary epithelial cells. Microb. Pathog. 40(6):279-285.

Tashakkori, N., B. Khoramian, M. Farhoodi Moghadam, M. Heidarpour, K. Mashayekhi, and N. Farzaneh. 2020. Evaluating the effectiveness of two bovine mastitis vaccines and their influences on oxidant and antioxidant capacities of milk. Trop. Anim. Health Prod. 52(3):1493-1501.

Tassi, R., T. N. McNeilly, J. L. Fitzpatrick, M. C. Fontaine, D. Reddick, C. Ramage, M. Lutton, Y. H. Schukken, and R. N. Zadoks. 2013. Strain-specific pathogenicity of putative host-adapted and nonadapted strains of *Streptococcus uberis* in dairy cattle. J. Dairy Sci. 96(8):5129-5145.

Taylor, B. C., J. D. Dellinger, J. S. Cullor, and J. L. Stott. 1994. Bovine milk lymphocytes display the phenotype of memory T cells and are predominantly CD8+. Cell. Immunol. 156(1):245-253.

Thomas, L. H., W. Haider, A. W. Hill, and R. S. Cook. 1994. Pathologic findings of experimentally induced *Streptococcus uberis* infection in the mammary gland of cows. Am. J. Vet. Res. 55(12):1723-1728.

Thomas, L. H., J. A. Leigh, A. P. Bland, and R. S. Cook. 1992. Adherence and colonization by bacterial pathogens in explant cultures of bovine mammary tissue. Vet. Res. Commun. 16(2):87-96.

Tomazi, T., A. C. C. H. Tomazi, J. C. C. Silva, L. Bringhenti, M. L. M. C. Bravo, M. X. Rodrigues, and R. C. Bicalho. 2021. Immunization with a novel recombinant protein (YidR) reduced the risk of clinical mastitis caused by *Klebsiella* spp. and decreased milk losses and culling risk after *Escherichia coli* infections. Journal of Dairy Science 104(4):4787-4802.

Tomita, G. M., S. C. Nickerson, W. E. Owens, and B. Wren. 1998. Influence of route of vaccine administration against experimental intramammary infection caused by *Escherichia coli*. J. Dairy Sci. 81(8):2159-2164.

1312 Tomita, G. M., C. H. Ray, S. C. Nickerson, W. E. Owens, and G. F. Gallo. 2000. A comparison of
1313 two commercially available *Escherichia coli* J5 vaccines against *E. coli* intramammary challenge. J.
1314 Dairy Sci. 83(10):2276-2281.
1315 Tuchscherr, L., B. Loffler, F. R. Buzzola, and D. O. Sordelli. 2010. *Staphylococcus aureus*
1316 adaptation to the host and persistence: role of loss of capsular polysaccharide expression. Future
1317 Microbiol. 5(12):1823-1832.
1318 Tuchscherr, L. P., F. R. Buzzola, L. P. Alvarez, R. L. Caccuri, J. C. Lee, and D. O. Sordelli. 2005.
1319 Capsule-negative *Staphylococcus aureus* induces chronic experimental mastitis in mice. Infect.
1320 Immun. 73(12):7932-7937.
1321 Tyler, J. W., J. S. Cullor, B. I. Osburn, R. B. Bushnell, and B. W. Fenwick. 1988. Relationship
1322 between serologic recognition of *Escherichia coli* 0111:B4 (J5) and clinical coliform mastitis in cattle.
1323 Am. J. Vet. Res. 49(11):1950-1954.
1324 van Dijk, W. C., H. A. Verbrugh, M. E. van Erne-van der Tol, R. Peters, and J. Verhoef. 1981.
1325 *Escherichia coli* antibodies in opsonisation and protection against infection. J. Med. Microbiol.
1326 14(4):381-389.
1327 Vangroenweghe, F., L. Duchateau, and C. Burvenich. 2020. Short communication: J-5
1328 *Escherichia coli* vaccination does not influence severity of an *Escherichia coli* intramammary
1329 challenge in primiparous cows. J. Dairy Sci. 103(7):6692-6697.
1330 Varhimo, E., P. Varmanen, A. Fallarero, M. Skogman, S. Pyorala, A. Livanainen, A. Sukura, P.
1331 Vuorela, and K. Savijoki. 2011. Alpha- and beta-casein components of host milk induce biofilm
1332 formation in the mastitis bacterium *Streptococcus uberis*. Vet. Microbiol. 149(3-4):381-389.
1333 Vautor, E., G. Abadie, A. Pont, and R. Thiery. 2008. Evaluation of the presence of the bap gene
1334 in *Staphylococcus aureus* isolates recovered from human and animals species. Vet. Microbiol. 127(3-
1335 4):407-411.
1336 Verdier, I., G. Durand, M. Bes, K. L. Taylor, G. Lina, F. Vandenesch, A. I. Fattom, and J. Etienne.
1337 2007. Identification of the capsular polysaccharides in *Staphylococcus aureus* clinical isolates by PCR
1338 and agglutination tests. J. Clin. Microbiol. 45(3):725-729.
1339 Vreede, R. W., J. H. Marcelis, and J. Verhoef. 1986. Antibodies raised against rough mutants of
1340 *Escherichia coli* and *Salmonella* strains are opsonic only in the presence of complement. Infect.
1341 Immun. 52(3):892-896.
1342 Ward, P. N., T. R. Field, W. G. Ditcham, E. Maguin, and J. A. Leigh. 2001. Identification and
1343 disruption of two discrete loci encoding hyaluronic acid capsule biosynthesis genes hasA, hasB, and
1344 hasC in *Streptococcus uberis*. Infect. Immun. 69(1):392-399.
1345 Ward, P. N., T. R. Field, C. D. Rapier, and J. A. Leigh. 2003. The activation of bovine plasminogen
1346 by PauA is not required for virulence of *Streptococcus uberis*. Infect. Immun. 71(12):7193-7196.

1347 Warren, H. S., S. F. Amato, C. Fitting, K. M. Black, P. M. Loiselle, M. S. Pasternack, and J. M.
1348 Cavaillon. 1993. Assessment of Ability of Murine and Human Antilipid-a Monoclonal-Antibodies to
1349 Bind and Neutralize Lipopolysaccharide. J. Exp. Med. 177(1):89-97.
1350 Watson, D. L. 1981. Immunologically-specific resistance to infection with particular reference
1351 to staphylococcal mastitis. Adv. Exp. Med. Biol. 137:579-590.
1352 Watson, D. L. 1992a. Staphylococcal mastitis vaccine. Vaccine 10(5):359.
1353 Watson, D. L. 1992b. Vaccination against experimental staphylococcal mastitis in dairy heifers.
1354 Res. Vet. Sci. 53(3):346-353.
1355 Watson, D. L. and J. W. Kennedy. 1981. Immunisation against experimental staphylococcal
1356 mastitis in sheep - effect of challenge with a heterologous strain of *Staphylococcus aureus*. Aust. Vet.
1357 J. 57(7):309-313.
1358 Wedlock, D. N., B. M. Buddle, J. Williamson, S. J. Lacy-Hulbert, S. A. Turner, S. Subharat, and A.
1359 Heiser. 2014. Dairy cows produce cytokine and cytotoxic T cell responses following vaccination with
1360 an antigenic fraction from *Streptococcus uberis*. Vet. Immunol. Immunopathol. 160(1-2):51-60.
1361 Whitnack, E., A. L. Bisno, and E. H. Beachey. 1981. Hyaluronate capsule prevents attachment of
1362 group A streptococci to mouse peritoneal macrophages. Infect. Immun. 31(3):985-991.
1363 Williams, M. R. and A. W. Hill. 1982. A role for IgM in the in vitro opsonisation of
1364 *Staphylococcus aureus* and *Escherichia coli* by bovine polymorphonuclear leucocytes. Res. Vet. Sci.
1365 33(1):47-53.
1366 Wilson, D. J. and R. N. Gonzalez. 2003. Vaccination strategies for reducing clinical severity of
1367 coliform mastitis. Vet. Clin. North Am. Food Anim. Pract. 19(1):187-197, vii-viii.
1368 Wilson, D. J., Y. T. Grohn, G. J. Bennett, R. N. Gonzalez, Y. H. Schukken, and J. Spatz. 2007.
1369 Comparison of J5 vaccinates and controls for incidence, etiologic agent, clinical severity, and survival
1370 in the herd following naturally occurring cases of clinical mastitis. J. Dairy Sci. 90(9):4282-4288.
1371 Wolf, S. L., J. S. Hogan, and K. L. Smith. 2004. Iron uptake by *Escherichia coli* cultured with
1372 antibodies from cows immunized with high-affinity ferric receptors. J. Dairy Sci. 87(7):2103-2107.
1373 Yancey, R. J., Jr. 1993. Recent advances in bovine vaccine technology. J.Dairy Sci. 76(8):2418-
1374 2436.
1375 Yao, D., H. Zhang, X. Wang, S. Yu, Y. Wei, W. Liu, J. Wang, X. Chen, Z. Zhang, H. Sun, L. Yu, J. Ma,
1376 C. Tong, B. Song, and Y. Cui. 2016. Identification and characterization of CD4(+) T-cell epitopes on
1377 GapC protein of *Streptococcus dysgalactiae*. Microb. Pathog. 91:46-53.
1378 Yu, L., Z. Fan, J. Ma, C. Tong, B. Song, Z. Zhu, and Y. Cui. 2014. Cross-protective effect of a novel
1379 multi-antigen-chimeric vaccine against *Streptococcus* and *Staphylococcus aureus* infection in mice. J.
1380 Med. Microbiol. 63(Pt 12):1732-1740.

Zhang, S. Y., S. Piepers, R. X. Shan, L. J. Cai, S. L. Mao, J. Q. Zou, T. Ali, S. De Vliegher, and B. Han. 2018. Phenotypic and genotypic characterization of antimicrobial resistance profiles in *Streptococcus dysgalactiae* isolated from bovine clinical mastitis in 5 provinces of China. J. Dairy Sci. 101(4):3344-3355.

Ziegler, E. J., H. Douglas, J. E. Sherman, C. E. Davis, and A. I. Braude. 1973. Treatment of *E. coli* and *klebsiella* bacteremia in agranulocytic animals with antiserum to a UDP-gal epimerase-deficient mutant. J. Immunol. 111(2):433-438.

Ziegler, E. J., J. A. McCutchan, J. Fierer, M. P. Glauser, J. C. Sadoff, H. Douglas, and A. I. Braude. 1982. Treatment of Gram-negative bacteremia and shock with human antiserum to a mutant *Escherichia coli*. N. Engl. J. Med. 307(20):1225-1230.

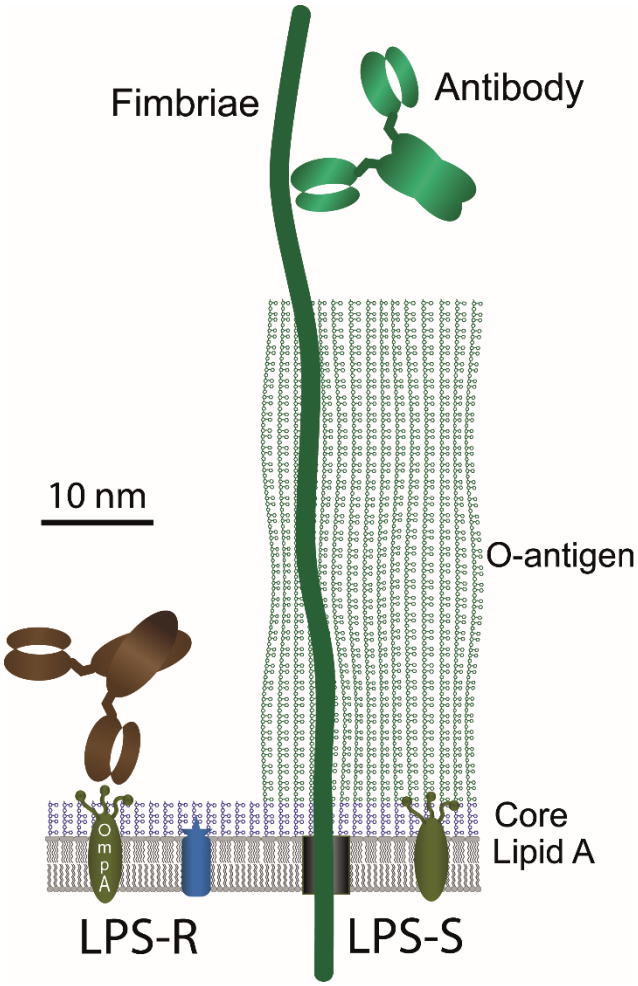

Figure 1. Steric hindrance for antibody accessibility to E. coli outer membrane of rough (LPS-R) or smooth (LPS-S) strains. Antibodies can access outer membrane proteins such as OmpA on rough strains, but are prevented from reacting with these proteins by the shielding effect of the 0-antigen of smooth strains. However, bacterial structures protruding from the smooth LPS, such as fimbriae, are accessible to antibodies. The IgG molecule of antibody is shown to scale for comparison with the bacterial components. The LPS-S of *E. coli* O32 with 15 repeats of the O-antigen unit is shown.

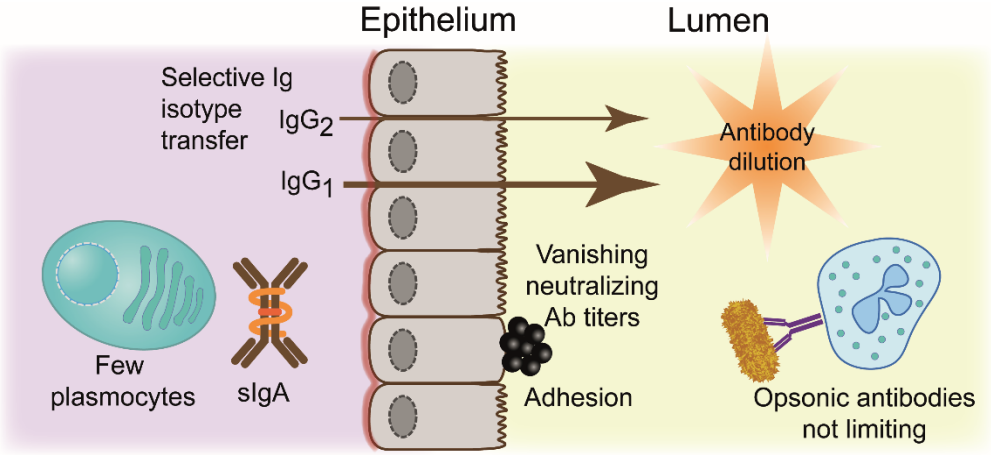

Figure 2. Vaccine-induced antibodies and antibody-dependent MG defenses face several challenges. In healthy glands, passive transudation of antibodies from blood to milk is low. Active transepithelial transfer favors the IgG_1 isotype, which is not opsonic for neutrophils. Local production of antibodies, such as secretory IgA (sIgA) is limited, as resident plasmocytes are few in the mammary parenchyma. In addition, antibodies that pass the epithelial barrier are diluted in high volumes of milk. In the absence of apical mucus capable of retaining antibodies on the luminal surface of the epithelium, antibody concentrations is locally not sufficient to prevent adhesion of bacteria or interfering with nutrient acquisition. Furthermore, elevated antibody concentrations to common mastitis-causing bacteria in blood and milk are transient after vaccination. Finally, there are few examples of increased opsonic activity towards mastitis bacteria following vaccination. This latter failure is largely because natural antibodies in milk and blood already have fairly good opsonic activity, so opsonic antibodies are usually not the limiting factor for protection.

1422 Table 1. Brief summary of key features of illustrative mastitis coliform vaccine trials

1423

Vaccine antigens	Targeted or putative effect	Efficacy	Pitfalls or knowledge gaps	Salient references
E. coli J5 bacterins	Antibodies to LPS core antigen	Decreased coliform mastitis severity in field experiments	Variable effect on incidence of cases Unknown mechanism	(Gonzalez et al., 1989) (Cullor, 1991) (Hogan et al., 1992a)
Salmonella Re-17 bacterin toxoid	Antibodies to LPS core antigen	Decreased coliform mastitis severity in field experiment	Unknown mechanism	(McClure et al., 1994)
E. coli J5 bacterin	Antibodies to LPS core antigens	Discrepant results: reduction or not of severity in experimental infection	Unknown mechanism	(Hogan et al., 1992c) (Hill, 1991)
E. coli J5 bacterin, hyperimmunization	Antibodies to coliform outer membrane antigens in the IgG_2 isotype	Decreased occurrence of severe mastitis compared to usual schedule	Variable among herds Unknown mechanism	(Erskine et al., 2007)
E. coli J5 bacterin with killed S. aureus (StartVac)	Antibodies to coliform outer membrane antigens	Decreased mastitis severity in a field study	No reduction in incidence of case. Unknown mechanism	(Bradley et al., 2015)
Enterobactin FepA	Iron acquisition	Growth reduction in dry mammary secretion	Likely not active in lactation, not tested in vivo	(Lin et al., 1999)
Siderophore receptor FecA	Iron acquisition	None in experimental infection	Antibody titer insufficient in milk	(Takemura et al., 2002) (Wolf et al., 2004)
Whole E. coli (P4), intramammary booster with bacterial extract	Antibody and cell-mediated responses	Reduction in severity, likely independent of antibodies, related to Th17 response	Heterologous protection not tested	(Herry et al., 2017)
Klebsiella Siderophore receptors and porin proteins (KlebVax™)	Iron acquisition and multiple bacterial functions With antibodies	Effective in one small scale study, ineffective in a large scale study		(Gorden et al., 2018, Tomazi et al., 2021)
Klebsiella recombinant YidR	Unknown bacterial functions With antibodies	Reduced incidence of *Klebsiella* clinical mastitis	No effect on risk of death if clinical Little antibody response to whole bacteria and activity unknown	(Tomazi et al., 2021)

1424

1425 Table 2. Brief summary of key features of illustrative mastitis *S. aureus* vaccine trials
1426

Vaccine antigens	Targeted or putative effect	Efficacy	Pitfalls or knowledge gaps	Salient references
Whole killed bacteria and toxoid	Opsonization and neutralizing antibodies	Reduction in severity, intramammary challenge	No self-cure, homologous challenge	(Derbyshire, 1960)
Bacterial lysate (5 strains) Lysigin® (Boehringer Ingelheim Vetmedica)	Antibodies	Some reduction in severity and incidence of IMI	Variable results	(Middleton et al., 2006) (Middleton et al., 2009)
Live vaccine, subcutaneous	Opsonization by IgG_2 antibodies	Better reduction in severity than killed vaccine, boosted recruitment of neutrophils	Challenge of ewes Mechanism not identified	(Watson and Kennedy, 1981) (Colditz and Watson, 1982)
Killed vaccine, "in vivo" antigen and dextran sulfate	Opsonization by IgG_2 antibodies	Reduced severity		(Watson, 1992b)
Capsular polysaccharides (CP5, CP8, teichoic acid)	Opsonization by antibodies, cell-mediated immunity	Slight increase in opsonization,	No protection study	(Lee et al., 2005)
Slime on killed bacteria, StartVac® (Hipra)	Opsonization, adhesion	Reduction in bacterial shedding in milk	Mechanism not identified Little effect on severity and incidence of new IMI	(Prenafeta et al., 2010) (Schukken et al., 2014)
Live VraG mutant SCV	Antibodies & cell-mediated immunity	Humoral and cell-mediated response of Th1/Th17 type	Mouse model, no challenge	(Côté-Gravel et al., 2016)
Protein A (SpA)	antibodies	Increased spontaneous cure of *S. aureus* IMI after experimental challenge	No field trial Mechanism not identified	(Pankey et al., 1985)
FnBP and ClfA	Antibodies and cell-mediated immunity	Increased spontaneous cure of *S. aureus* IMI after experimental challenge	No field trial Mechanism not identified	(Shkreta et al., 2004)
Recombinant IsdB and IsdH	Antibodies interfering with iron acquisition, opsonization	IgG2 antibodies and antigen-specific lymphoproliferation	No protection study in cows	(Ster et al., 2010)
GapB and GapC	Antibodies	Immunogenic in mice	No protection study in cows	(Kerro-Dego et al., 2006)

1427 SCV, small coony variant; VraG, *S. aureus* ABC transporter; *Staphylococcus aureus* surface proteins: FnBP, fibronectin-binding protein; ClfA, clumping facor A;
1428 IsdB, IsdH, *S. aureus* iron-regulated surface proteins; GapB, GapC: proteins with homology to glyceraldehyde-3-phosphate dehydrogena

1429