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A critical appraisal of mastitis vaccines for dairy cows

Pascal Rainard1*, Florence B. Gilbert1, Pierre Germon1, and Gilles Foucras2

1ISP, INRAE, Université de Tours, UMR1282, Nouzilly, France
2IHAP, 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.
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 \textit{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 IgG1 to \textit{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 \textit{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 \textit{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 development 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
is responsible for the toxicity of LPS, monoclonal antibodies to lipid A have been developed for the therapy of Gram-negative sepsis. However, their efficiency has been doubted as they did not consistently show neutralizing activity (Warren et al., 1993). The treatment of calves with J5 antiserum did not protect against shock induced by intravenous LPS injection (Morris et al., 1986). Antibodies to the core part of LPS have also been tested for protective activity. Coupling the core glycolipid to a carrier protein improved the immunogenicity and resulted in higher antibody titers than those elicited by a J5 bacterin vaccine (Cross et al., 2014). However, there are five different *E. coli* core antigens (K-12, R1, R2, R3 and R4) that can induce core-specific antibodies. A mixture of those core antigens conjugated to a protein carrier was used to vaccinate cows. Compared to control cows, SCC and *E. coli* shedding in milk did not differ in vaccinated animals after intramammary challenge, although high titers of antibodies to the core type of the challenge strain had been elicited (Brade et al., 2013). It is not even certain that the J-5 vaccines induce antibodies to the core LPS, as one study suggested that the raised antibodies were not directed to rough LPS but to outer membrane proteins such as OmpA (Chaiyotwittayakun et al., 2004).

Several studies have investigated the effect of J5 vaccines on the opsonic activity of serum and milk of vaccinated cows. The focus has been on the induction of antibodies that are opsonic for neutrophils of dairy ruminants, i.e. IgG2 and IgM antibodies, as these antibodies play a major role in the opsonization of *E. coli* (Williams and Hill, 1982, Hill et al., 1983a). Most natural opsonic antibodies to *E. coli* are of the IgM isotype and usually there is little IgG2 activity in the serum and milk of unimmunized cows (Williams and Hill, 1982). Natural infection of the mammary gland (MG) elicits opsonic antibodies, but they are serotype specific (Hill et al., 1983a). Immunization with smooth bacteria can induce opsonic antibodies in serum and milk (Rainard, 1983, Herry et al., 2017), but these antibodies are mainly serotype-specific, 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 IgG2 sub-isotype
was difficult, requiring more than three administrations to reach significantly higher than initial
titers (Chaiyotwittayakun et al., 2004). Increased IgG2 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 IgG2 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 IgG1 and IgG2 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 IgG2 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 (Gareaux 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 with the recombinant 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 IgG2 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
resistant when cultured in medium supplemented with casein hydrolysate (strains 0140J and ST10), and bacteria susceptible to phagocytosis under both conditions (strains EF20 and CC21) (Leigh and Field, 1991). A previous study, involving the most susceptible strain (EF20) and a resistant strain (0140J), had shown that both could produce a hyaluronic capsule and that the susceptibility to the bactericidal activity of neutrophils was not dictated by the capsule (Leigh et al., 1990). In effect, strain EF20, which loses its capsule at the stationary phase of culture, was equally susceptible to phagocytosis at the exponential and stationary phases, and the decapsulation of strain 0140J with hyaluronidase did not render it susceptible to phagocytosis. Those two strains had shown different pathogenicity for the lactating MG, as the strain EF20 failed to induce mastitis most of the time, whereas the strain 0140J was usually successful (Hill, 1988). Surprisingly, the strain EF20 was eliminated from inoculated glands in the absence of neutrophil influx, thus challenging the presupposed view that the difference in pathogenicity of the two strains was linked to their differing resistance to phagocytosis. It should be noted that the two strains were equally efficient at inducing mastitis in dry MG. Further studies with strain 0140J showed that the presence of the capsule appears to contribute to resistance to phagocytosis as neutrophils killed 0140J acapsular mutants (Ward et al., 2001). Nevertheless, other studies have indicated that although the hyaluronic acid capsule contributes to resistance to phagocytosis, it was not required for strain 0140J to induce clinical mastitis (Field et al., 2003). Intriguingly, the authors explained the inefficiency of the MG phagocytic defense by a potent antiphagocytic activity exerted by the strain 0140J culture supernate. This inefficiency is consistent with in vivo data that has shown that bacterial numbers in milk remain high despite a massive influx of neutrophils (Field et al., 2003). The identity of this putative inhibitor and its mode of action have not been established. Among antiphagocytic streptococcal factors, hyaluronic acid has been reported to prevent attachment of streptococci to macrophages (Whitnack et al., 1981). It is worth noting that not all S. uberis isolates are able to produce a
capsule even at their primary in vitro culture (Matthews et al., 1994b), but that most of the
strains isolated from mastitis possess the genes (hasABC) required for capsule synthesis (Field
et al., 2003).

Contrary to neutrophils, macrophages obtained from dried MGs were able to
phagocytose a strain resistant to phagocytosis by neutrophils, in the presence of IgG1 or IgG2
antibodies from normal serum, independently of complement (Grant and Finch, 1996). This
observation is in keeping with the experimental infection of the lactating MG with S. uberis
showing that bacteria were seen within macrophages but not within neutrophils (Thomas et al.,
1994). Mammary gland macrophages were able to take up capsulated or unencapsulated
mastitis isolates of S. uberis, but the capsule reduced phagocytosis and intracellular killing
(Almeida and Oliver, 1993). In these studies, the bactericidal efficacy of MG macrophages
appeared to be limited even in the presence of serum, consistent the idea that MG macrophages
have depressed phagocytic and pro-inflammatory functions, especially milk macrophages
during lactation (Denis et al., 2006).

All these results suggest that phagocytosis by neutrophils and macrophages (at least
during lactation) is not an effective defense of the MG against S. uberis (Leigh, 1999).
Consequently, several other avenues have been followed to develop effective vaccines. One of
them stemmed from the observation that effective vaccine trials showed protection of the gland
without inflammation and prevented bacterial growth in vivo (Hill et al., 1994). S. uberis are
fastidious bacteria that are auxotrophic for at least eight amino-acids (Leigh, 2000). One way
streptococci acquire their nutrients in the MG is by activating milk plasminogen and binding
the activated plasmin to their surface, thus being able to use peptides from hydrolyzed milk
proteins for their growth and multiplication (Leigh and Lincoln, 1997). The plasminogen
activator PauA is produced by most mastitis isolates of S. uberis and is highly conserved across
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
in Montanide ISA (mineral oil; Seppic, France). The vaccine is administered by the intramuscular route on three occasions, two months before calving, about 40 days later, finally about 15 days after calving. A trial involving experimental infection with a strain different from the vaccine strain has been conducted with 13 vaccinated and 12 control cows. The initial response to the challenge was similar in the two groups, but the clinical score was lower in the vaccinated group 3 days after challenge, and bacterial shedding after six days. Milk production losses were reduced in the vaccinated group. Nevertheless, milk leukocytosis was similar and high (more than $10^6$ cells/mL during the first 8 days) in the challenged quarters in both groups. Antibodies to LTA increased in the vaccinated group, and immune serum was somewhat active in reducing biofilm formation. Since control cows only received PBS injection, a potential non-specific immunostimulation due to the presence of MPLA and adjuvant in vaccinated cows could have contributed to the results obtained. The results of a field study were presented at the National Mastitis Council Conference in Milan (Puig et al., 2018). Involving 401 vaccinated and 380 control cows in six herds, the study showed a reduction in clinical mastitis cases in vaccinated animals compared to controls (6.1% vs 13.5%) through reduction in infection severity, but the impact on the milk yield was small (36.8 vs 36.4 L/day). The effect of vaccination on phagocytosis by neutrophils or adherence to MECs was not reported.

In all the studies mentioned above, the contribution of vaccine-induced antibodies to MG defense was either doubtful (opsonic antibodies) or not unquestionably established (prevention of adherence and invasion). Most attempts to improve the immune response to *S. uberis* have focused on humoral immunity. However, a few observations point to a role of cell-mediated immunity in the control of *S. uberis* MG infection. Blood lymphocytes of cows protected after vaccination with live *S. uberis* proliferated in vitro when exposed to killed vaccine bacteria (Hill et al., 1994). Spontaneous resolution of infection coincided with a measurable interleukin IL-17A response in the milk of cows experimentally infected with *S.
uberis, suggesting the involvement of IL-17A-producing lymphocytes (Tassi et al., 2013). Gamma Interferon (IFN-γ), another cytokine mainly produced by T lymphocytes, is found in the milk of S. uberis-infected quarters (Bannerman et al., 2004). Memory lymphocytes of CD8+ CD45RO+ phenotype proliferating when exposed to S. uberis crude antigen extract in the presence of antigen-presenting cells were found in the blood and mammary secretion (dry glands) of most cows, particularly from infected quarters (Denis et al., 2011): CD8 T cell lines derived by exposure to S. uberis antigen released IFN-γ and had substantial killing activity towards S. uberis in vitro. Immunization of cows by the subcutaneous route with an S. uberis crude extract (twice before drying-off and once before calving) elicited an antibody response, a moderate IL-17A and IFN-γ release in blood upon stimulation with antigen, and cells that could exert a cytotoxic activity towards monocytes that had phagocytosed S. uberis (Wedlock et al., 2014). The protective effect of these humoral and cell-mediated immune responses was not determined.

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

**Streptococcus agalactiae Mastitis Vaccines**

Clinical and subclinical mastitis due to Streptococcus agalactiae are frequent in countries that develop their dairy industry, such as Brazil, China or Portugal (Almeida et al., 2016, Bi et al., 2016, Carvalho-Castro et al., 2017, Pang et al., 2017), and are reemerging in
countries where dairy farming is developed but farming conditions change as in Northern
Europe (Mweu et al., 2012, Lyhs et al., 2016). This situation could rekindle interest in vaccine
design after a long period of neglect owing to the effectiveness of conventional control
measures in ridding herds of *S. agalactiae* MG infection and keeping them free from infection

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

Early attempts to develop efficient vaccines against *S. agalactiae* mastitis relied on
bacterins, with variable results. An encouraging outcome was obtained with an autogenous
vaccine in combination with antibiotic therapy (Johnson and Norcross, 1971). Another study
showed that vaccination or natural infections were not protective despite the induction of
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
reduced the challenge-induced inflammation as assessed by the concentrations of cells in the MG secretion (Bolton et al., 2004). This preliminary study was not continued. A chimeric protein comprising *S. dysgalactiae* GapC and two *S. aureus* proteins (IsdB and TRAP) elicited protection in a mouse lethal challenge after two vaccine injections, the first in complete Freund adjuvant, the second in incomplete Freund adjuvant (Yu et al., 2014). One asset of GapC is that this protein shows a high sequence identity across several bacterial species so that cross-protection can be expected. Indeed, cross-protection was obtained by immunizing mice with a genetically modified *E. coli* expressing *S. dysgalactiae* GapC (11–150 sequence) in a lethal mouse challenge with either *S. dysgalactiae*, *S. uberis* or *S. agalactiae* (1.5 to 2 x 10⁹ CFU by intraperitoneal injection) (Song et al., 2017). Translation to the cow will need challenges less distant from natural infection of the MG. GapC-induced cross-protection is not systematic, as immunization of cows with recombinant *S. uberis* GapC did not protect against challenges with *S. dysgalactiae* (Fontaine et al., 2002). Another potential asset of GapC is that it elicits specific CD4+ T cell immune responses preferentially towards Th1/Th17 polarization (Yao et al., 2016). This line of research is worth pursuing.

**Staphylococcus aureus** VACCINES

**Whole-bacterial Cell Vaccines**

Despite the implementation of the standard mastitis prevention program, *S. aureus* mastitis remains endemic in most countries and is the most frequent cause of subclinical mastitis of dairy cows in many regions (Omore et al., 1996, Petzer et al., 2009, Kalmus et al., 2011, Mistry et al., 2016, Poutrel et al., 2018). Mastitis by *S. aureus* remains a major problem to the dairy industry owing to its contagiousness, persistence in infected glands, resistance to treatment and threat to public health (Rainard et al., 2018). The development of an efficacious vaccine has long been a research target. Some success has been achieved with bacterins combined with
detoxified staphylococcal toxins (formalin-treated culture supernate) to prevent the most severe forms of mastitis of small ruminants (Derbyshire and Smith, 1969). This result can be considered acceptable as the prevalence of *S. aureus* in small ruminant herds is usually limited and the main issue is with severe, often gangrenous mastitis (Bergonier and Berthelot, 2003, Contreras et al., 2003, Gelasakis et al., 2015). In cows, *S. aureus* mastitis is usually subclinical, so what is expected of an efficient vaccine is to reduce the incidence of new infections and limit their spread within herds (Middleton, 2008). As most infections persist over long periods, another indication would be the elimination of chronic infections. The results obtained with bacterin plus toxoids have been inconsistent (Slanetz et al., 1965, Brock et al., 1975). The addition of toxoids to killed bacteria was necessary to obtain the best protection (Derbyshire, 1960). In this regard, several studies have suggested that the alpha and beta toxins are involved in the pathogenesis of mastitis (Bramley and Neave, 1975, Yancey, 1993). An improvement over this first approach has been sought by taking into consideration the production of bacterial antigens during infection. Live vaccines confer better protection than do killed vaccines, an edge that was attributed to the production of “in vivo antigens” and to the elicitation of antibodies in the IgG2 isotype (Watson, 1981). In addition, the live vaccine boosted the recruitment of neutrophils into the MG during the initiation phase of the inflammatory response (Colditz and Watson, 1982). A preparation of bacteria grown under suitable in vitro conditions stimulating the production of a pseudocapsule or slime, administered by the systemic (subcutaneous) route with an appropriate adjuvant favoring the production of antibodies in the IgG2 isotype (dextran sulfate), was proposed as a strategy to get protection (Watson, 1992a). The live vaccine lead has recently been refined by engineering a defective *VraG* mutant in a genetically stable small colony variant (SCV) *S. aureus* (Côté-Gravel et al., 2016). SCVs have altered metabolism and slow growth, associated with reduced production of toxins but increased 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) are 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, Vautoir et al., 2008, Dhanawade et al., 2010). The slime vaccine approach was pursued
in a trial involving cows that were vaccinated with *S. aureus* bacterins with either high or low slime content (Prenafeta et al., 2010). Two doses of bacterins were administered 45 days apart to primiparous gestating cows before calving, followed by an intramammary challenge with a heterologous strain of *S. aureus*. Compared to unvaccinated heifers, only those vaccinated with the high slime-producing bacterin shed less colony-forming units (CFU) of bacteria in their milk on day 1 post-challenge, but there was no difference in clinical signs between groups. Antibodies to slime were associated with the reduction in milk CFU numbers. This line of research led to the release of a licensed vaccine in Europe in 2009 (Startvac®. Hipra SA).

A field study was conducted in two large dairy herds by using the StartVac® vaccine according to the label directions (Schukken et al., 2014). Cows received three doses of the vaccine, at 45 days before the expected parturition date, 35 days later, and at 52 days in milk. The vaccine consists of killed *E. coli J5* bacteria and killed *S. aureus* slime-producing strain SP140 emulsified in liquid paraffin. It is administered by the intramuscular route. Over the 21-month observation period, vaccination resulted in a moderate reduction in the incidence of new staphylococcal IMI, and complex modeling of the IMI dynamics yielded a reduction of the “basic reproduction ratio” of 45% for *S. aureus* and 35% for coagulase-negative staphylococci. In a field study involving seven farms, the vaccine administered following the label regimen did not reduce the incidence or prevalence of clinical or subclinical mastitis but was associated with a significant reduction in the severity of clinical cases and milk losses, with a return on investment of 2.57 to 1 (Bradley et al., 2015). In that study, most clinical cases (25%) were due to *E. coli*, clinical cases caused by *S. aureus* or coagulase-negative staphylococci CoNS accounting for only 2.5 and 5.6% of cases, respectively. Another field study conducted in two large farms in which *S. aureus* mastitis prevalence was high did not show any beneficial effect on udder health, milk production or culling rate (Landin et al., 2015). Another trial carried out in a herd in which *S. aureus* was the predominant pathogen led the
authors to conclude that the vaccine was not an appropriate tool to manage the *S. aureus* problem (Freick et al., 2016).

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

**Subunit Vaccines**

Overall, the commercial vaccines against *S. aureus* mastitis tend to reduce the severity of clinical mastitis but do not solve the issue of chronic subclinical mastitis. The current bacterin vaccines are not very different from the vaccine approach advocated by Dennis Watson (Watson, 1992a). Many other attempts to improve on these vaccines have been based on the use of bacterial components supposed to play a role in the pathogenesis or to be the target of efficient immune defenses of the host (Scali et al., 2015). An early attempt was the evaluation of staphylococcal protein A to protect cows from repeated exposure to *S. aureus* by dipping the 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
from the heme proteins (Skaar and Schneewind, 2004). Cattle immunization with IsdB and
IsdH induced strong antibody responses and proliferation of CD4+ but not CD8+ cells in a
PBMC stimulation assay (Ster et al., 2010). Protection in cattle induced by these antigens has
not been established.

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

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

**CONCLUSIONS**

This review aims at giving an objective account of the present state of mastitis vaccines and current research. It is clear that the achievements are not up to expectations, whatever the bacteria involved. There are several obstacles to the development of efficacious vaccines against mammary pathogens. Some result from the MG physiology, particularly in lactation, as
dairy cows have been selected to secrete large amounts of milk which blunts and waters down immune defenses. Others pertain to the pathogens, such as their diversity and their adaptation to the MG niche. The complexities of the host-pathogen interaction within the cow’s MG represent another major obstacle to the development of efficacious mastitis vaccines of the host-pathogen interaction within the cow’s mammary gland represent a major obstacle to the development of efficacious mastitis vaccines. These difficulties may be confounded by a possible misdirection of most of past research, biased towards antibody-dependent defenses (Figure 2). There is a need for an in-depth analysis of the reasons that could explain the lack of success of past attempts, with a view to proposing new ways of getting out of the mastitis vaccine predicament. One way is to explore how cell-mediated immunity could strengthen MG defenses, and how this immunity could be harnessed by vaccination. Eliciting type 3 immunity in the MG is an attractive option (Rainard et al., 2020). It will be useful to select adjuvants that orient the immune response towards protection and to validate reliable correlates of protection. The route of administration is also likely to be of major importance. In addition, lessons learned from successes and failures of vaccines directed at other diseases could help identify the peculiarities of the MG niche and the different ways bacteria cause mastitis, hence helping to focus on relevant vaccine targets.

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

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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 O-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.
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<td>Decreased coliform mastitis severity in field experiment</td>
<td>Unknown mechanism</td>
<td>(McClure et al., 1994)</td>
</tr>
<tr>
<td>E. coli J5 bacterin</td>
<td>Antibodies to LPS core antigens</td>
<td>Discrepant results: reduction or not of severity in experimental infection</td>
<td>Unknown mechanism</td>
<td>(Hogan et al., 1992c) (Hill, 1991)</td>
</tr>
<tr>
<td>E. coli J5 bacterin, hyperimmunization</td>
<td>Antibodies to coliform outer membrane antigens in the IgG₂ isotype</td>
<td>Decreased occurrence of severe mastitis compared to usual schedule</td>
<td>Variable among herds Unknown mechanism</td>
<td>(Erskine et al., 2007)</td>
</tr>
<tr>
<td>E. coli J5 bacterin with killed S. aureus (StartVac)</td>
<td>Antibodies to coliform outer membrane antigens</td>
<td>Decreased mastitis severity in a field study</td>
<td>No reduction in incidence of case. Unknown mechanism</td>
<td>(Bradley et al., 2015)</td>
</tr>
<tr>
<td>Enterobactin FepA</td>
<td>Iron acquisition</td>
<td>Growth reduction in dry mammary secretion</td>
<td>Likely not active in lactation, not tested in vivo</td>
<td>(Lin et al., 1999)</td>
</tr>
<tr>
<td>Siderophore receptor FecA</td>
<td>Iron acquisition</td>
<td>None in experimental infection</td>
<td>Antibody titer insufficient in milk</td>
<td>(Takemura et al., 2002) (Wolf et al., 2004)</td>
</tr>
<tr>
<td>Whole E. coli (P4), intramammary booster with bacterial extract</td>
<td>Antibody and cell-mediated responses</td>
<td>Reduction in severity, likely independent of antibodies, related to Th17 response</td>
<td>Heterologous protection not tested</td>
<td>(Herry et al., 2017)</td>
</tr>
<tr>
<td>Klebsiella Siderophore receptors and porin proteins (KlebVax™)</td>
<td>Iron acquisition and multiple bacterial functions With antibodies</td>
<td>Effective in one small scale study, ineffective in a large scale study</td>
<td></td>
<td>(Gorden et al., 2018, Tomazi et al., 2021)</td>
</tr>
<tr>
<td>Klebsiella recombinant YidR</td>
<td>Unknown bacterial functions With antibodies</td>
<td>Reduced incidence of Klebsiella clinical mastitis</td>
<td>No effect on risk of death if clinical Little antibody response to whole bacteria and activity unknown</td>
<td>(Tomazi et al., 2021)</td>
</tr>
</tbody>
</table>
Table 2. Brief summary of key features of illustrative mastitis *S. aureus* vaccine trials

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

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