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1 **A critical appraisal of mastitis vaccines for dairy cows**

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8

9 **ABSTRACT**

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

25

26

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

28

29

30 INTRODUCTION

31 Mastitis is one of the most costly diseases in the dairy industry, costing US dairy industry up to
32 \$2 billions per year and from €17 to €198 per cow at the farm level in Europe (Hogeveen et al.,
33 2011). In addition to its economic impact, mastitis is also of concern regarding animal welfare
34 and farmer serenity. Mastitis, even subclinical, affects milk quality and can be a source of
35 foodborne pathogens, such as livestock- associated Methicillin Resistant *S. aureus* (LA-MRSA)
36 (Goerge et al., 2017, Garcia et al., 2019). Mastitis is the most common reason for antimicrobials
37 use in dairy farms, and a prudent use of antibiotics is recommended (Ruegg, 2017). As the
38 widespread prophylactic use of antibiotics is no longer sustainable, and considering its
39 importance as a mastitis control practice, an additional or replacement procedure is needed.
40 This is only partly achieved by internal teat sealants along with selective dry cow therapy
41 (Bradley et al., 2010). Efficacious vaccines, by reducing the incidence of new infections and
42 the occurrence of clinical cases, would be an appropriate and convenient way to fulfill this need.
43 Most mastitis cases are caused by the so-called major pathogens, *Staphylococcus aureus*,
44 *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Escherichia coli*,
45 and *Klebsiella pneumoniae*. Documented reviews on *S. aureus* vaccines are available that
46 present in detail the results obtained in field experiments, their merits and limitations
47 (Middleton et al., 2009, Scali et al., 2015, Côté-Gravel et al., 2019). Vaccines directed at other
48 major pathogens are less well covered (Keane, 2019). Vaccine development faces a number of
49 hurdles. Some relate to the return on investment, which is conditioned by the size of the market
50 and by the acceptability of the vaccine, which largely depends on its proven effectiveness.

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

58

59 **COLIFORM MASTITIS VACCINES**

60 ***Escherichia coli* J5 Mastitis Vaccines**

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

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

88 Nevertheless, vaccines based on J5 mutant strains attracted the interest of mastitis
89 researchers and were used as a component of the control of clinical coliform mastitis in
90 parturient and early lactation cows. The observation that dairy cows with low ELISA titers of
91 IgG₁ to *E. coli* J5 were associated with five times the rate of clinical coliform mastitis compared
92 to animals with higher titers (Tyler et al., 1988) elicited interest in immunization with rough
93 mutants of Gram-negative bacteria. Several field studies were carried out, mainly with the
94 mutant strain J5 but also with a rough mutant of *Salmonella enterica* Typhimurium Re-17
95 strain, as reviewed by (Wilson and Gonzalez, 2003). In the late 1980s, evidence emerged that
96 vaccination of cows with J5 bacterins had some efficacy against clinical coliform mastitis. The
97 bacterins were administered by the subcutaneous route on several occasions, often at drying-
98 off, with a booster before calving and another after calving, with significant reductions in the
99 incidence of clinical mastitis in vaccinated compared to unvaccinated cows (Gonzalez et al.,
100 1989, Cullor, 1991, Hogan et al., 1992a). A vaccine based on the use of killed *Salmonella* Re-

101 17 produced a comparable reduction in clinical cases of coliform mastitis and mortality rate
102 (McClure et al., 1994), raising interrogation about the underlying mechanisms other than the
103 developement of cross-reactive antibody immunity, as discussed below.

104 An early study of the financial return on the use of the J5 vaccine indicated that when
105 more than 1% of cow lactations were affected by clinical coliform mastitis, the vaccination
106 would be profitable (DeGraves and Fetrow, 1991). The efficiency of a recently licensed vaccine
107 against coliform and staphylococcal mastitis (Startvac®, Hipra SA) has been investigated in a
108 field study: there was no decrease in the incidence rate of clinical mastitis, but mastitis severity
109 was significantly reduced, with lessened milk losses resulting in a return on investment of 2.57
110 to 1 (Bradley et al., 2015). It has been proposed that through the reduction in milk production
111 losses and replacement of culled or dead cow, J5 vaccination is profitable in herds that
112 experience cases of clinical mastitis by Gram-negative bacteria beyond a threshold of more than
113 4% of lactating cows per month (Wilson and Gonzalez, 2003). An overview of the main
114 coliform vaccine trials is given in Table 1.

115

116 **Supposed Mechanisms of Protection by J5 Vaccines**

117 Remarkably, the relative success of rough Gram-negative bacteria vaccines remains
118 unexplained in terms of their underlying mechanisms. The initial rationale for these vaccines
119 was that they could elicit antibodies capable of neutralizing the toxic activity of the lipid A
120 moiety of LPS and opsonizing mastitis-associated coliform bacteria. Early studies in mice and
121 humans indicated that passive transfer of antibodies induced by J5 immunization protected
122 against endotoxic shock (Ziegler et al., 1982). However, the neutralization of endotoxic activity
123 is controversial. Several attempts of passive transfer of antibodies to J5 were unsuccessful,
124 possibly because of insufficient antibody concentration, so that the use of J5 antiserum to
125 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. IgG₂ 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 IgG₂
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

151 bacteria (Linton et al., 1979, Sanchez-Carlo et al., 1984, Lipman et al., 1995). To obviate the
152 narrow specificity of opsonic antibodies, J5 bacterins were used to induce cross-reactive
153 opsonic antibodies. It appeared that eliciting antibodies to J5 bacteria in the IgG₂ sub-isotype
154 was difficult, requiring more than three administrations to reach significantly higher than initial
155 titers (Chaiyotwittayakun et al., 2004). Increased IgG₂ antibodies to J-5 obtained by
156 hyperimmunization correlate with a decreased occurrence of clinical mastitis (Erskine et al.,
157 2007). However, antibody titers tend to dwindle in a few months (Erskine, 2012).

158 The opsonic activity of antibodies raised with J5 bacterins is a matter of controversy.
159 Studies showed either some increase in opsonic activity following vaccination (Hogan et al.,
160 1992b) or not (Vreede et al., 1986). The antibodies bound to their antigen target have to be
161 accessible to the phagocytes, and specifically to their immunoglobulin receptors, for ingestion
162 to be triggered. First, the antigen targets must be accessible to antibodies. The accessibility of
163 lipid A, core antigens and outer membrane proteins has been doubted, owing to the shielding
164 effect of the side chains of LPS (O-antigen) of smooth strains (Greisman and Johnston, 1997).
165 This shielding has been confirmed with mastitis strains: immunization of cows with J5 induced
166 cross-reactive antibodies to rough strains but these antibodies, except those directed at fimbriae,
167 reacted very poorly with smooth strains and did not improve on the opsonic activity of pre-
168 existing natural antibodies (Figure 1) (Rainard et al., 2021). Absorption of J5 immune serum
169 with smooth strains did not reduce the antibody reactivity with rough strains, demonstrating the
170 efficient shielding by the O-antigen polysaccharide layer toward outer membrane antigens. In
171 accordance, immunization of rabbits with *E. coli* J5 did not augment the opsonization titer to
172 smooth strains (van Dijk et al., 1981). Nevertheless, J5 bacterins induce cross-reactive
173 antibodies, even though their specificity has not been characterized (Ziegler et al., 1973), except
174 for the outer membrane protein OmpA and type 1 fimbriae (Rainard et al., 2021). The role
175 played by these cross-reacting antibodies in protection against infection has been evaluated in

176 vaccination and challenge experiments. Several studies did not find noticeable protective effect
177 following systemic or intramammary J5 vaccination (Hill, 1991, Smith et al., 1999, Tomita et
178 al., 2000, Steele et al., 2019, Tashakkori et al., 2020, Vangroenweghe et al., 2020). One study
179 found that the J5 vaccine was associated with faster clearance of *E. coli* in milk and less
180 reduction in milk yield following an intramammary challenge with a strain that did not induce
181 clinical mastitis (Wilson et al., 2007). Of note, the IgM and IgG₂ milk titers were not different
182 from titers of control cows just before challenge, and higher IgM antibody titers against J5 at
183 12 h post-challenge tended to be associated with higher milk production losses, suggesting that
184 the vaccine effect was probably independent of opsonic antibodies.

185 The vaccine formulation is likely to account for part of the discrepant results of
186 experimental challenges. The comparison of two J5 vaccines using two different adjuvants
187 yielded different outcomes, one showing some protection and the other none compared to
188 control cows (Hogan et al., 2005). The route of administration could also influence the
189 efficacy of the vaccine. The common way of J5 vaccine administration is subcutaneous
190 injection in the neck. A study compared this route to the area drained by the supramammary
191 lymph node, without much difference in efficacy, with a comparable increase in antibody
192 titers to J5 (Tomita et al., 1998). In another vaccine trial, the two J5 vaccines tested failed to
193 improve serum and mammary secretion IgM antibody titers but increased IgG₁ and IgG₂
194 titers, with no change in clinical status of challenged cows among treatment groups (Tomita et
195 al., 2000). The *E. coli* strains used for the challenge are also likely to affect the results of
196 vaccine trials. In all of these experiments, the role of anti-J5 antibodies was not established.
197 One study showed a slight augmentation of phagocytosis of a smooth *E. coli* strain after
198 vaccination with J5 that was attributed to a slight augmentation of IgM antibodies to the
199 smooth strain in the serum of vaccinated cows, although there was no increase of titers to J5
200 (Hogan et al., 1992b). The specificity of the opsonic antibodies was not determined. It

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

211 All things considered, the importance of opsonic antibodies as a defense mechanism
212 induced by J5 vaccines is questionable. The observation that early lactation pooled whey (5–10
213 days post-partum) was opsonic for all mastitis strains of *E. coli* tested suggests that since early
214 lactation milk contains sufficient opsonins, severe *E. coli* mastitis at this stage of lactation is
215 not due to opsonic deficiency (Hill et al., 1983b). In other words, opsonic antibodies are not a
216 limiting factor in defense against *E. coli* mastitis even in early lactation when coliform mastitis
217 tends to be severe. This conclusion is supported by the absence of reinforcement of milk
218 neutrophils phagocytic activity at the onset of infection in the quarters of vaccinated cows
219 challenged with the homologous strain compared to control cows, although antibody titers to
220 this strain were markedly augmented in the IgG₂ and IgM isotypes (Herry et al., 2017). Another
221 factor contributing to reducing the potential benefit of vaccine-induced antibodies is the
222 difficulty in maintaining elevated concentrations because of dilution in milk. It can be
223 concluded that the role of J5 vaccine-induced opsonic antibodies remains dubious and that the
224 experimental challenge trials displayed limited protection and did not yield many clues as to
225 the mechanism behind the activity of the J5 vaccine observed in field experiments.

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

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

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

260

261 **Sub-unit Vaccines against Coliforms**

262 Another approach to vaccination against coliform mastitis has been explored. Blocking
263 the growth of coliform bacteria in mammary secretions has the potential to prevent IMI rather
264 than reducing only its severity. Iron is an essential element for the survival and multiplication
265 of coliform bacteria. Most iron in mammary secretions is bound to lactoferrin during the dry
266 period and to citrate during lactation (Reiter, 1978). Coliform bacteria can acquire iron in bodily
267 fluids by utilizing high-affinity iron acquisition systems (Garenaux et al., 2011). Two of those
268 systems have been targeted to develop a mastitis vaccine. The enterobactin iron acquisition
269 system is common in coliforms isolated from mastitis. The ferric enterobactin receptor FepA,
270 which is an iron-regulated outer membrane protein, binds ferric enterobactin, an efficient iron
271 chelator (siderophore). Cows were immunized with purified FepA, and the serum IgG fraction
272 tested for bacteriostatic activity (Lin et al., 1999): the growth of all the *E. coli* and *Klebsiella*
273 *pneumoniae* isolates tested was inhibited by 4 mg/mL of the IgG preparation in the presence à
274 0.5 mg/mL apolactoferrin. Of note, purified IgG from cows immunized with *E. coli* J5 had little
275 inhibitory effect on the growth of *E. coli* or *K. pneumoniae* mastitis isolates (Lin et al., 1999).

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

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

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

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

337

338 **STREPTOCOCCAL VACCINES**

339 ***Streptococcus uberis* Mastitis Vaccines**

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

350 lower bacterial concentration and more than 10 times lower leukocyte concentrations in milk
351 than unvaccinated animals. Interestingly, although specific antibacterial antibodies were
352 elicited in the IgG₂ and IgM isotypes, the opsonic activity of serum or milk was not improved,
353 despite a modest initial phagocytic activity (50% bacterial survival). Contrary to vaccination
354 with a surface extract of the challenge strain, which did not induce protection, the live vaccine
355 induced a proliferative response of blood lymphocytes to *S. uberis* antigen. A further study
356 showed that vaccination with live bacteria protected against the homologous strain but was less
357 effective against a heterologous strain (Finch et al., 1997). Another attempt indicated that local
358 vaccination (intramammary) with killed bacteria without adjuvant administered six times one
359 week apart during the dry period protected the gland against homologous challenge by
360 preventing bacterial growth without inflammation (Finch et al., 1994). Again, the opsonic
361 activity of milk or serum of vaccinated cows was not improved. Furthermore, in experimental
362 MG infections with virulent strains of *S. uberis*, intense recruitment of neutrophils into
363 mammary tissue and milk occurred concomitantly with high concentrations of bacteria, which
364 were not frequently observed associated with neutrophils (Hill et al., 1994, Thomas et al., 1994).
365 Those findings were surprising. Indeed, the phagocytic killing of bacteria by neutrophils is
366 considered an essential defense of the MG, a position supported by cogent arguments with
367 regard to mastitis-associated *E. coli* and *S. aureus* (Paape et al., 2002). Accordingly, the prompt
368 and intense recruitment of activated neutrophils at the onset of infection is assumed to have a
369 positive effect on the outcome of infection (Craven and Williams, 1985, Rainard and Riollot,
370 2003). These views may not apply to *S. uberis* mastitis, an oddity that prompted investigations
371 on the interactions of *S. uberis* with phagocytic cells.

372 The investigation of a small panel of five *S. uberis* clinical isolates exposed to bovine
373 neutrophils in the presence of skim milk displayed three phenotypes: bacteria that resisted
374 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 IgG₁ or IgG₂
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

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

431 However, PauA would not be a requirement for *S. uberis* to grow in milk (Ward et al.,
432 2003), which casts some doubt on the effectiveness of a vaccine based exclusively on this
433 antigen. It has been shown that the glyceraldehyde-3-phosphate dehydrogenase GapC
434 contributes to the binding of plasmin at the bacterial surface (Cunningham, 2000). Vaccination
435 of cows with recombinant *S. uberis* GapC or CAMP factor was reported to induce protection
436 after homologous challenge (Fontaine et al., 2002), but peculiarities of the challenge model
437 (low virulence of the *S. uberis* strain, size or viability of the inoculum) made this experiment
438 unconvincing (Leigh, 2002). Field studies with PauA, GapC, or CAMP subunit vaccines are
439 awaited.

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

450 mechanism in the pathogenesis of *S. uberis* mastitis (Almeida et al., 2015), another considering
451 that adhesion is not required and cannot play a major role in the initiation of infection (Leigh,
452 1999). Adherence and invasion of MAC-T cells depend on the presence of the surface *S. uberis*
453 adhesion molecule (SUAM) (Almeida et al., 2006). Antibodies obtained by immunizing cows
454 with recombinant SUAM reduced the adherence and internalization of *S. uberis* by MAC-T
455 cells (Prado et al., 2011). Infection of the MG with a mastitis isolate and its isogenic mutant
456 devoid of SUAM showed that the defective mutant induced mastitis of lesser intensity with
457 fewer bacterial shedding and milk leukocytosis (Almeida et al., 2015). However, bacterial
458 shedding and milk leukocytosis were still sizable, indicating that SUAM was not the only
459 virulence factor at play during MG infection. In an experimental *S. uberis* intramammary
460 challenge involving 40 cows half of which were vaccinated with SUAM and adjuvant, all the
461 cows developed mastitis (Siebert et al., 2017). These results suggest that SUAM alone would
462 not suffice to design an efficient vaccine, and would need to be associated with other antigen
463 targets.

464 A possible way *S. uberis* could resist MG immune defenses and antimicrobial treatments
465 is the formation of biofilm. Biofilms are dense aggregates of surface-adherent microorganisms
466 embedded in a complex matrix mainly comprising exopolysaccharides. Most if not all *S. uberis*
467 mastitis isolates can grow in biofilm under suitable culture conditions (Crowley et al., 2011,
468 Dieser et al., 2017). In particular, casein proteolytic peptides contribute to increased biofilm
469 formation, conditions likely to occur during MG infection (Varhimo et al., 2011). A recently
470 licensed vaccine produced by Laboratorios Hipra S. A. (UBAC®) is based on a slime
471 preparation as antigen (Collado et al., 2018). The Biofilm Adhesion Component (BAC)
472 comprises an extract of biofilm produced by a mastitis isolate cultivated on a solid surface and
473 extracted by autoclaving. This complex antigen contains lipoteichoic acid (LTA). The antigen
474 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

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

558 These encouraging preliminary studies await their translation to the field. One possible
559 pitfall is the capacity of *S. agalactiae* strains to modify the expression of their surface antigens,
560 with possible eclipse or loss of capsular and protein production (Jensen, 1980, Rosini et al.,
561 2015). It will probably be necessary to combine several of these antigens to circumvent that
562 issue.

563

564 ***Streptococcus dysgalactiae* Mastitis Vaccines**

565 *S. dysgalactiae* subspecies *dysgalactiae* ranks highly among bacteria responsible for
566 clinical and subclinical mastitis (Sampimon et al., 2009, Lundberg et al., 2014, Zhang et al.,
567 2018). The dearth in research on the pathogenesis and immune response to this pathogen,
568 relatively to the extensive research on pathogens of similar importance such as *S. uberis* or *S.*
569 *aureus*, is surprising (Klaas and Zadoks, 2018). Consequently, we know little about the MG
570 defenses specific to this pathogen. In an early vaccine trial, formalin-killed bacteria were
571 administered by the subcutaneous route in the region of the supramammary lymph node (Stark
572 and Norcross, 1970). Repeated, frequent immunizations were necessary to get partial protection
573 which was attributed to the elicited antibodies. Recently, several groups used the dehydrogenase
574 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 10⁹ 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 IgG2 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

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

638 The “in vivo antigen” approach was patented (Watson, 1992a) but has not find a way
639 into widespread commercialization. Another patented bacterin vaccine preparation, comprising
640 three *S. aureus* strains grown in a medium favoring the production of exopolysaccharides,
641 showed encouraging results in a small-scale experiment, but without published follow-up
642 (Leitner et al., 2003). More recently, the “slime track” has been followed and developed (Pérez
643 et al., 2009). The production of exopolysaccharides by *S. aureus* mastitis isolates is inducible
644 by growth in special laboratory media rich in salt and sugars or in milk whey (Baselga et al.,
645 1994). Among these exopolysaccharides there are true capsular polysaccharides, which form
646 thin and discontinuous microcapsules of mainly two serotypes, CP5 and CP8 (Sutra et al.,
647 1990a, Poutrel and Sutra, 1993), with a variable expression between strains and within bacterial
648 populations of most mastitis isolates (Poutrel et al., 1997). Specific antibodies can be elicited
649 by immunizing cows with capsular polysaccharides conjugated to a protein carrier (Gilbert et

650 al., 1994, Lee et al., 2005). The anti-capsular antibodies are opsonic (Kampen et al., 2005) but
651 are not required to opsonize mastitis isolates. Moreover, a number of isolates from chronic
652 infections do not produce capsules because they repress their production or have mutated in the
653 capsule operon (Tuchscherr et al., 2005). Those nontypable variants or mutants are supposed to
654 have an increased capacity to adhere to and invade epithelial cells than their capsulated
655 counterparts (Tuchscherr et al., 2010). On the other hand, the growth of mastitis isolates in
656 exopolysaccharide-promoting media interferes with phagocytosis by neutrophils (Sutra et al.,
657 1990b). Apart from capsular polysaccharides, the contribution of inducible slime is likely.
658 Bacterins comprising strong slime-producing *S. aureus* induce antibodies against poly-N-
659 acetyl- β -glucosamine (PNAG), a major component of slime, and have conferred protection of
660 lactating ewes against infection and mastitis (Pérez et al., 2009). Bacterins were better inducers
661 of antibodies to PNAG than was purified slime, even when slime was incorporated into
662 liposomes. After an intramammary challenge with a live heterologous *S. aureus* strain, the
663 shedding of bacteria was reduced by two orders of magnitude in the groups vaccinated with the
664 high slime-producing bacteria compared to controls and animals in the groups vaccinated with
665 the low slime-producing bacteria. Surprisingly, bacterial concentrations in the milk of ewes
666 immunized with crude or purified PNAG exceeded concentrations in milk of control ewes.
667 Clinical data and mammary tissue lesions tended to correlate negatively with the antibody titers
668 and bacterial shedding reduction in milk (Pérez et al., 2009). Accordingly, antibodies induced
669 by the deacylated form of PNAG (dPNAG) are opsonic (Maira-Litran et al., 2005). The study
670 suggests that antibodies to slime, including PNAG, are induced when associated with bacterial
671 bodies, and that they protect against, or correlate with protection to, severe mastitis in ewes.
672 This protection manifested itself by a marked reduction in the severity of the symptoms. It
673 should be noted, however, that not all mastitis isolates are high producers of slime (Oliveira et
674 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

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

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

746 The capacity to acquire iron or growth in milk is an important attribute of mastitis-
747 associated bacteria. The expression of several *S. aureus* iron-regulated genes is upregulated
748 during growth in vivo (Allard et al., 2006). Among these genes, those of the iron-regulated
749 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. aureus* 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 unsurmountable 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.

800

801

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

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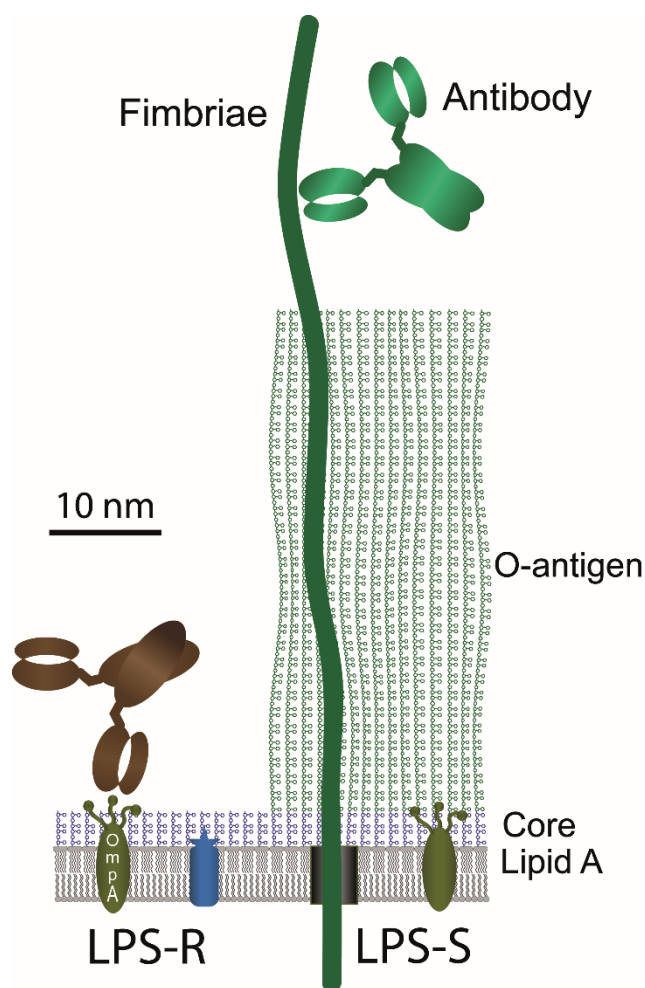
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1394 **Figure 1.** Steric hindrance for antibody accessibility to *E. coli* outer membrane of rough
 1395 (LPS-R) or smooth (LPS-S) strains. Antibodies can access outer membrane proteins such as
 1396 OmpA on rough strains, but are prevented from reacting with these proteins by the shielding
 1397 effect of the O-antigen of smooth strains. However, bacterial structures protruding from the
 1398 smooth LPS, such as fimbriae, are accessible to antibodies. The IgG molecule of antibody is
 1399 shown to scale for comparison with the bacterial components. The LPS-S of *E. coli* O32 with
 1400 15 repeats of the O-antigen unit is shown.

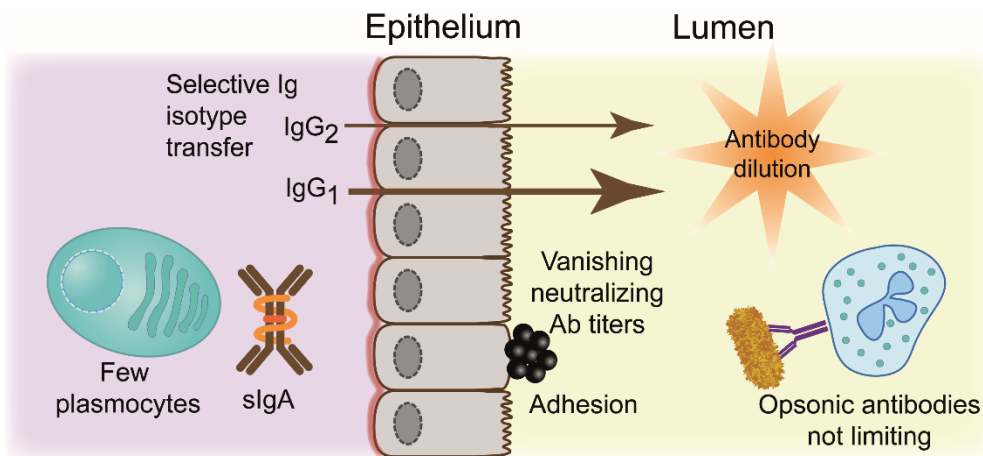
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1408 **Figure 2.** Vaccine-induced antibodies and antibody-dependent MG defenses face several
1409 challenges. In healthy glands, passive transudation of antibodies from blood to milk is low.
1410 Active transepithelial transfer favors the IgG₁ isotype, which is not opsonic for neutrophils.
1411 Local production of antibodies, such as secretory IgA (sIgA) is limited, as resident
1412 plasmocytes are few in the mammary parenchyma. In addition, antibodies that pass the
1413 epithelial barrier are diluted in high volumes of milk. In the absence of apical mucus capable
1414 of retaining antibodies on the luminal surface of the epithelium, antibody concentrations is
1415 locally not sufficient to prevent adhesion of bacteria or interfering with nutrient acquisition.
1416 Furthermore, elevated antibody concentrations to common mastitis-causing bacteria in blood
1417 and milk are transient after vaccination. Finally, there are few examples of increased opsonic
1418 activity towards mastitis bacteria following vaccination. This latter failure is largely because
1419 natural antibodies in milk and blood already have fairly good opsonic activity, so opsonic
1420 antibodies are usually not the limiting factor for protection.

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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 <i>Klebsiella</i> 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 <i>S. aureus</i> 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 <i>S. aureus</i> 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-Deogo et al., 2006)

1427 SCV, small coony variant; VraG, *S. aureus* ABC transporter; *Staphylococcus aureus* surface proteins: FnBP, fibronectin-binding protein; ClfA, clumping factor A;

1428 IsdB, IsdH, *S. aureus* iron-regulated surface proteins; GapB, GapC: proteins with homology to glyceraldehyde-3-phosphate dehydrogenase

