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infected cows. In this regard, STARTVAC® is the first vaccine registered in the world that confers protection against mastitis caused by coagulase negative staphylococci. Antibodies to the PNAG exopolysaccharide of the SAAC, induced by immunisation with STARTVAC®, could be one of the factors responsible for conferring cross-protection against infections from CNS.

6. Perspectives of biofilm in mastitis

The ability to form biofilm is an important virulence factor of *S. aureus* involved in bovine and ovine mastitis. Although other virulence factors may be involved in the pathogenesis of mastitis, the PNAG or SAAC-specific antibodies may prevent the establishment of infection of *S. aureus* in the mammary gland by binding to the exopolysaccharide extracellular matrix (before the establishment of the biofilm), thereby facilitating polymorphonuclear neutrophil-mediated phagocytosis and elimination of infection. From this point of view, vaccination with STARTVAC® offers an option for reducing intramammary infections from *S. aureus* and CNS.

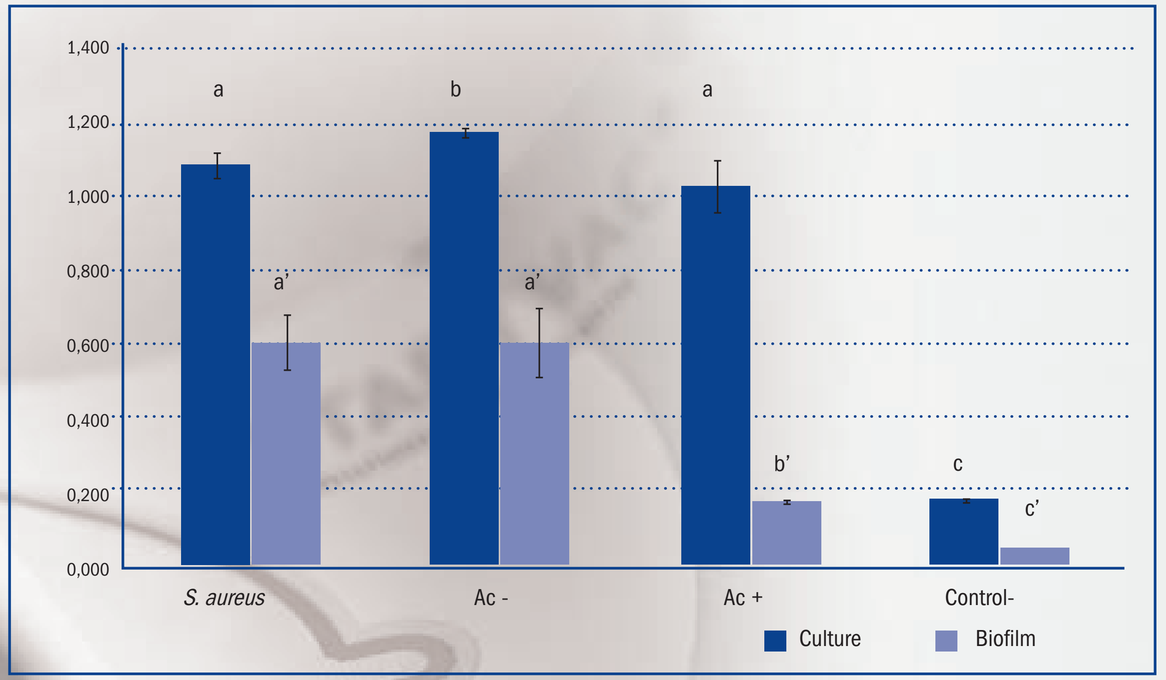

Figure 5. Inhibition of biofilm formation of *S. aureus* in microplate mediated by anti-SAAC antibodies. The graph plots the OD of bacterial growth at the end of the microplate incubation (in blue) and the OD of the biofilm after staining of adherent cells (in light blue). In this study, a biofilm producing strain of S. aureus was incubated without antibodies ("*S. aureus*" columns), in the presence of serum without anti-SAAC antibodies ("Ac- columns") or in the presence of a hyperimmune serum with anti-SAAC antibodies ("Ac+ columns"). The "Control" columns correspond to the wells with uninoculated culture medium. The columns with the same letter do not differ significantly between each other (P <0.05). Error bars indicate the standard deviation of the mean.

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Bibliographic references

1. Amorena, B., Baselga, R. and Albizu, I., 1994. Use of liposome-immunopotentiated exopolysaccharide as a component of an ovine mastitis staphylococcal vaccine. Vaccine. 2:243-249.

2. Baselga, R., Albizu, I., De La Cruz, M., Del Cacho, E., Barberan, M. and Amorena, B., 1993. Phase variation of slime production in *Staphylococcus aureus*: implications in colonization and virulence.

3. Cerca, N., Jefferson, K.K., Maira-Litrán, T., Pier, D.B., Kelly-Quintos, C., Goldmann, D.A., Azeredo, J. and Pier, G.B., 2007. Molecular basis for preferential protective efficacy of antibodies directed to the poorly acetylated form of staphylococcal poly-*N*-acetyl-β-(1-6)-glucosamine. Infect. Immun. 75:3406-3413.

4. Corrigan, R.M., Rigby, D., Handley, P. and Foster, T.J., 2007. The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. Microbiology. 153:2435-2446.

5. Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W. and Götz, F., 1999. The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect. Immun. 67:5427-5433.

6. Cucarella, C., Tormo, M.A., Úbeda, C., Trotonda, M.P., Monzón, M., Peris, C., Amorena, B., Lasa, I. and Penadés, J.R., 2004. Role of biofilm-associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus*. Infect. Immun. 72:2177-2185.

7. Dhanawade, N.B., Kalorey, D.R., Srinivasan, R., Barbuddhe, S.B. and Kurkure, N.V., 2010. Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis. Vet. Res. Commun. 34:81-9.

8. Donlan, R.M. and Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews. 15:167-193.

9. Hall-Stoodley, L., Costerton, J.W. and Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nature Reviews Microbiology. 2: 95-108.

10. Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., López, A.J., Foster, T.J., Penadés, J.R. and Lasa, I. 2009. Protein A-mediated multicellular behaviour in *Staphylococcus aureus*. J. Bacteriol. 191:832-843.

11. McDevitt, D., Francois, P., Vaudaux, P. and Foster, T.J., 1994. Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. Mol. Microbiol. 11:237-248.

12. Nordhaug, M.L., Nesse, L.L., Norcross, N.L. and Gudding, R., 1994. A field trial with an experimental vaccine against *Staphylococcus aureus* mastitis in cattle. 1. Clinical parameters. J. Dairy Sci. 77:1267-1275.

13. Oliveira, M., Nunes, S.F., Carneiro, C., Bexiga, R., Bernardo, F. and Vilela, C.L., 2007. Time course of biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. Vet. Microbiol. 124:187-191.

14. O'Neill, E., C. Pozzi, P. Houston, D. Smyth, H. Humphreys, D.A. Robinson, A. Loughman, T.J. Foster and J.P. O'Gara., 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J. Bacteriol. 190:3835-50.

15. Patty, J.M., Jonsson, H., Guss, B., Switalski, L.M., Wiberg, K. et al. 1992. Molecular characterization and expression of a gene encoding a *Staphylococcus aureus* collagen adhesion. J. Biol. Chem. 267:1766-1772.

16. Pérez, M.M., Prenafeta, A., Valle, J., Penadés, J., Rota, C., Solano, C., Marco, J., Grilló, M.J., Lasa, I., Irache, J.M., Maira-Litran, T., Jiménez-Barbero, J., Costa, L., Pier, G.B., de Andrés, D., Amorena, B., 2009. Protection from *Staphylococcus aureus* mastitis associated with poly-*N*-acetyl β-1,6 glucosamine specific antibody production using biofilm-embedded bacteria. Vaccine. 27, 2379-2386.

17. Prenafeta, A., March, R., Foix, A., Casals, I. and Costa, LL., 2009. Study of the humoral immunological response after vaccination with a *Staphylococcus aureus* biofilm-embedded bacterin in dairy cows: possible role of the exopolysaccharide specific antibody production in the protection from *Staphylococcus aureus* induced mastitis. Vet. Immun. Immunopathol. 134:208-217.

18. Schroeder, K., Jularic, M., Horsburgh, S.M., Hirschhausen, N., Neumann, C., Bertling, A., Schulte, Foster, S., Kehrel, B.E., Peters, G. and Heilmann, C., 2009. Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation. PLoS ONE. 4(10):7567.

19. Tung, H., Guss, B., Hellman, U., Persson, L., Rubin, K. et al., 2000. A bone sialoprotein-binding protein from *Staphylococcus aureus*: a member of the staphylococcal Sdr family. Biochem J. 345(3):611-619.

20. Vasudevan, P., Nair, M.K.M., Annamalai, T. and Venkitanarayanan, K.S., 2003. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet. Microbiol. 92:179-185.

21. Watson, D.L. and Watson, N.A., 1989. Expression of a pseudocapsule by *Staphylococcus aureus*: influence of cultural conditions and relevance to mastitis. Research in Veterinary Science. 47:152-157.

22. Watson, D.L. and Davies, H.I., 1993. Influence of adjuvants on the immune response of sheep to a novel *Staphylococcus aureus* vaccine. Vet. Microbiol. 34:139-153.

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General aspects of biofilm and its implication in ruminant mastitis

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1. Biofilm as a survival mechanism

Biofilm can be defined broadly as a dynamic and well structured microbial community, attached to a solid surface and aggregated by an extracellular matrix. The ability to form biofilm is a widespread feature among prokaryotes (both in the archaeal and bacterial domain) and it has been found in fossil formations dating back 3.2 billion years. From an evolutionary standpoint, the formation of biofilm probably conferred an adaptive advantage by providing homeostasis against extreme conditions and fluctuations of the primitive earth (temperature, pH and exposure to UV radiation). In addition to offering protection from physical and chemical environmental factors, biofilm facilitates extracellular catalytic functions (because cells remain close to each other) and promotes the concentration of nutrients on the surface (Hall-Stoodley *et al.*, 2004). Biofilm resistance to antimicrobial agents (e.g. antibiotics) may be due to difficulty in penetration of the antimicrobial agent through the extracellular matrix, to the decreased growth rate of biofilm cells (β-lactam antibiotics are effective in Gram-positive cells that are actively dividing) or the existence of resistant phenotypes among a genetically heterogeneous population.

2. Biofilm in natural environments and its implication in infections

Biofilm formation is ubiquitous in natural environments. These types of biological structures are found at the bottom of rivers or on the surface of stagnant water; in extreme environments, from hot springs to glaciers in the Antarctic; in showers or baths favoured by the warm moist environment; inside water ducts or industrial gas and oil pipes; in symbiosis with plants, etc.

Biofilm formation is also implicated in the pathogenesis of many human infections. The adhesion of *Staphylococcus* or *Streptococcus* to the proteins of the basal membrane of the damaged heart epithelium is a cause of endocarditis. In the case of cystic fibrosis patients, decreased ciliary activity of the respiratory mucosa and mucus hyperviscosity promote colonisation and biofilm formation by *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*. Another well known example of biofilm is the subgingival plaque of *Streptococcus mutans*.

Biofilm formation has also been described in uropathogenic strains of *Escherichia coli*. Finally, biofilm is an important virulence factor involved in the development of implant-related infections of intravenous catheters, heart valves, prostheses, peritoneal dialysis catheters, endotracheal tubes, etc., which are mainly caused by the adhesion of *S. aureus* and *Staphylococcus epidermidis* to the surface of these implants (Hall-Stoodley *et al.*, 2004).

The contribution of biofilm to pathogenesis is attributed to its resistance to antibiotics and phagocytosis, thereby facilitating chronic infections. On the other hand, detachment of biofilm bacteria cells is a cause of septicaemia and new colonisations, while the production of endotoxins and exotoxins produce inflammation and tissue damage.

One of the difficulties in eliminating infections associated with biofilm production is resistance to antibiotics. Table 1 shows that bacterial death within biofilm requires a greater amount of antibiotic than the concentration of bactericidal activity in planktonic bacterial cells (free or in suspension).

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Table 1. Sensitivity to antibiotics of different bacterial genera growing in planktonic form (free or in suspension) or in biofilm (Donlan and Costerton, 2002).

Microorganism	Antibiotic	Reference Organism Antibiotic MIC or MBC of planktonic phenotype (µg/ml)	Concentration effective against biofilm phenotype (µg/ml)
S. aureus NCTC 8325-4	Vancomycin	2 (MBC)	20
Pseudomonas aeruginosa ATCC 27853	Imipenem	1 (MIC)	1024
E. coli ATCC 25922	Ampicillin	2 (MIC)	512
P. pseudomallei	Ceftazidime	8 (MBC)	800
Streptococcus sanguis 804	Doxycycline	0.063 (MIC)	3.15

MIC: Minimum Inhibitory Concentration
MBC: Minimum Bactericidal Concentration

3. Development of *S. aureus* biofilm

Biofilm formation by *S. aureus* is a well characterised process that occurs in two steps. First, bacterial cells adhere to a surface specifically or by physicochemical interactions. In the former case, specific *S. aureus* adhesion proteins bind to the extracellular matrix factors of the host, such as fibronectin binding proteins (FnBPA, FnBPB) (O'Neill *et al.*, 2008), fibrinogen binding proteins (ClfA, ClfB) (McDevitt *et al.*, 1994), collagen binding protein (Can) (Patti *et al.*, 1992) or bone sialoprotein binding protein (BBP) (Tung *et al.*, 2000). In a second step, adherent bacterial cells multiply, interact with each other and accumulate in layers, embedded by an extracellular matrix secreted by the bacterial cell itself (Figure 1). The main constituent of the *S. aureus* and *S. epidermidis* extracellular matrix, responsible for the intercellular interactions, is the exopolysaccharide poly-N-acetyl-β -1,6-glucosamine (PNAG), synthesized by enzymes encoded in the *icaADBC* operon (Cramton *et al.*, 1999). Likewise, some *S. aureus* proteins have been described that can be involved in intercellular interaction and biofilm formation: Bap (Cucarella *et al.*, 2001), protein A (Merino *et al.*, 2009), SasC (Schroeder *et al.*, 2009) and SasG (Corrigan *et al.*, 2007).

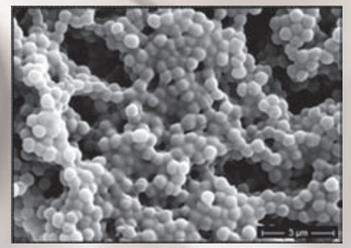

Figure 1. micrograph by scanning electron microscopy of a biofilm of *S. aureus* growing in vitro (www.erc.montana.edu).

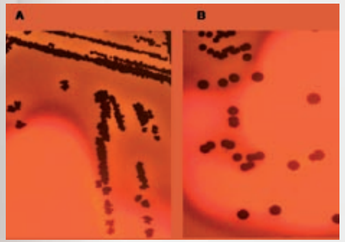

Figure 2. Determination of the ability to form biofilm by characterising colonial morphology in agar plates with Congo red. Slime positive or biofilm-producing isolates form colonies that have rough and irregular outlines in Congo red plates (A), whereas slime negative or non-biofilm producing isolates form colonies with shiny, smooth and well-defined boundaries (B).

4. Implication of biofilm in ruminant mastitis caused by *S. aureus*

In bovine and ovine mastitis caused by staphylococci, bacterial cells attach to the epithelial cells of the mammary gland and grow into colonies surrounded by an extracellular matrix, thereby forming the biofilm. Because of its size, biofilm is not capable of being phagocytised by polymorphonuclear neutrophils or macrophages and, moreover, it confers resistance to antibiotics, thereby promoting the chronicity of infection.

Various studies demonstrate the presence of the *icaADBC* operon, which encodes the enzymes responsible for the biosynthesis of the PNAG exopolysaccharide, the main component of the extracellular matrix of the biofilm, in 94.36% (Cucarella *et al.*, 2004) or 100% (Vasudevan *et al.*, 2003) of *S. aureus* isolated from bovine mastitis.

Apart from this genetic capacity, a number of studies have also demonstrated the ability of bovine mastitis isolates to form biofilm *in vitro*. In this regard, Vasudevan *et al.* (2003) found that 91% of isolates of *S. aureus* from bovine mastitis had the ability to form biofilm *in vitro* by determination of colonial morphology on agar plates with Congo red (Figure 2), whereas 69% showed adhesion in a microplate (Figure 3). In another study, Oliveira *et al.* (2007) characterised 80.8% of isolates of *S. aureus* and 75.9% of isolates of *S. epidermidis* in bovine mastitis as *in vitro* producers of biofilm. Dhanawade *et al.* (2010) found that 48.03% of the strains of *S. aureus* isolated from bovine mastitis had the ability to form biofilm *in vitro* by the culture test on agar plates with Congo red.

While the genetic capacity and *in vitro* biofilm production in *S. aureus* isolates from bovine mastitis seems clear, is there any evidence of biofilm production of *S. aureus* in the mammary gland? Watson *et al.* (1989) observed by electron microscopy the production of a polysaccharide extracellular matrix (called pseudocapsule by the authors) in *S. aureus* cells isolated directly from the milk of sheep and cows with clinical mastitis. Shortly afterwards, Baselga *et al.* (1993) demonstrated the production of an exopolysaccharide matrix in *S. aureus* cells by immunohistochemical analysis of mammary gland parenchymal tissue samples from sheep experimentally infected with *S. aureus* by intramammary route. The *in vivo* exopolysaccharide expression has also been shown indirectly by observing the production of specific antibodies against PNAG (Perez *et al.*, 2009) and against SAAC (Slime Associated Antigenic Complex; Prenafeta *et al.*, 2010) in sheep and cows, respectively, experimentally infected with *S. aureus* by intramammary route.

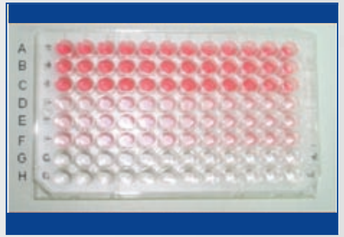

Figure 3. Analysis of biofilm production in microplate (adhesion test). After an incubation period of the *S. aureus* isolate in the wells of the microplate, the plate is emptied, the wells washed, and the cells that have adhered fixed, stained and the optical density (OD) of the wells determined by means of an ELISA plate reader. If the isolate formed biofilm during growth, the OD of the wells is high (rows A, B and C), whereas the OD is not significant if the isolate did not produce biofilm (rows D, E and F) compared with the uninoculated negative control wells (rows G and H).

5. Vaccines against the biofilm of *S. aureus* to combat mastitis in ruminants

Given that biofilm formation is an important virulence factor of *S. aureus* in the pathogenesis of mastitis in sheep and cows, the efficacy of different experimental vaccines has been tested, showing different levels of protection. Watson *et al.* (1993) and Nordhaug *et al.* (1994) used vaccines based on whole *S. aureus* inactivated cells embedded in their own extracellular matrix called pseudocapsule.

The experimental vaccines in a study by Amorena *et al.* (1994) consisted of a mixture of slime (biofilm exopolysaccharide matrix) in liposomes, toxoid and various inactivated *S. aureus* isolates. More recently, knowing that the PNAG exopolysaccharide is the major component of the extracellular matrix of the *S. aureus* biofilm, Perez *et al.* (2009) conducted an efficacy trial against an intrammamary challenge with a virulent *S. aureus* strain in sheep, using bacterins (whole and inactivated bacterial cells), crude extract, or purified PNAG, with different adjuvants, as vaccines. The results of this study showed that bacterins from strong biofilm-producing bacteria induced the highest titres of specific antibodies to PNAG and conferred the greatest protection against an intramammary challenge, compared to vaccines containing bacterins from weak biofilm-producing bacteria, crude extract or purified PNAG.

The study by Prenafeta *et al.* (2010) clarifies the role of SAAC-specific antibodies in protecting against the mastitis caused by *S. aureus* in an experimental infection in cows. SAAC is an isolated cell fraction from *S. aureus* strains that produce biofilm. The presence of this extracellular component has been determined for all isolates of *S. aureus* characterised as slime producers in agar plates with Congo red (Figure 4) and its production is directly related to the *in vitro* biofilm formation (Table 2). The chemical characterisation showed that the SAAC is comprised of 58% (w/v) polysaccharide and 42% (w/v) protein. Contents of 6.1% of glucosamine and galactosamine were found in the polysaccharidic fraction, suggesting that these sugars may correspond to deacetylated forms of PNAG. It is noteworthy that antibodies to deacetylated forms of PNAG are those with the greatest capacity for opsonization (specific antibody binding to antigen) and protection against infection by *S. aureus* (Cerca *et al.*, 2007).

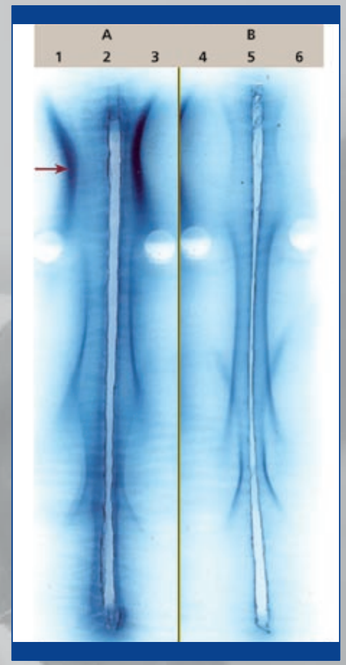

Figure 4. Analysis by immunoelectrophoresis in agarose gel for bacterial extracts from a strain of an *S. aureus* biofilm producer (A: wells 1 and 3) and non-producing strain (B: wells 4 and 6), using a polyclonal serum against whole bacteria (lines 2 and 5). The arrow shows the line of immunoprecipitation for the SAAC antigen, which is present only in strains characterised as exopolysaccharide producers in agar plates with Congo red.

Table 2. Determination of a slime producing phenotype (+/-), biofilm formation capacity in microplate (OD of the biofilm in the test) and production of SAAC (mg SAAC/mg total protein) in isolates of *S. aureus*. The correlation between the production of SAAC and the ability to form biofilm in microplate is significant (R = 0.882).

Isolate *S. aureus*	Slime producing phenotype	OD in the biofilm test (SD[1])	Production of SAAC (SD[1])
SA1H	+	1.444 (0.04)	54.0 (0.012)
SA2H	+	1.597 (0.02)	63.3 (0.015)
SA3H	+	0.385 (0.03)	20.8 (0.011)
SA4H	+	1.499 (0.04)	60.5 (0.012)
SA5H	+	1.521 (0.03)	27.6 (0.015)
SA6H	-	0.088 (0.01)	2.2 (0.011)
SA7H	+	1.030 (0.02)	26.5 (0.012)
SA8H	-	0.388 (0.06)	Nd[2]
SA9H	-	0.200 (0.02)	Nd[2]
SA10H	-	0.145 (0.01)	0.1 (0.010)
SA11H	-	0.130 (0.01)	Nd[2]
SA12H	-	0.235 (0.01)	Nd[2]
SA13H	+	0.632 (0.02)	6.9 (0.013)

[1]SD: standard deviation of the mean.
[2]Nd. : Not detected.

The main defence mechanism of the mammary gland against infections is antibody-mediated opsonization and subsequent phagocytosis by polymorphonuclear neutrophils. However, we must not exclude that the biofilm-specific antibodies also act in a direct manner in protection, binding to cells and preventing bacterial adherence to epithelium and intercellular interaction that leads to the formation of biofilm. In this regard, in an *in vitro* study, it was shown that antibodies to SAAC are capable of inhibiting biofilm formation without the presence of neutrophils (Figure 5 shows the results of a study conducted by the author).

One of the advantages of using PNAG or the SAAC component as vaccine antigens, unlike capsular antigens, is that no serotypes have been reported among isolates of *S. aureus*. Therefore, the antibodies induced by vaccination with these antigens confer cross-protection regardless of the capsular type of *S. aureus*.

STARTVAC® (HIPRA) is the first vaccine against bovine mastitis registered throughout the European Union via the EMEA (European Medicines Agency). This vaccine contains inactivated cells of a high biofilm-producing *S. aureus* strain with a high content of cell-associated SAAC. Moreover, the vaccine contains the inactivated *E. coli* J5 strain and a suitable adjuvant to boost the immune response.

In both preclinical testing and in clinical trials conducted during the development of the vaccine, immunisation with STARTVAC® induced high and long-lasting titres of anti-SAAC antibodies in blood and milk. Clinical trials carried out with STARTVAC® on six farms (198 cows vaccinated with STARTVAC® and 188 unvaccinated control cows) showed that vaccination significantly reduced the incidence of clinical and subclinical mastitis and severity of clinical symptoms of mastitis caused by *S. aureus*, coliforms and coagulase negative staphylococci (CNS) (reduction in somatic cell counts, decreased clinical signs and reduction of antibiotic treatment in infected animals). Additionally, vaccination with STARTVAC® increased the spontaneous cure rate in