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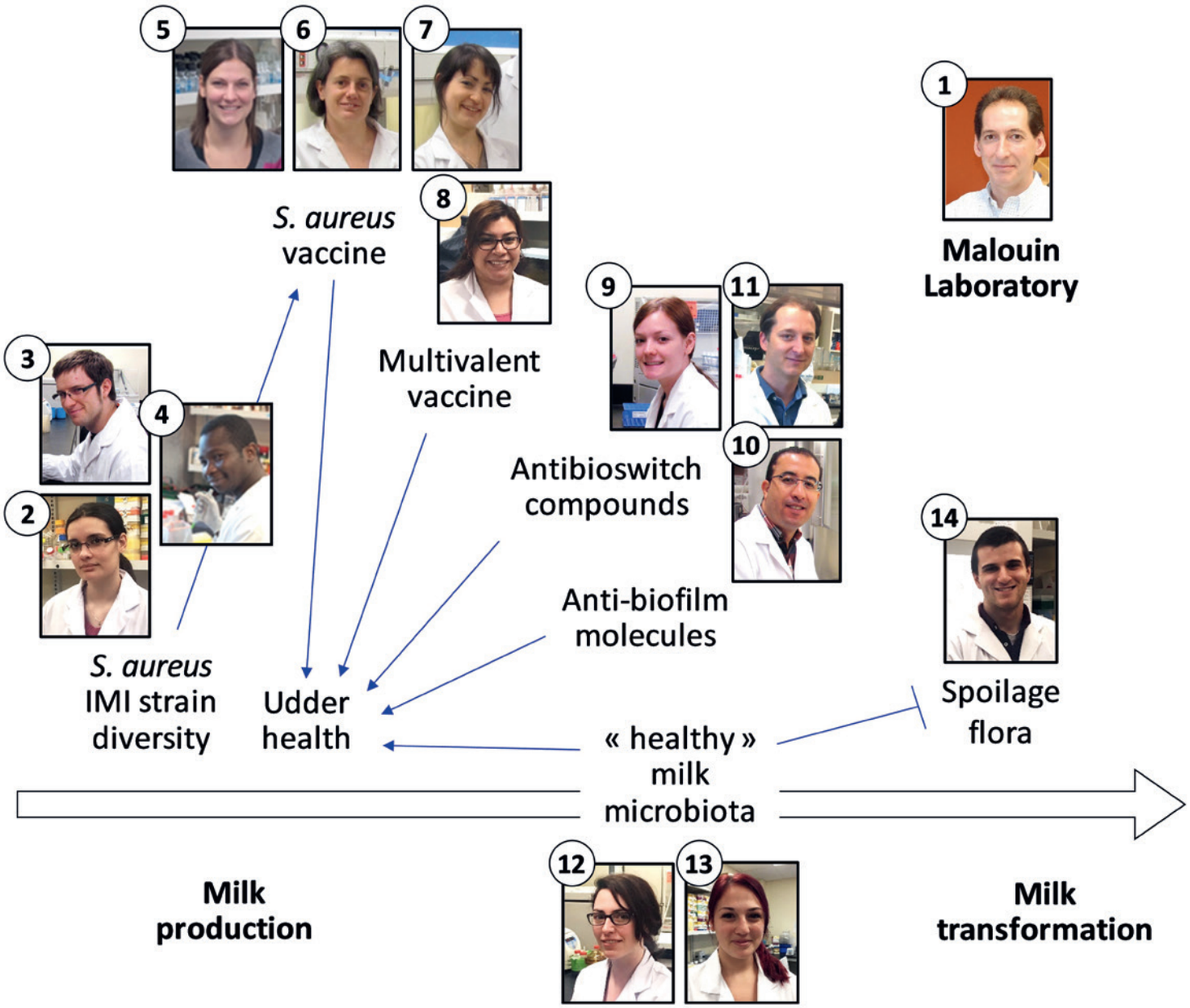
Novel control and treatment approaches for *Staphylococcus aureus* intramammary infections

Bovine mastitis is an inflammation of the udder, which most often results from an intramammary infection (IMI). *Staphylococcus aureus*, a bacterium frequently responsible for IMIs, is of particular interest since it is transmittable from cow to cow during milking and is therefore considered contagious. *S. aureus* IMIs often remain asymptomatic (subclinical mastitis), a situation that allows *S. aureus* to disseminate within the herds. Furthermore, staphylococcal IMI commonly lead to chronic mastitis disease due to the persistence of the original infective bacteria despite antibiotic treatment. Mastitis decreases milk production and milk quality. Due to the cost of treatment and the loss of milk, which must be discarded due to the possible presence of antibiotic residues, it is the most costly disease of dairy cows.

Novel methodologies to prevent or treat *S. aureus* IMI are needed. In Canada, the Bovine Mastitis and Milk Quality Research Network and also the Op+Lait strategic network for milk of optimal quality unite a critical mass of research scientists who work together to provide knowledge and solutions on *S. aureus* mastitis. Furthermore, Op+Lait supports research activities that not only focus on udder health, but also examine the entire path between milk production at the farm and milk transformation at the plant to ensure that practices at the farm result in a milk composition that is compatible to the milk transformation processes. Indeed, historically, there has been a gap between dairy production and transformation. On the one hand, milk is produced and is transferred off farm, and on the other hand, milk is received and used for transformation at the plant. Op+Lait now allows some coordination between research activities that are aimed at udder health at the farm and milk transformation at the plant. In this context, the laboratory of Professor François Malouin diligently works on multiple research projects aiming at improving udder health while sustaining optimal milk quality for transformation. His team of 14 graduate students, research professionals and trainees investigate the various aspects of *S. aureus* pathogenesis and virulence, i.e., the strategies and tools utilized by the bacterium to colonize and multiply in the mammalian host. Indeed, understanding how the bacterium bypasses the natural host defences against infection is key in the process of identifying the most needed tools used by the bacterium to cause disease. Consequently, such essential bacterial tools can also be viewed as the bacterial weaknesses and thus become ideal targets for antibiotic or vaccine development. Promoting a natural immunity against *S. aureus* by vaccination ultimately represents the best prevention measure. Besides, more and more research indicates that the milk microbiota – the microbial community residing naturally in milk – is a natural defence against the invasion of pathogens. Hence, Professor Malouin's research activities also investigate how vaccination can maintain a "healthy microbiota" useful for both protection against infection and subsequent milk transformation. Also, concerned with the "One-Health" concept, Professor Malouin specifically develops new classes of antibiotics for the control of mastitis, meaning that to reach optimal health for people, animals, and the environment, the food-animal production industry must be equipped with an efficient antibiotic arsenal that is used moderately and that is not impacting on the environment or the effectiveness of antibiotic classes currently used in human medicine. Some of Professor Malouin's specific research activities are described in more detail below.

Vaccine development

Until recently, despite years of research in veterinary and human



Professor F. Malouin's laboratory established an original research program that tackles S. aureus intramammary infections at multiple levels. The program includes S. aureus strains characterization and vaccine development. It also includes the investigation of new treatment options using new classes of antibiotics having non-traditional modes of action, such as antibiotics that target regulation of bacterial gene expression (anti-bioswitch compounds) and anti-biofilm molecules. All of this keeping in mind the milk industry continuum from milk production to milk transformation and making sure that new control measures for udder health preserve a healthy raw milk microbiota that can control the spoilage microflora. (1) Professor F. Malouin; (2) Graduate student É. Demontier; former students (3) S. Pichette-Jolette, (4) A. K. Veh and (5) M. Allard; (6) Research associate C. Ster; (7) Doctoral student J. Côté-Gravel and Master student (8) D. V. Bran-Barrera; former students (9) V. Belley and (10) A. Asli; (11) Research associate E. Brouillette; (12) Master student M. Cyrenne, and Trainees (13) J. Beaulieu and (14) A. Dubé-Duquette.

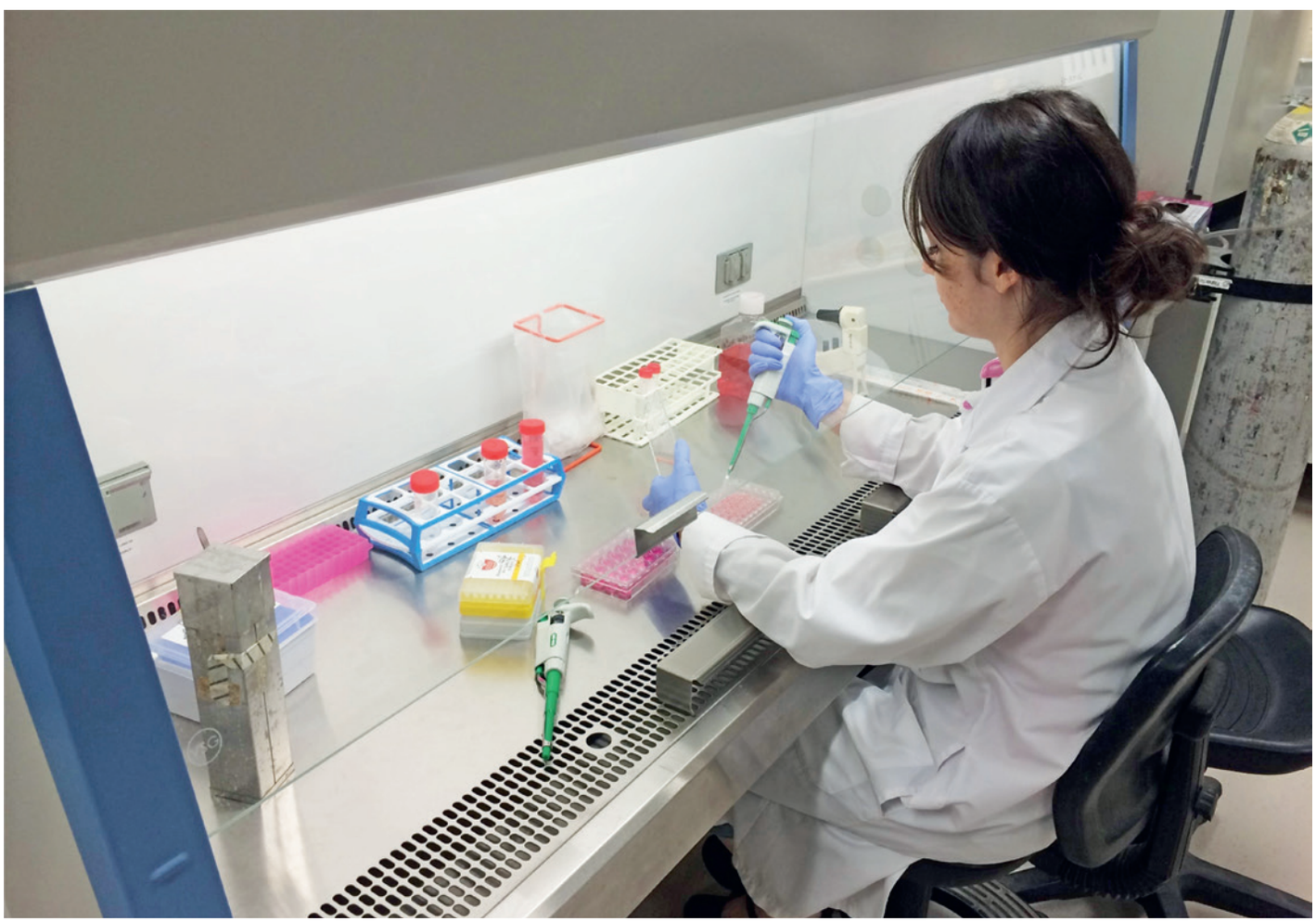
medicine, the scientific community has achieved very limited success in terms of vaccine development against *S. aureus* and there are several reasons for this. First, *S. aureus* is an outstanding pathogen that is well acquainted with its mammalian host and possesses tools that allow evasion from immune responses such as the production of superantigen toxins that can disturb the normal response of immune cells, as well as other toxins that can destroy them, such as hemolysins and leucocidins. In addition, *S. aureus* can coordinate its attack against the host by a phenomenon known as "quorum sensing", which allows the bacterial invader to wait until it is sufficiently populous to collectively unload

toxins. The well-known persistence of *S. aureus* infections is also attributable to its ability to colonize the host within a protective extracellular matrix called "biofilm", which provides further protection against the host immune response and antibiotic action (Ster *et al.*, 2017; Goetz *et al.*, 2016). Another persistent form of *S. aureus* is known as its small-colony variant (SCV), which develops slowly but has an increased capacity to produce biofilms and even hide within host cells to remain incognito. SCVs have been found in bovine IMIs as well as in chronic pulmonary infections in humans (Atalla *et al.*, 2008; Mitchell *et al.*, 2013).

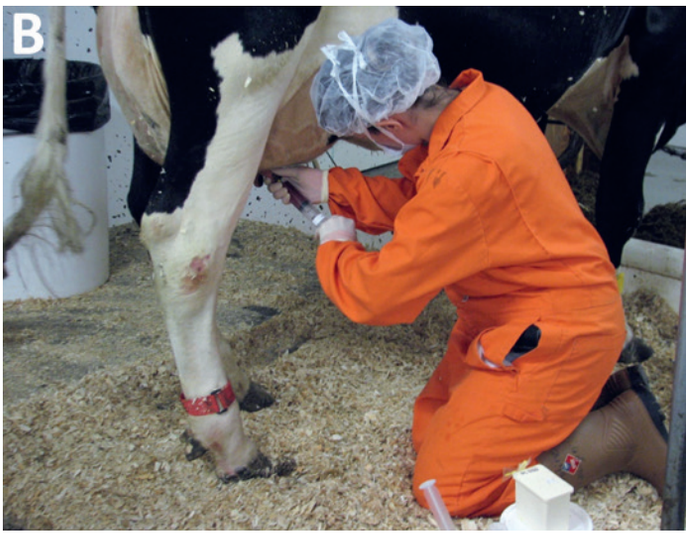
In terms of vaccine development, another obstacle to overcome is the diversity of *S. aureus* strains that can proficiently cause IMIs and mastitis. Indeed, an efficient vaccine must ideally allow the host to develop immunity (protection) against all *S. aureus* strains that can be encountered in the herd.

Hence, many of Professor Malouin's efforts are aimed at the characterization of *S. aureus* strains that cause mastitis. For example, the ability of strains to produce certain toxins or high amounts of biofilms may help to predict the duration of *S. aureus* IMI or the probability that a *S. aureus* IMI become persistent. Such knowledge would help herd management decisions and allow prudent use of antibiotics. Thus far, such research activities, in collaboration with Professors J.P. Roy and S. Dufour (Université de Montréal, QC, Canada) led to some associations between strain characteristics and persistence of IMIs (Veh *et al.*, 2015). These findings also further exemplified the existing diversity of *S. aureus* strains and stressed the need for a vaccine development strategy that accounts for this diversity.

With the advent of new molecular biology technologies allowing identification of genes (tools) that are expressed and used by the bacteria in specific environments, the Malouin laboratory could specifically determine which *S. aureus* tools were most needed by the bacterium during infection of the bovine mammary gland. Using a diversity of *S. aureus* strains and multiple cows, and by following and collecting milk and bacteria from sustained experimental IMIs (14 days), the laboratory



Several assay models are used to evaluate novel therapeutic approaches. Here, bovine mammary gland epithelial cells are cultured and used to study the interactions of bacteria with host cells. Promising therapies are subsequently evaluated in a mouse mastitis model and ultimately by means of experimental infections in cows.



The biosafety level 2 barn (A) at Agriculture and Agri-Food Canada (Sherbrooke, QC, Canada) can house 20 cows that can be used for experimental S. aureus IMIs (B) and evaluation of novel vaccines or therapies.

was able to identify bacterial tools that were most likely important for the survival and persistence of the bacteria in the mammary gland (Allard *et al.*, 2013). Selecting tools that were commonly expressed by all strains and that were needed for the entire infection process, Professor Malouin's team formulated a vaccine that contained such purified and inactivated bacterial tools to stimulate an immune response targeting the weaknesses of the invader. As a result, vaccinated cows could better respond to an experimental challenge (infection) with *S. aureus* (lower somatic cell or bacterial counts in milk), maintained milk production as well as milk quality compared to non-vaccinated cows. Bayer Animal Health (Leverkusen, Germany) and Université de Sherbrooke entered into a global license agreement to advance a novel bovine mastitis vaccine against *S. aureus* based on this innovation.

Currently, the Malouin laboratory builds on this vaccine technology to target other bacterial species capable of causing IMIs and mastitis, notably the streptococci. The team seeks to develop multivalent vaccines against multiple mastitis pathogens. Additional steps include validating that such multivalent vaccines protect against the target pathogens, preserve a "healthy" raw milk microbiota, and maintain high milk quality for subsequent transformation. Also, keeping in mind that one of the difficulties for the host immune response to counteract *S. aureus* is its ability to hide within host cells, the Malouin laboratory continues to explore new vaccine development strategies that specifically address this problem. Since SCVs can penetrate host cells and reside in the host without causing a destructive infection, Professor Malouin's team hypothesized that these SCV properties could be attenuated further by creating a laboratory-derived *S. aureus* strain that could be used as a live vaccine. Indeed, live-attenuated bacteria, which better represent natural infections, elicit broad and robust immune responses that can target both extra and intracellular forms of *S. aureus* (Côté-Gravel *et al.*, 2016).

Development of antibiotics and anti-biofilm molecules

As mentioned earlier, the Malouin laboratory was able to identify *S. aureus* genes (tools) needed (expressed) during infection of the mammary gland (Allard *et al.*, 2013). Of particular interest was a bacterial gene (*guaA*) for which expression was controlled by a molecular structure called a riboswitch. Such a riboswitch can prevent the expression of *guaA* when the bacteria do not need it. To exert its suppressive effect on the expression of the bacterial essential gene, the riboswitch must associate with a ligand (guanine in this case), helping the molecular switch to turn off the expression of *guaA*. Malouin and his colleague, Professor D. Lafontaine (Université de Sherbrooke), thus envisaged that mimicking the guanine ligand by a molecule that is not useful to the bacterium, but that can exert the same negative regulation, would prevent expression of the essential gene *guaA* and strongly impair the bacterial infection process (Mulhbacher *et al.*, 2010). Such a ligand mimic was named PC1 and was indeed able to shut down *guaA* expression to the point of killing the bacteria. Because this new antibiotic had a completely new mode of action compared to traditional antibiotics, PC1 was affectionately named an "anti-bioswitch". Besides, the relevance of such a discovery for treatment of *S. aureus* IMIs needed to be demonstrated. For such a demonstration, the Malouin laboratory developed relevant assay models in the mouse. The mouse mastitis model has been reviewed (Brouillette and Malouin, 2005) and used extensively by Malouin's group over the years for evaluation of various therapeutic treatments (Brouillette *et al.*, 2004; Malouin *et al.*, 2005; Karaolis *et al.*, 2007; Diarra *et al.*, 2013; Asli *et al.*, 2017). The therapeutic efficacy of PC1 was thus first demonstrated in the mouse mastitis model (Mulhbacher *et al.*, 2010), and the antibacterial activity of PC1 in experimental therapies for *S. aureus* IMIs in cows was subsequently demonstrated (Ster *et al.*, 2013) in collaboration with P. Lacasse (Agriculture and Agri-Food Canada, Sherbrooke, QC, Canada). Current research efforts in collaboration with Professor E. Marsault (Université de Sherbrooke, QC, Canada) include the design

of PC1 derivatives and product formulations that further improve the therapeutic efficacy of novel anti-bioswitch compounds.

Additional efforts to develop new therapeutic agents that could specifically be used for the treatment of bovine mastitis without impacting the effectiveness of antibiotic classes currently used in human medicine focus on yet another important *S. aureus* property, its biofilm formation. As mentioned above, the biofilm is defined as a community of bacteria embedded in a self-produced organic polymer matrix that offers protection against host immune defences and antibiotic action. Therapies that directly tackle biofilm represent interesting alternatives to traditional antibiotics.

The Malouin laboratory, in collaboration with Professors M. Jacques (Université de Montréal) and R. Brzezinski (Université de Sherbrooke) found that polycationic polymers such as chitosan (a natural molecule derived from the shell of crustaceans) could be used to treat biofilm-embedded *S. aureus*. In addition, chitosan in combination with low amounts of antibiotics demonstrated its potential to reduce antibiotic use in dairy farms. Indeed, the combination of chitosan with antibiotics improved the killing of bacteria within preformed biofilms and in the mouse model of mastitis (Asli *et al.*, 2017).

In conclusion, Professor Malouin's team established an original research program that tackles *S. aureus* bovine mastitis at multiple levels by either stimulating host resistance to infection through vaccination or by developing innovative classes of antibiotics having non-traditional modes of action such as the anti-bioswitch compounds or anti-biofilm molecules. All of this keeping in mind the milk industry continuum from the dairy farm to the milk transformation plant by making sure that new control measures for udder health maintain a healthy raw milk microbiota that is optimal for milk transformation. M²

References

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

Asli, A., E. Brouillette, C. Ster, M. G. Ghinet, R. Brzezinski, P. Lacasse, M. Jacques, F. Malouin. 2017. Antibiofilm and antibacterial effects of specific chitosan molecules on *Staphylococcus aureus* isolates associated with bovine mastitis. PLoS ONE 12(5): e0176988.

Atalla, H., C. Gyles, C. L. Jacob, H. Moisan, F. Malouin and B. Mallard. 2008. Characterization of a *Staphylococcus aureus* small colony variant (SCV) associated with persistent bovine mastitis. Food. Patho. Dis. 5:785-799.

Brouillette, E. and F. Malouin. 2005. The pathogenesis and control of *Staphylococcus aureus*-induced mastitis: Study models in the mouse. 2005. Microb. Infect. 7:560-568.

Brouillette, E., G. Grondin, C. Lefebvre, B.G. Talbot, F. Malouin. 2004. Mouse mastitis model of infection for antimicrobial compound efficacy studies against intracellular and extracellular forms of *Staphylococcus aureus*. Vet. Microbiol. 101:253-262.

Côté-Gravel, J., E. Brouillette, N. Obradovic, C. Ster, B.G. Talbot, F. Malouin. 2016. Characterization of a *vraG* mutant in a genetically stable *Staphylococcus aureus* small-colony variant and preliminary assessment for use as a live-attenuated vaccine against intramammary infections. PLoSONE 11(11): e0166621.

Diarra, M.S., G. Block, H. Rempel, B.D. Oomah, J. Harrison, J. McCallum, S. Boulanger, E. Brouillette, M. Gattuso, F. Malouin. 2013. In vitro and in vivo antibacterial activities of cranberry press cake extracts alone or in combination with β-lactams against *Staphylococcus aureus*. BMC Compl. Alter. Med. 13:90.

Goetz, C., S. Dufour, M. Archambault, F. Malouin, M. Jacques. 2016. Importance et contrôle de biofilms formés par les staphylocoques lors d'infections intra-mammaires chez la vache laitière: une revue bibliographique. Rev. Med. Vet. 167(7-8): 215-229.

Karaolis, D.K.R., T.K. Means, D. Yang, M. Takahashi, T. Yoshimura, E.B.G. Muraille, D. Philpott, J.T. Schroeder, M. Hyodo, Y. Hayakawa, B.G. Talbot, E. Brouillette, F. Malouin. 2007. Bacterial c-di-GMP is an immunostimulatory molecule. J. Immunol. 178:2171-2181.

Malouin, F., E. Brouillette, A. Martinez, B. J. Boyll, J. L. Toth, J. L. Gage, N. E. Allen. 2005. Identification of antimicrobial compounds active against intracellular *Staphylococcus aureus*. FEMS Immunol. Med. Microbiol. . 45:245-252.

Mitchell, G., K. Pepin Gaudreau, A. Fugère, E. Brouillette, E.H. Frost, A. M. Cantin, F. Malouin. 2013. SigB is a dominant regulator of virulence in *Staphylococcus aureus* small-colony variants. PLoS ONE 8(5): e65018.

Mulhbacher, J., E. Brouillette, M. Allard, L.-C. Fortier, F. Malouin, D. Lafontaine. 2010. Novel riboswitch ligand analogs as selective inhibitors of guanine-related metabolic pathways. PLoS Pathogens 6(4):e1000865.

Ster, C., M. Allard , S. Boulanger, M. Lamontagne Boulet, J. Mulhbacher, D.A. Lafontaine, É. Marsault, P. Lacasse, F. Malouin. 2013. Experimental treatment of *Staphylococcus aureus* bovine intramammary infection using a guanine riboswitch ligand analog. J. Dairy Science. 96 :1000-1008.

Ster, C., V. Lebeau, J. Leclerc, A. Fugère, K.A. Veh, J.-P. Roy, F. Malouin. 2017. In vitro antibiotic susceptibility and biofilm production of *Staphylococcus aureus* isolates recovered from bovine intramammary infections that persisted or not following extended therapies with cephapirin, pirlimycin or ceftiofur. Vet. Res. 48:56-65.

Veh, K.A., R. C. Klein, C. Ster, G. Keefe, P. Lacasse, D. Scholl, J.-P. Roy, D. Haine, S. Dufour, B. G. Talbot, A. O. B. Ribon, and F. Malouin. 2015. Genotypic and phenotypic characterization of *Staphylococcus aureus* causing persistent and non-persistent subclinical bovine intramammary-quarter infections during lactation or at dry-off. J Dairy Sci. 98(1):155-68.