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INVITATION PUBLIC DEFENSE

In vitro regulation of *Staphylococcus aureus* growth and virulence by bovine non-*aureus* staphylococci: potential implications for udder health

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21/12/2021

PROMOTERS

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Curriculum Vitae

Bruno Toledo Silva was born on 17 October 1990 in Paraguaçu Paulista, São Paulo, Brazil. After completing secondary school, he joined a five-year bachelor program in Veterinary Medicine at the Faculty of Veterinary Medicine of the State University of North Paraná (Brazil) and in December 2012 he graduated with distinction as a veterinarian. His undergraduate research examined the *in vitro* antimicrobial activity of *Punica granatum* L. extracts against *Staphylococcus aureus* isolated from bovine milk. Thereafter, he started a two-year Master program in "Veterinary Clinical Sciences" at the Faculty of Veterinary Medicine of the University of São Paulo (Brazil). During this period (2013-2015) he participated in several studies related to bovine colostrum, udder health, bovine respiratory diseases, and veterinary clinical immunology. While still actively involved in research, Bruno worked as a clinician in small animal practices from 2015 to 2017. Still in 2016, Prof. dr. Fernando Nogueira de Souza encouraged Bruno to apply for a Brazilian PhD scholarship jointly with Prof. dr. Sarne De Vliegher. At the end of 2017 the scholarship was granted and he enrolled for the doctoral program "Doctor of Veterinary Medicine" at the "Mastitis and Milk Quality Research Unit" of the Department of Internal Medicine, Reproduction, and Population Medicine at the Faculty of Veterinary Medicine of Ghent University. His research focused on the *in vitro* interactions between bovine non-*aureus* staphylococci and *Staphylococcus aureus* under the supervision of Prof. dr Sarne De Vliegher, Prof. dr. Fernando Nogueira de Souza and Prof. dr. Freddy Haesebrouck. His research experience resulted in several scientific publications along the years, including those from his current PhD work, as well as presentations at international conferences, including the National Mastitis Council - Annual Meeting in 2021, where he was selected for the Scholar program. Bruno has supported, helped, and guided several bachelor's and master's students into their research interests throughout his academic years at the Universities. He also followed several specialization courses and received the diploma of the "Doctoral Schools of Life Science and Medicine" from Ghent University.

How to attend?

The public defense will take place on Tuesday the 21st of December at 17:00 and can be followed via Microsoft Teams. The link will be shared with registered individuals. If you wish to attend the defense, please send an email to Bruno Toledo Silva before the 17th of December: Bruno.Silva@UGent.be.

Members of the Examination Committee

Prof. dr. L. Duchateau
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Prof. dr. K. Hermans
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Dr. J. Maris
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Summary of the thesis

Staphylococcus aureus remains one of the most common causative agents of bovine mastitis because of its pathogenicity, contagiousness, capability to persist in the mammary gland, colonization of skin, and poor cure rates when causing intramammary infections with the currently available therapies. Therefore, alternative treatments and preventive measures are desirable to control *S. aureus* mastitis, especially when caused by methicillin-resistant strains (MRSA). *In vitro* and *in vivo* studies have suggested beneficial traits of non-*aureus* staphylococci (NAS) originating from bovine milk and teat apices (TA) against major pathogens such as *S. aureus*. More recent studies have speculated that interactions between *S. aureus* and other colonizing staphylococci via the quorum-sensing (QS) system might affect the ability of *S. aureus* to produce virulence factors such as biofilm, and cause infection, suggesting that NAS could be used as potential sources of metabolites to prevent or target *S. aureus* infections. The general aim of this thesis was to study some elements of the regulation of *S. aureus* growth and virulence, including biofilm formation, by bovine NAS, gauging for new venues for preventive or therapeutic measures against bovine *S. aureus* mastitis.

The first study explored the ability of NAS isolates originating from bovine milk samples and TA (*S. chromogenes, S. epidermidis*, and *S. simulans*) to regulate the QS system of *S. aureus* and inhibit the growth of *S. aureus in vitro*, and whether those effects were associated. In co-culture with *S. aureus* it was observed that the 3 NAS species in general downregulated the expression of *rnaIII*, the effector molecule of the QS system. However, this downregulating effect was more pronounced in *S. chromogenes* and *S. simulans* isolates than in *S. epidermidis* isolates. The growth-inhibition of *S. aureus* by NAS resulted in a small underestimation of the downregulating effect of NAS on *rnaIII* expression of *S. aureus*. This study also assessed the capacity of NAS culture supernatants, NAS supernatants treated with proteinase K, and NAS cells themselves to regulate *S. aureus agr*-related virulence factors (*rnaIII, hla,* and *spa*). The culture supernatants of the NAS isolates as well as the proteinase K treated supernatants expressed greater regulatory activity over the *S. aureus* virulence genes than the washed NAS cells suspended in sterile water.

Biofilm formation is commonly found in *S. aureus* strains causing subclinical mastitis in dairy cows, a virulence factor that is also regulated by the *agr* QS system. In the second study the capacity of NAS to influence biofilm formation as well as dispersion of a pre-established biofilm of the strain *S. aureus* 8325-4 (*agr*-positive) was studied. Therefore, specific NAS traits such as species, origin (milk and TA), biofilm genotype (*bap, ica, aap*, or *agr*), biofilm phenotype (weak, moderate or strong producers), and *in vitro* growth inhibition capacity (data from the first study; total or partial growth-inhibition) were taken into account. The capacity of the *agr*-positive *S. aureus* strain to form biofilm was increased more in the presence of *S. chromogenes* than in the presence of *S. simulans* and *S. epidermidis* isolates and in the presence of NAS isolates that did not harbor biofilm related genes. Conversely, biofilm dispersion of this *S. aureus* strain was suppressed by NAS as a group, an effect that was more pronounced by isolates from TA. Next, we analyzed whether the capacity of the NAS isolates to repress the *agr* system of *S. aureus* (data from the first study; weak, moderate, or strong regulation) was related to biofilm formation and biofilm dispersion by an *agr*-positive and -negative *S. aureus* strain. In general, the effects observed on biofilm formation and dispersion of both *S. aureus* strains were not associated with the capacity of NAS to repress the *agr* system.

A semiquantitative method was used in our studies to determine the ability of NAS originating from milk and TA to inhibit the growth of *S. aureus*. Still, this method lacks information on the degree and variability of the growth-inhibitory effects of the different bovine NAS to draw final conclusions. This issueurged us to develop a novel, quantitative method (third study). With the new method, the growth inhibition of *S. aureus*, *Escherichia* (*E.*) *coli*, and *Streptococcus* (*S.*) *uberis* from milk samples of dairy cows by NAS isolates (*S. chromogenes* and *S. simulans*) originating from bovine milk samples was tested and the results were compared with those from the semiquantitative method. The *in vitro* growth inhibition of the major mastitis pathogens, including *E. coli*, was confirmed for all NAS, an effect that varied highly among NAS isolates, a finding that was not evident from the semiquantitative method. Finally, the new method was applied on another subset of NAS isolates originating from bovine milk samples and TA include in the first and second study, to check growth-inhibitory effect towards methicillin-sensitive (MSSA) and -resistant *S. aureus* (MRSA) isolates originating from milk. Specific NAS traits such as species, origin, and the ability to repress the *agr* system of *S. aureus* (data from the first study; none, weak, moderate, or strong regulation) as well as the antimicrobial resistance profile of *S. aureus* (MSSA versus MRSA) were considered in the analyses. Overall, *S. simulans*, NAS originating from TA, and NAS with strong or moderate capacity to repress the *agr* system required lower concentrations to inhibit *S. aureus* growth (including MRSA isolates). The inhibitory effects of TA isolates was more pronounced in *S. epidermidis* than in *S. simulans* and *S. chromogenes*. We conclude that the semiquantitative method can be used to screen a large group of NAS isolates for their inhibitory activity towards major pathogens, especially Gram-positive, whereas the novel method allows us to quantify the intra- and inter-species differences between NAS isolates.

Whilst our findings indicate that bovine-associated NAS are able to regulate the growth and virulence of *S. aureus in vitro*, more research is required to further elucidate the mechanisms behind these effects. Also, the role of NAS in the bovine mammary gland it is still unclear, more precisely their interaction with the host and with other udder associated/invading bacteria.