Understanding *Staphylococcus aureus* and Associated Inter-Bacterial Interactions to Develop Prophylactics and Therapeutics for Bovine Clinical Mastitis

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This thesis is dedicated to my family & my partner
for supporting me since day one.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABREVIATIONS

ABR	Antibiotic resistance
AMR	Antimicrobial resistance
bMEC	Bovine mammary epithelial cell
CC	Clonal complex
CDS	Coding sequences
CFU	Colony-forming unit
CM	Clinical mastitis
CoNS	Coagulase-negative staphylococci
CP	Capsular polysaccharide
GLM	General linear model
HGT	Horizontal gene transfer
IMI	Intramammary infection
LAB	Lactic acid bacteria
MAG	Metagenome-assembled genome
MGE	Mobile genetic element
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NAS	Non-*aureus* Staphylococci
NGS	Next-generation sequencing

NRPs	Non-ribosomally synthesized antimicrobial peptides
ORF	Open reading frame
OTU	Operational taxonomic unit
PVL	Panton-Valentine leukotoxin
QQ	Quorum-quenching
QS	Quorum-sensing
R-M	Restriction-modification
SAg	Staphylococcal superantigen
SCC	Somatic cell count
SCM	Subclinical clinical mastitis
SCV	Small-colony variant
SEC	Staphylococcal enterotoxin C
ST	Sequence type
TAS	Targeted amplicon sequencing
TSST	Toxic shock syndrome toxin
T7SS	Type VII protein secretion system
VRSA	Vancomycin-resistant *Staphylococcus aureus*
vWbp	von Willebrand factor-binding protein
WGS	Whole-genome sequencing

ABSTRACT

Bovine mastitis impacts on animal welfare and causes considerable economic damage to the Canadian dairy industry, resulting in an annual loss of $600 million. Among mastitis pathogens, *Staphylococcus aureus* is the most common etiological agent responsible for both clinical and subclinical mastitis. Since clinical mastitis results in a substantial amount of antibiotics being used in veterinarian medicine, strains of antimicrobial-resistant (AMR) *S. aureus* emerging from dairy farms is a public health concern. More recently, medically important antibiotics are being phased out of use in Canadian agriculture. Therefore, early diagnostic tests and novel prophylactic/therapeutic strategies for treating and preventing bovine mastitis need to be developed to maintain the sustainability of the dairy industry.

This thesis characterizes the genetic potential of bovine-associated *S. aureus* isolates and studies bacterial interactions between *S. aureus* and bovine commensal bacteria. We have examined *S. aureus* clinical mastitis using three different approaches: comparative genomics, inter-bacterial antagonism assays, and characterizing changes in the raw milk microbiome associated with infection.

Host-species specific and clonal complex exclusive genes were identified by comparing *S. aureus* genomes isolated from humans or dairy cows. Phylogenomic analysis revealed that the major clonal complexes (CCs) found in Canadian dairy cows were CC151 and CC97. Bovine intramammary infection (IMI)-associated *S. aureus* isolates showed distinct clades compared to human-originated *S. aureus* isolates. Host-specific genes and clonal lineage-specific genes were mostly located in mobile genetic elements (MGEs). The genome sequences of three suspicious isolates showed either acquisition or loss of the host-specific genes, providing evidence of recent host-jumping events, and a snapshot of genomic characteristics in early host spillover events. We

specifically investigated restriction-modification (R-M) genes in bovine IMI-associated *S. aureus*. The distribution of CC exclusive genes including virulence and AMR genes were closely correlated with the presence of R-M systems in *S. aureus*, suggesting that R-M systems may contribute to shaping *S. aureus* clonal diversification by providing a genetic barrier to horizontal gene transfer.

To screen bovine commensal bacteria for antagonistic activity against *S. aureus*, a new screening strategy more effective and efficient than traditional methods was developed. Two recombinant plasmids (pQS1 and pQS3) were engineered to carry either gfp_{bk} or *mCherry* controlled by a quorum-sensing promoter (*agr*P3) of *S. aureus*. The stability test showed that pQS1 and pQS3 remained highly stable in *S. aureus* strains (CC151 and CC97) for at least 24 hours in batch culture conditions without selection pressure. This high stability allows co-culturing of *S. aureus* with other bacteria and monitoring *S. aureus* growth and quorum sensing simultaneously. Using the pQS plasmid transformants of *S. aureus*, we found bacteria with *S. aureus* growth-inhibiting activity (n = 13) and quorum-quenching activity (n = 13), originally isolated from dairy milk. These newly identified antagonistic bacteria and their functional biomolecules are promising candidates for future development of probiotic drugs and prophylactics/therapeutics for bacterial infections including *S. aureus*.

For the first time, this study characterized changes in the microbial community present in raw milk before, during, and after *S. aureus*-induced clinical mastitis and compared microbial communities in milk from quarters that did and did not become infected. The microbiota of healthy quarters was distinguishable from quarters with *S. aureus* clinical mastitis up to two weeks before infection was detected via visual inspection. During *S. aureus* mastitis, healthy and sick quarters had significantly different alpha- and beta-diversity, while two weeks after clinical

mastitis, the milk microbiota in healthy and sick quarters returned to being similar. Microbial network analysis showed that 11 genera present in milk samples had a negative impact on the relative abundance of *Staphylococcus* genus, yet none of these genera showed a significant correlation with somatic cell count (SCC). Interestingly, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, and *Aerococcus urinaeequi* were each highly abundant in milk from quarters with low inflammation, suggesting their potential roles as a microbial barrier against mastitis pathogens colonizing the bovine mammary glands.

In summary, the knowledge generated in each of the research chapters aid in the development of new early diagnostic and prophylactic/therapeutic strategies for reducing *S. aureus* clinical mastitis in dairy cattle. Ultimately, this knowledge will help minimize both the antibiotic use in agriculture and the economic burden on Canadian dairy farms. Furthermore, understanding *S. aureus* would help to produce a holistic picture of transmission and prevention and be highly beneficial in a sustained One Health approach.

RÉSUMÉ

La mammite bovine cause des dommages économiques considérables à l'industrie laitière canadienne, entraînant une perte annuelle de 600 millions de dollars. Parmi les agents pathogènes de la mammite, *Staphylococcus aureus* est l'agent étiologique le plus courant responsable de la mammite clinique et subclinique. Étant donné que la mammite clinique entraîne l'utilisation de quantités importantes d'antibiotiques en médecine vétérinaire, les souches de S. *aureus* résistants aux agents antimicrobiens (RAM) émergeant des fermes laitières constituent un important problème de santé publique. Plus récemment, l'utilisation d'importants antibiotiques médicaux a été progressivement abandonnée dans l'agriculture canadienne. Par conséquent, des tests diagnostiques précoces et de nouvelles stratégies prophylactiques/thérapeutiques pour le traitement et la prévention de la mammite bovine doivent être développés pour maintenir la durabilité de l'industrie laitière.

Cette thèse caractérise le potentiel génétique de *S. aureus* associé aux bovins et étudie les interactions bactériennes entre *S. aureus* et les bactéries commensales bovines. Nous avons examiné la mammite clinique à *S. aureus* via trois approches différentes : la génomique comparative, les tests d'antagonisme interbactérien et le microbiome du lait.

Premièrement, les gènes spécifiques à l'hôte et ceux aux complexes clonaux exclusifs ont été étudiés en comparant les génomes isolés de *S. aureus* chez l'homme et chez les vaches laitières. L'analyse phylogénomique a révélé que les principaux complexes clonaux (CC) trouvés chez les vaches laitières canadiennes étaient CC151 et CC97. Les isolats de *S. aureus* associés à une infection intramammaire bovine (IIM) ont montré des clades distincts par rapport aux isolats de *S. aureus* d'origine humaine. Les gènes spécifiques à l'hôte et les gènes spécifiques à la lignée clonale étaient principalement situés dans des éléments génétiques mobiles (ÉGM). Les séquences du

génome de trois isolats suspects ont montré soit l'acquisition soit la perte des gènes spécifiques à l'hôte, fournissant la preuve d'un saut d'hôte récent et un aperçu des caractéristiques génomiques dans les premiers événements de débordement de l'hôte. Nous avons spécifiquement étudié les gènes de restriction-modification (R-M) chez *S. aureus* bovin associé à l'IIM. La distribution des gènes exclusifs CC, y compris la virulence et les gènes RAM, était étroitement corrélée à la présence de systèmes R-M chez *S. aureus*, ce qui suggère que les systèmes R-M peuvent contribuer à façonner la diversification clonale de S. aureus en fournissant une barrière génétique au transfert horizontal de gènes.

Deuxièmement, pour contrôler les bactéries commensales bovines ayant une activité antagoniste contre *S. aureus*, nous avons développé une stratégie de contrôle plus efficace et performante que les méthodes traditionnelles. Nous avons génétiquement modifié deux plasmides recombinants (pQS1 et pQS3) pour transporter gfp_{bk} ou *mCherry* contrôlé par un promoteur de détection de quorum (*agr*P3) de *S. aureus*. Le test de stabilité a montré que pQS1 et pQS3 restaient très stables dans les souches de *S. aureus* (CC151 et CC97) pendant au moins 24 heures dans des conditions de culture en série sans pression de sélection. Cette stabilité élevée permet la co-culture de *S. aureus* avec d'autres bactéries et la surveillance simultanée de la croissance et du quorum de *S. aureus*. En utilisant les transformants plasmidiques pQS de *S. aureus*, nous avons trouvé des bactéries ayant une activité inhibitrice de croissance de *S. aureus* (n = 13) et une activité d'extinction de quorum (n = 13), initialement isolées du lait de vache. Ces bactéries antagonistes nouvellement identifiées et leurs biomolécules fonctionnelles sont des candidats prometteurs pour le développement futur de médicaments probiotiques et de prophylactiques/thérapeutiques pour les infections bactériennes, y compris *S. aureus*.

Enfin, pour la première fois, cette étude a caractérisé les changements de la communauté microbienne présente dans le lait cru avant, pendant et après la mammite clinique induite par *S. aureus* et a comparé les communautés microbiennes dans le lait de quartiers infectés et non infectés. Le microbiote des quartiers sains se distinguait des quartiers atteints de mammite clinique à *S. aureus* jusqu'à deux semaines avant que l'infection ne soit détectée par inspection visuelle. Au cours de la mammite à *S. aureus*, les quartiers sains et malades présentaient une diversité alpha et bêta significativement différente, tandis que deux semaines après la mammite clinique, le microbiote du lait dans les quartiers sains et malades était similaire. L'analyse du réseau microbien a montré que 11 genres présents dans les échantillons de lait avaient un impact négatif sur l'abondance relative du genre *Staphylococcus*, mais aucun de ces genres n'a montré de corrélation significative avec le nombre de cellules somatiques. Fait intéressant, *Staphylococcus xylosus*, *Staphylococcus epidermidis* et *Aerococcus urinaeequi* étaient chacun très abondants dans le lait des quartiers à faible inflammation, ce qui suggère leur rôle potentiel dans une barrière microbienne contre les agents pathogènes de la mammite dans les glandes mammaires bovines.

En résumé, les connaissances générées dans chacun des chapitres de recherche aident au développement de nouvelles stratégies de diagnostiques précoces et prophylactiques/thérapeutiques pour réduire la mammite clinique à S. aureus chez les bovins laitiers. Ultimement, ces connaissances contribueront à réduire à la fois l'utilisation d'antibiotiques en agriculture et le fardeau économique pour les fermes laitières canadiennes. De plus, la comprehension de la pathophysiologie de *S. aureus* sera extremement avantageux dans le contexte d'une One Health approche.

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CONTRIBUTION OF AUTHORS

The work reported in all chapters of this study was conducted by Soyoun Park, Ph.D. candidate, in consultation with her supervisor Dr. Jennifer Ronholm. Soyoun Park conceived and designed the experiments, performed the experiments, collected and analyzed results, and drafted this thesis and the manuscripts for scientific presentation and publications. Dr. Jennifer Ronholm is the thesis supervisor who guided and supervised the Ph.D. candidate in performing the research as well as in reviewing and editing manuscripts. Dr. Simon Dufour also provided guidance and reviewed manuscripts for publication. All co-authors read and approved the final manuscripts that were published and submitted.

Bovine-associated *Staphylococcus aureus* isolates used in the comparative genomic study (Chapter 3) were obtained from the Mastitis Pathogen Culture Collection. The whole genomes of 27 *S. aureus* isolates were sequenced at McGill University by Soyoun Park, Dongyun Jung, and Jannia Ruffini. Dr. François Malouin and his team also provided additional raw sequences of bovine-associated *S. aureus* isolates. Forest Dussault and Bridget O'Brien contributed to WGS assembly and gene identification, respectively. Soyoun Park performed the formal analysis, investigation, data curation, and data visualization.

Soyoun Park conceptualized, designed, constructed, and evaluated the new screening strategy and the expression of reporter proteins in *S. aureus*. Roberto Maldonado contributed to *S. aureus* competent cell preparation, Hanny Maéva Gohou conducted plasmid stability test. Emily Kretschmann and Chloe Duvernay isolated commensal bacteria from raw milk and Adam Classen participated antagonistic assay.

Dr. Jennifer Ronholm and Dr. Simon Dufour conceptualized this study (Chapter 5). All raw milk samples were collected and provided by Dr. Simon Dufour and Daryna Kurban in Faculté de

Médecine Vétérinaire at Université de Montréal. Soyoun Park and Dongyun Jung organized, archived, and tracked milk samples. Soyoun Park performed bacterial DNA extraction, library preparations, TAS sequencing, data analysis, and data visualization. Shotgun metagenomic sequencing was performed at Genome Québec, Montreal, Québec, Canada. Ianina Altshuler provided original analysis code and contributed to microbial network analysis.

CHAPTER 1. INTRODUCTION

In this thesis, the genomic characteristics of *Staphylococcus aureus*, commensal bacteria with antagonistic activity against *S. aureus*, and raw milk microbiota have been examined through a comparative genomic study, competition assays, and a longitudinal cohort study, respectively. All knowledge produced in this study benefits the understanding of complex microbial interactions that occur during the onset of *S. aureus* clinical infections and disease susceptibility in dairy cattle. This knowledge will likely be translated into the development of new prophylactic and therapeutic strategies for *S. aureus*-associated bovine clinical mastitis. This first chapter consists of a general introduction describing the current problem along with the research rational, specific thesis objectives, and original contributions made towards the completion of this work.

1.1 General Introduction

Bovine mastitis imposes considerable economic losses in the Canadian dairy industry, including a loss of $66,178 CAD per 100 cows per year for the average dairy farmer (Aghamohammadi et al., 2018). Given the number of dairy cows in Canada estimated by the Canadian Dairy Commission, bovine mastitis costs the industry approximately $600 million every year. To reduce economic losses and increase successful treatment rates for bovine mastitis, early medical intervention and prevention strategies are needed. Among mastitis pathogens, *S. aureus* is known to be the most common etiological agent responsible for both clinical and subclinical mastitis (Levison et al., 2016). *S. aureus* is extremely difficult to remove from the mammary gland, once it is established, due to long-term persistence resulting from phenotypic dynamics, biofilm formation, capsule production, antibiotic resistance, and intracellular colonization (Garzoni & Kelley, 2009; Grunert et al., 2018; Saini, McClure, Leger, et al., 2012; Zapotoczna et al., 2016). Recently, an increasing

number of community-associated methicillin-resistant *S. aureus* (CA-MRSA) is believed to result from extensive use of antimicrobials in animal production and there has been an emergence of livestock-associated methicillin-resistant *S. aureus* (LA-MRSA). Although there are ongoing efforts to reduce antibiotic use on dairy farms, there is still a significant amount being used to treat and prevent bovine mastitis (Kromker & Leimbach, 2017). In response to this global phenomenon, World Health Organization (WHO) acted on antimicrobial-resistant bacteria in 2017, grouping *S. aureus* into high-priority pathogens for research. More recently, medically important antimicrobials are being phased out of usage in Canadian agriculture (Canada, 2015). Hence, early diagnostics and novel therapeutics are required to maintain economic prosperity of the dairy industry while preserving animal welfare standards and minimizing antibiotic use.

Whole-genome sequencing (WGS) of *S. aureus* can provide unprecedented amounts of information about characteristics of differentiate strains. *S. aureus* isolates from bovine mastitis are well-equipped with host adaptation and virulence factors involved in colonization, stress response, and disease progression in the mammary gland. Once these features are identified, additional analyses and experimental work can be conducted to elucidate their roles in bovine mastitis. Host-specific virulence factors are crucial to identify because they play a critical role in disease manifestation and severity compared to other determinants. Furthermore, comparative genomics and pan-genome analysis can be conducted using WGS data to elucidate evolutionary relationships and pathogenesis of *S. aureus* in bovine mastitis. Therefore, whole-genome and pan-genome studies in *S. aureus* isolates derived from a specific disease and animal host are needed to understand the pathogenic potential in a particular host species. Ultimately, knowledge derived from WGS can lead to new strategies for pathogen reduction and successful medical treatment.

To reduce the use of antibiotics, *S. aureus* antagonistic bacteria such as lactic acid bacteria (LAB), a group of bacteria capable of producing antimicrobial biomolecules, have been actively

studied as alternatives to antibiotics. The effect of LAB on bovine mastitis is comparable to those of commercial antibiotics in field studies (Klostermann et al., 2008; Mignacca et al., 2017). Moreover, interference of the *agr* system, a global regulator responsible for *S. aureus* quorum-sensing (QS), has also been demonstrated to reduce *S. aureus* pathogenesis (Gov et al., 2001). Therefore, commensal bacteria with antagonistic and/or quorum-quenching (QQ) activity against *S. aureus* may be promising prophylactics to control *S. aureus* bovine mastitis.

Commensal and symbiotic bacteria protect their hosts from infection by competing with invading pathogens and enhancing the host immune system through the production of crucial signals such as short-chain fatty acids (Kamada et al., 2013; Rooks & Garrett, 2016). Previous work has demonstrated that the complex microbiota in the bovine udder is associated with udder health and milk quality (Quigley et al., 2013). Distinct differences in microbial diversity between healthy and mastitic milk clearly indicate a relationship between the microbiome and the health status of the cow (Oikonomou et al., 2012). In addition, mastitis infections can alter the composition of a healthy microbiome, and this alteration can contribute to further mastitis development (Falentin et al., 2016). Although such evidence emphasizes the importance of the microbiome, a great deal of research regarding the milk microbiome and bovine mastitis has solely focused on the microbiome at the time of mastitis or after mastitis in artificially infected animals. Therefore, a longitudinal study focusing on the udder microbiome composition throughout a healthy state in addition to during and after mastitis, is critical to characterizing the dynamics of milk microbial communities. Specifically, understanding microbial dynamics prior to mastitis must be investigated to elucidate the relationship between microbiome and mastitis susceptibility – and possibly to identify udder symbionts capable of preventing colonization by mastitis pathogens.

1.2 Rationale

Bovine mastitis in dairy cattle results in huge economic losses for the Canadian Dairy industry and decreases the welfare of dairy cattle. In response, dairy producers treat cattle with both therapeutic and prophylactic intramammary antibiotics on a routine basis. However, agricultural use of antibiotics is an important driver of rapidly increasing AMR human infections, and the use of antibiotics in agriculture are rapidly becoming controversial and unsustainable. Novel methods for treating and preventing bovine mastitis, that do not rely on the continued use of antibiotics, are urgently required.

1.3 Research Hypothesis

The bovine udder microbiome houses a rich mix of symbiotic and commensal bacteria – some of which have antagonistic relationships with important mastitis pathogens and these natural antagonistic interactions can be exploited in the development of novel mastitis prophylactics/therapeutics.

1.4 Research Objectives

The ultimate objective of this study is to produce knowledge for the development of novel, natural, therapeutic technologies to control bovine mastitis and the dissemination of *S. aureus,* including antibiotic-resistant strains. To address our research hypothesis and meet the goal of this thesis, we formulated three discrete objectives associated with *S. aureus* genomes, antagonistic interactions between commensal bacteria and *S. aureus*, and changes in microbiome composition.

1. Perform a comparative genomic analysis to understand the evolutionary relationships between bovine mammary pathogenic *S. aureus* and human pathogenic *S. aureus*.

2. Develop a new strategy using a highly stable plasmid and fluorescent gene(s) to screen bacterial isolates for antagonistic activity toward *S. aureus*
3. Characterize dynamics of the mammary gland microbial community before *S. aureus* mastitis infection, during the infection, and after infection resolution in dairy cows.

1.5 Contributions

In fulfilment of the thesis-specific objectives, a number of contributions were made, including published papers, presentations, and awards. These accomplishments are listed below.

Original research articles included in this thesis

1. (*Submitted Mar 9, 2022*) **Park, S.**, Jung, D., Altshuder, I., Kruban, D., Dufour, S., Ronholm, J. The Effect of Milk Microbiome Composition on Host Susceptibility to *Staphylococcus aureus* Clinical Mastitis in Dairy Cattle. *Animal Microbiome*.
2. **Park, S.**, Jung, D., O'Brien, B., Ruffini, J., Dussault, F., Dubé-Duquette, A., Demontier, É., Lucier, J.F., Malouin, F., Dufour, S., Ronholm, J. (2021) Comparative Genomic Analysis of *Staphylococcus aureus* Isolates Associated with Either Bovine Intramammary Infections or Human Infections Demonstrates the Importance of Restriction-Modification Systems in Host Adaptation. *Microbial Genomics*. DOI: 10.1099/mgen.0.000779
3. **Park, S.**, Classen, A., Gohou, H., Maldonado, R., Kretschmann, E., Duvernay, C., Kim, G.J., Ronholm, J. (2021) A New, Reliable, and High-Throughput Strategy to Screen Bacteria for Antagonistic Activity against *Staphylococcus aureus*. *BMC Microbiology*. DOI: 10.1186/s12866-021-02265-4
4. **Park, S.** & Ronholm, J. (2021) *Staphylococcus aureus* in Agriculture: Lessons in Evolution from a Multispecies Pathogen. *Clinical Microbiology Reviews*. DOI: 10.1128/CMR.00182-20

5. **Park, S.**, Jung, D., Dufour, S., Ronholm, J. (2020) Draft Genome Sequences of 27 *Staphylococcus aureus* Strains and 3 *Staphylococcus* Species Strains Isolated from Bovine Intramammary Infections. *Microbiology Resource Announcements*. DOI: 10.1128/MRA.00300-20

Original research articles not included in this thesis

6. Demontier, E., Dubé-Duquette, A., Brouillette, E., Larose, A., Ster, C., Lucier, J.F., Rodrigue, S., **Park, S.**, Jung, D., Ruffini, J., Ronholm, J., Dufour, S., Roy, J.P., Ramanathan, S., Malouin, F. (2021) Relative virulence of *Staphylococcus aureus* bovine mastitis strains representing the main Canadian spa types and Clonal Complexes as determined using in vitro and in vivo mastitis models. *Journal of Dairy Science*. DOI: 10.3168/jds.2020-19904
7. Jung, D., **Park, S.**, Ruffini, J., Dussault, F., Dufour, S., Ronholm, J. (2021) Comparative genomic analysis of *Escherichia coli* isolates from cases of bovine clinical mastitis identifies nine specific pathotype marker genes. *Microbial Genomics*. DOI: 10.1099/mgen.0.000597
8. Jung, D., **Park, S.**, Ruffini, J., Dufour, S., Ronholm, J. (2021) Draft Genome Sequences of 113 Mammary Pathogenic *Escherichia coli* strains Isolated from Intramammary Infections. *Microbiology Resource Announcements*. DOI: 10.1128/MRA.01464-20
9. Yu, Z., Jung, D., **Park, S.**, Hu, Y., Huang, K., Rasco, B., Wang, S., Ronholm, J., Lu, X., Chen, J. (2020) Smart Traceability for Food Safety. *Critical Reviews in Food Science & Nutrition*. DOI: 10.1080/10408398.2020.1830262

Oral presentations

10. **Park, S**., Jung, D., Kurban, D., Dufour, S., Ronholm, J. "Longitudinal Study on the Milk Microbiota of Dairy Cows Diagnosed with *Staphylococcus aureus* Clinical Mastitis" 61st

National Mastitis Council Annual Meeting. February 2, 2022. Virtual Meeting

Poster presentations

11. **Park, S**., Jung, D., Kurban, D., O'Brien, B., Ruffini, J., Altshuder, I., Classen, A., Gohou, H., Maldonado, R., Kretschmann, E., Duvernay, C., Dussault, F., Dubé-Duquette, A., Demontier, É., Lucier, J.F., Malouin, Kim, GJ., Dufour, S., Ronholm, J. "Understanding *Staphylococcus aureus* and Associated Inter-Bacterial Interactions to Develop Prophylactics and Therapeutics for Bovine Clinical Mastitis" 71th Annual Conference of the Canadian Society of Microbiologists. Guelph, ON. June 26-29, 2022.
12. **Park, S**., Jung, D., Kurban, D., Dufour, S., Ronholm, J. "A longitudinal cohort study of milk microbiota associated with *Staphylococcus aureus* bovine clinical mastitis" 70th Annual Conference of the Canadian Society of Microbiologists. Virtual Meeting. June 16, 2021.
13. **Park, S.**, Jung, D., O'Brien, B., Ruffini, J., Dussault, F., Dubé-Duquette, A., Demontier, É., Lucier, J.F., Malouin, F., Dufour, S., Ronholm. J. "L'antagonisme intra-espèce de *Staphylococcus aureus* isolé d'une infection intramammaire chez les vaches laitiéres" OP+lait Annual Meeting. Virtual Meeting. October 28, 2020.
14. **Park, S.**, Jung, D., O'Brien, B., Ruffini, J., Dussault, F., Dubé-Duquette, A., Demontier, É., Lucier, J.F., Malouin, F., Dufour, S., Ronholm. J. "Comparative genomic study of *Staphylococcus arueus*: Lessons from its gene contents" Mastitis Network 2020 Annual Scientific Meeting. Virtual Meeting. October 6, 2020.
15. Jung, D., **Park, S.**, Ruffini, J., Dussault, F., Dufour, S., Ronholm, J. "Comparative genomic analysis of mammary pathogenic *E. coli* and bovine commensal *E. coli*" Mastitis Network 2020 Annual Scientific Meeting. Virtual Meeting. October 6, 2020.
16. **Park, S.** & Ronholm, J. "The milk storage effect on the milk microbiome and an optimized

bacteria DNA extraction protocol: pave the way for a longitudinal study to control *Staphylococcus aureus* mastitis" 69th Annual Conference of the Canadian Society of Microbiologists, Sherbrooke, ON. June 2019

17. **Park, S.** & Ronholm, J. "The milk storage effect on the milk microbiome and an optimized bacterial DNA extraction protocol: pave the way for a longitudinal study to control *Staphylococcus aureus* mastitis" Mastitis Network, 2019 Annual Scientific Meeting, Montreal, QC, May 2019

Awards and accomplishments

During my Ph.D., I was awarded a research grant and personal funding:

1. Fonds de recherche – Nature et technologies (FRQNT) (2021-2022): $21,000/year
2. Schulich Fellowship, McGill University (2020-2021): $25,000
3. OP$^+$lait Complements, OP$^+$lait (2019-2021): $20,000
4. Cross-disciplinary Exchanges Awards, OP$^+$lait (2019): $5,000
5. CREATE in Milk Quality Program Scholarship (2018-2022): $37,500
6. The McGill Sustainability Systems Initiative (MSSI) Ideas Fund (2019): $7,000

I was also fortunate enough to receive a series of awards from conferences and competitions:

1. The FRQNT Relève étoile Louis-Berlinguet Mars (2022): $1,000
2. Research Communication Award, McGill Graduate Research Symposium (2021): $583
3. Finalist, 3MT competition, McGill University (2021)
4. Poster Presentation Award, Mastitis Network 2020 Annual Scientific Meeting (2020): $250

1.6 Thesis Chapter Preface

This thesis is composed of 7 chapters. The first chapter consists of a general introduction, research hypothesis, and specific objectives of this project. Chapter 2 consists of a comprehensive literature review of relevant topics necessary to understand this research and includes a published literature review article. Chapters 3 through 5 encompass the original research contributions that were published or submitted to peer-reviewed scientific journals. Each chapter contains an abstract, introduction, materials and methods, results, and discussion and conclusion section. Finally, Chapter 6 is a general discussion and Chapter 7 is overall summary, conclusion, and future perspectives.

CHAPTER 2. BACKGROUND & LITERATURE REVIEW

2.1 Bovine Mastitis and *Staphylococcus aureus*

This Chapter 2.1 (specifically 2.1.2-2.14) includes some parts of a published literature review (Park & Ronholm, 2021).

2.1.1 Bovine mastitis

Bovine mastitis is the inflammation of bovine udder, usually caused by a bacterial infection, that results in considerable annual losses, estimated at $35 billion globally, for the dairy industry (Wellenberg et al., 2002). Inflammation of the cow udder changes milk composition and quality, reduces milk production and animal welfare, and increases discarded milk and culling (Ruegg, 2017). To quantify the level of inflammation in mammary glands, somatic cell count (SCC) is widely used as a reliable indicator of udder health. Although the SCC threshold for defining mastitis is not uniformly adopted, approximately 200,000 to 250,000 cells/mL is commonly used for discriminating healthy and abnormal milk (Ruegg, 2017). Subclinical mastitis (SCM) infections cause no visible changes to the milk or the appearance of the udder, but SCC will be >250,000 cells/mL in milk taken from udders with SCM. Clinical mastitis (CM) infections are accompanied by mastitis symptoms including and off-coloring of the milk, swelling and redness of the quarter, and sometimes fever. The severity of CM is often defined using the scoring system as follows: mild (abnormal milk), moderate (abnormal milk accompanied by swelling or redness of the udder), and severe (exhibiting systemic signs of illness in the cow, such as fever) (Pinzon-Sanchez & Ruegg, 2011).

There are several causes of mastitis including physical injury, viral infection, and fungal infection, but bacterial infections are the most common cause of bovine mastitis (Cheng & Han,

2020). Bacteria that are known to cause mastitis include a broad range of environmental and contagious pathogens, and encompass more than 137 species (Watts, 1988). Environmental pathogens such as *Escherichia coli* are mainly derived in the habitat of dairy cows, while contagious pathogens such as *Staphylococcus aureus* are spread from cow to cow via direct contact or through contaminated milking machines. In some cases, mostly during Gram-negative bacterial infections, intramammary infections (IMIs) recover spontaneously, while in other cases, as is common with *S. aureus* infections, culling or drug therapy is required if the cow does not recover (Ruegg, 2017).

The most common treatment method for bovine mastitis is intramammary infusion of antibiotics and parenteral drug administration (Kalmus et al., 2014; Sandgren et al., 2008). Despite of the emergence of antibiotic-resistant mastitis pathogens, treatment of active mastitis infection is highly dependent on antibiotics (Cheng & Han, 2020). On Canadian dairy farms, beta-lactam antibiotics and combinations are commonly used for dry cow therapy and clinical mastitis treatment (Saini, McClure, Léger, et al., 2012). However, among these antimicrobials, third generation cephalosporins are critically important in human medicine (Scott et al., 2019). In Canada, when treating mastitis with antibiotics, the milk produced during the treatment period and for a certain amount of time after the treatment (withdrawal period) is not fit for human consumption and needs to be discarded, and this creates economic losses for dairy farmers (Aghamohammadi et al., 2018). More recently, Canada has implemented on-farm AMR surveillance systems to describe the development and implementation of the Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR) in response to the growing concerns associated with AMR in food-producing animals (Fonseca et al., 2021).

To reduce the economic burden and emergence of antibiotic resistance on dairy farms, mastitis control programs were developed to prevent disease through good management. The National Mastitis Council developed a mastitis control program known as “The Five-Point Mastitis

Control Plan" that includes teat disinfection, treating clinical cases, dry-cow therapy, controlled culling, and milking machine check (Hillerton & Booth, 2018). Although mastitis control programs and modern dairy farm practices are relatively effective against contagious pathogens, environmental pathogens are still problematic (Klaas & Zadoks, 2018). Beside mastitis control programs, vaccines (e.g., Lysigin® and Startvac®) are commercially available for dairy cows, though none sufficiently protect against mastitis-inducing pathogens (El-Sayed & Kamel, 2021). Thus, researchers and dairy producers are obligated are interested in developing new strategies to minimize and technologies to prevent the occurrence of bovine mastitis.

2.1.2 Staphylococcus aureus bovine mastitis

S. aureus is a formidable bacterial pathogen responsible for infections in humans and various species of wild, companion, and agricultural animals. Bovine IMIs caused by *S. aureus* can vary greatly in terms of severity, transmissibility, and persistence; thus, *S. aureus* mastitis is somewhat unique compared to mastitis caused by other pathogens. *S. aureus* can cause both acute and chronic IMIs. The acute form of the disease is usually severe clinical mastitis, and the chronic form of *S. aureus* mastitis is usually subclinical (Piepers et al., 2009; Zhao & Lacasse, 2008). Chronic *S. aureus* IMIs are one of the most common reasons for premature culling in dairy herds (Piepers et al., 2009; Taponen et al., 2017). Both chronic and acute IMIs caused by *S. aureus* can result in damage to the mammary epithelium due to a release of metabolites by the pathogen and the release of lysosomal enzymes and oxidative products by phagocytes in the immune response (Kalińska et al., 2018; Zhao & Lacasse, 2008). In chronic cases of IMIs, *S. aureus* can evade clearing by both antibiotics and by the bovine immune system by forming biofilms and surviving intracellularly in both non-professional and professional phagocytes (Almeida et al., 1996; Bayles et al., 1998; Hebert et al., 2000).

S. aureus can cause contagious mastitis, where a single lineage quickly spreads throughout a dairy herd, as well as sporadic noncontagious mastitis, which usually does not spread beyond a single cow (Leuenberger et al., 2019). In addition to sequence type (ST), *S. aureus* can also be grouped at the subspecies level by genotyping. Studies conducted in European countries have found that genotype B (GTB) is highly contagious, while other genotypes cause sporadic noncontagious mastitis (Cosandey et al., 2016; Fournier et al., 2008; Leuenberger et al., 2019; Sartori et al., 2018). In dairy herds, a few dominant clones of contagious *S. aureus* tend to dominate, indicating transmissibility within the herd as well as the preference for phenotypic traits (Graber et al., 2009; Grunert et al., 2018; Haveri et al., 2007).

2.1.3 Host adaptation and virulence factors in Staphylococcus aureus

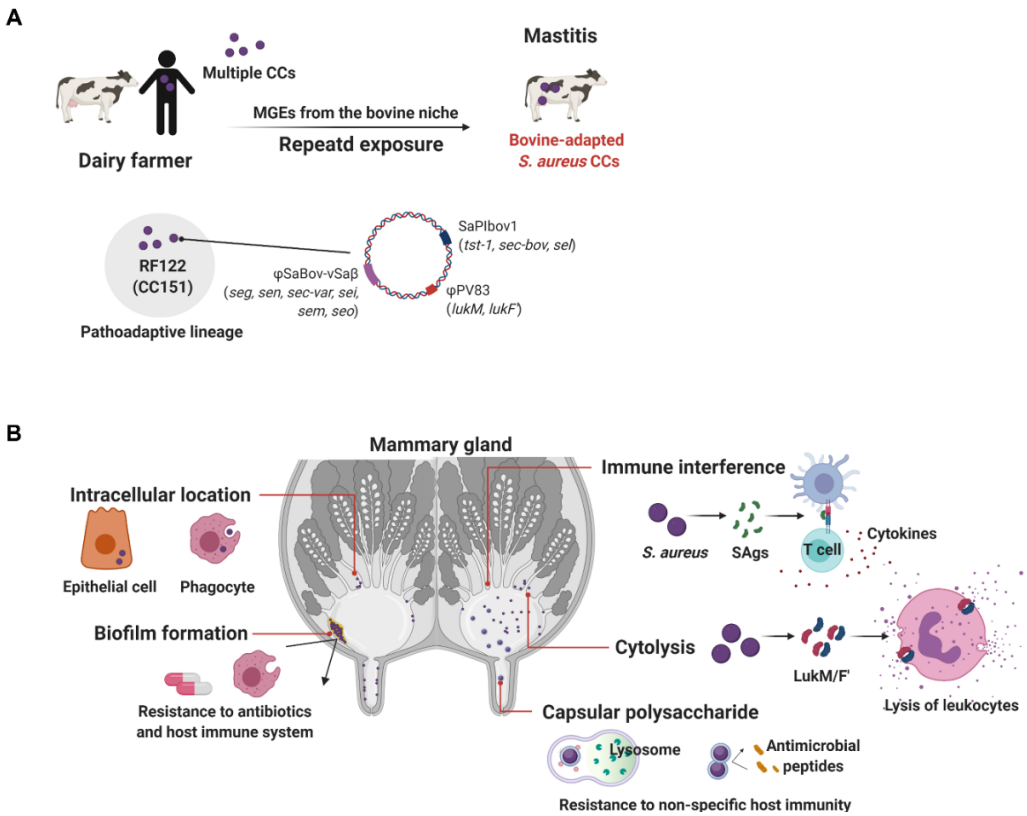

Figure 2.1 Adaptation and pathogenesis of *Staphylococcus aureus* pathogenesis in bovine. (A) Several bovine-adapted *S. aureus* clonal complexes (CCs) appear to have originated from humans

and became adapted to bovine hosts through multiple spillover events where various mobile genetic elements (MGEs) were acquired. (B) Various strategies of bovine-adapted *S. aureus* led to inflammation of cow udder and long-term persistence in mammary glands. This figure was created with BioRender.com (adapted from Park et al., 2021).

Bovine-associated *S. aureus* has been studied more than other livestock-associated *S. aureus* (Sakwinska et al., 2011). Molecular dating has estimated that human-to-bovine transmission of *S. aureus* happened post domestication, as early as 5,500 years ago and that human-to-bovine spillover has occurred on at least five independent occasions (Weinert et al., 2012; Zeder, 2009; Zeder, 2008). Globally dispersed bovine-adapted *S. aureus* lineages predominately belong to CCs: CC8, CC97, CC133, and CC151 (Boss et al., 2016; Matuszewska et al., 2020; Smith et al., 2014; Weinert et al., 2012). A notable trend in ruminant-adapted lineages is the loss of genes specifically involved in human infections, which likely increases their fitness in ruminants. For example, RF122 (CC151) is known as a highly virulent mastitis strain which has lost the functions of major human-associated surface proteins such as protein A and clumping factor A due to premature stop codons (Herron-Olson et al., 2007). Bovine-specific MGEs are also found in RF122 (Herron-Olson et al., 2007) (Figure 2.1).

Biofilm-producing *S. aureus* strains tend to be more successful in colonization and long-term persistence in the mammary gland (Cucarella et al., 2004; Hamed, 2018). Bacterial biofilms are more resistant to both antibiotic treatment and host immune defense mechanisms than their sessile counterparts (Melchior et al., 2006), and the ability to form intramammary biofilms may contribute to the persistence of *S. aureus* infections. In persistent *S. aureus* colonization in the mammary gland, intra-host selection of SigB-deficient pathotypes, which have an increased ability to form biofilms, appears to occur (Marbach et al., 2019); although, there are exceptions, including *S. aureus* NCTC8325 strains, which are SigB deficient yet still form very poor biofilms (Ma et al., 2017).

Intracellular survival of *S. aureus* in non-professional and professional phagocytes plays an important role in host adaptation in terms of persistence in the mammary gland. *S. aureus* can act as an intracellular pathogen in a variety of eukaryotic cells, including bovine mammary epithelial cells (Almeida et al., 1996). Viable intracellular *S. aureus* cells have also been isolated from alveolar cells and macrophages derived from the milk of chronically mastitic cows (Hebert et al., 2000). The ability of chronic *S. aureus* to act as an intracellular pathogen also explains how this pathogen can persist for long periods of time without causing apparent inflammation in both humans and animals (Garzoni & Kelley, 2009). The ability of *S. aureus* to express capsular polysaccharide (CP) appears to be inversely related to its ability to invade host cells since the absence of capsule appears to assist with the adherence and invasion of eukaryotic cells (Bardiau et al., 2016; Buzzola et al., 2007; Pohlmann-Dietze et al., 2000; Tuchscherr et al., 2005). Capsule-negative *S. aureus* strains are more prone to inducing chronic infection than capsule-positive strains (Tuchscherr et al., 2005). The expression of CP is regulated in a density-dependent manner via QS, indicating it has an important role in the progress of infections (Luong et al., 2002).

Panton-Valentine leukotoxin (PVL) is a bicomponent toxin that has a specific activity in leukocytes and is expressed by certain *S. aureus* strains (Turner & Sriskandan, 2015; Yamada et al., 2005). Functional leukotoxins require two components: a S subunit to bind a specific receptor and an F component, which can undergo oligomerization resulting in the formation of pores in the host cell, eventually leading to cellular death (Yamashita et al., 2014). One member of the leukotoxin family known as LukM and LukF-PV subunits (LukMF') is exclusively harbored by *S. aureus* of ruminant origin and associated with severe inflammation during bovine mastitis (Vrieling et al., 2015; Younis et al., 2005). Severe inflammation is a result of LukMF' having cytotoxicity against neutrophils, resulting in a strong inflammatory reaction and therefore clinical mastitis (Peton et al., 2014). LukM exhibits high specificity toward the specific chemokine receptor CCR1 on

bovine neutrophils (Fromageau et al., 2010; Vrieling et al., 2015; Zou et al., 2000). The genes encoding LukM and LukF-PV (*lukM-lukF-PV*) are encoded on a temperate phage φPV83 and can be transmitted to other *S. aureus* strains horizontally (Yamada et al., 2005). The level of production of LukMF' by *S. aureus in vivo* directly correlates with the severity of clinical mastitis (Hoekstra et al., 2018). Strains of *S. aureus* that express high levels of the LukMF' complex have a point mutation in the start codon of a repressor protein (*rot*), which is responsible for repressing the transcription of various toxins (Hoekstra et al., 2018). Strains that overexpress LukMF' are associated with the same genetic lineage (ST479) (Hoekstra et al., 2018).

Staphylococcal superantigens (SAgs) represent a family of immunostimulatory exotoxins secreted by staphylococcal species, including *S. aureus*, potentially leading to an uncontrollable cytokine storm (Tuffs et al., 2018). SAgs appear to play an important role in bovine mastitis, and most bovine *S. aureus* isolates contain five or more genes encoding SAgs, although the exact role of SAgs in the pathogenesis of mastitis is still unclear (Wilson et al., 2018). SAgs interfere with host immune response and thus may be important in persistent infections (Ferens & Bohach, 2000). SAgs induce the proliferation of T cells and the massive release of proinflammatory cytokines, this uncontrolled stimulation can impede the effectiveness of the immune response by disrupting the recruitment of effector cells (Figure 2.1) (Tuffs et al., 2018). The bovine pathogenicity island SaPIbov is found in bovine-associated lineages such as CC151 and CC133 (Wilson et al., 2018). SaPIbov contains several toxin proteins, including toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxin-like protein, and a bovine variant of staphylococcal enterotoxin C (SEC) (Fitzgerald et al., 2001).

2.1.4 Antibiotic-resistant Staphylococcus aureus

Globally, the use of antibiotics in agriculture exceeds that used for the treatment of human infections, and this has contributed to the growing antibiotic resistance (ABR) crisis (Van Boeckel et al., 2015). Concern over the growing number of ABR infections has led to certain localities placing restrictions on antibiotic use in agriculture. However, global standards governing antibiotic usage in agriculture do not exist, instead, there is an international patchwork of different regulatory approaches to antibiotic stewardship (Kirchhelle, 2018). The use of antibiotics in agriculture also drives the emergence of antibiotic-resistant bacteria, making the possible emergence of human-adapted ABR strains from agricultural practices concerning. Pathoadaptive clonal lineages of *S. aureus* – which are specialized to a specific agricultural host or group of hosts, have emerged and caused significant economic losses in the agricultural sector.

While penicillin was highly effective for treating *S. aureus* infections in 1928, today, > 90% of human-associated strains are resistant to this antibiotic (Olsen et al., 2006). Resistance to beta-lactam antibiotics is encoded by the *mecA* or *mecC* gene located on an MGE called staphylococcal cassette chromosome *mec* (SCC*mec*) (Ito et al., 2001). Based on the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), 13 SCC*mec* types (I to XIII) are currently recognized, based on combinations of the five identified *mec* complexes and the nine types of *ccr* complexes (Baig et al., 2018; Hiramatsu et al., 2013; IWG-SCC, 2009; Wu et al., 2015). The genetic content and the structural organization of SCC*mec* elements are diverse and found in several species (Lakhundi & Zhang, 2018). *S. aureus* strains contain SCC*mec* with *mecA* or *mecC* and are thus resistant to penicillin, methicillin, and all other beta-lactam antibiotics (Chambers & Deleo, 2009). Coagulase-negative staphylococci (CoNS) have been proposed as the donor of SCC*mec* (*mecA* and *mecC*) to multiple clones of methicillin-susceptible *S. aureus* (MSSA) (Bloemendaal et al., 2010; Tsubakishita et al., 2010; Wu et al., 2001).

The first reported LA-MRSA infection was from a dairy cow with mastitis in 1972 (Devriese & Hommez, 1975); since then, MRSA infection and colonization has been reported in a number of animals, including pigs, chickens, and rabbits (Khanna et al., 2008; Moreno-Grua et al., 2018; Nemeghaire et al., 2013). The use of antibiotics in food animal production plays an important role in the selection of MRSA isolates. For example, *S. aureus* CC398 was discovered in the 2000s and has become a rapidly emerging cause of human infections associated with livestock exposure (Armand-Lefevre et al., 2005; Voss et al., 2005). Phylogenetic reconstruction of the CC398 lineage suggests that CC398 was derived from human associated MSSA CC398 and was transmitted to livestock through reverse zoonoses. This CC diversified to gain methicillin resistance by acquiring three different SCC*mec* that included nine subtypes and was then reintroduced to humans (Kock et al., 2013; Price et al., 2012). CC398 has been found to infect a broad range of agricultural species, including pigs, cows, chickens, rabbits, sheep, buffalo, and turkey (Matuszewska et al., 2020). The rapid radiation and broad host spectrum of this lineage are likely associated with ST398 strains having only one type I R-M system, providing a less strict genetic barrier for foreign DNA acquisition (Schijffelen et al., 2010). The second example of the emergence of LA-MRSA being propagated through agriculture is the interhost transmission CA-MRSA from the CC97 lineage, which is common in both dairy cows and humans (Spoor et al., 2013).

Vancomycin is the first-line drug of choice for the treatment of MRSA infections. As the number of MRSA infections increases, a noticeable increase in the use of vancomycin has followed; thus, it is unsurprising that more than 50 vancomycin-resistant *S. aureus* (VRSA) isolates have been reported globally since the first case of VRSA was identified in the United States in 2002 (Bartley, 2002; Cong et al., 2020). Daptomycin and linezolid are commonly used for human VRSA infections, yet clinical treatments might not be effective and prolong hospitalization due to the deterioration of the primary diseases and scarcity of detailed clinical data and treatment guidelines

(Cong et al., 2020). VRSA dissemination for the last decade is low in humans and animals and is not comparable with the spread of MRSA. This low incidence can be explained because of the high fitness cost of VanA-type resistance, which leads to growth reduction and out-competition by other bacteria (Foucault et al., 2009). Increased cell thickness and modification in peptidoglycan synthesis can result in a relatively low level of vancomycin resistance in *S. aureus* (Cui et al., 2003). A high level of resistance to vancomycin is conferred by the *vanA* gene cluster that mediates and synthesis of D-Ala-D-lactase peptidoglycan precursors (Bugg et al., 1991). *S. aureus* acquires a Tn1546-containing plasmid harboring *vanA* genes from enterococci and then maintains this cluster either by retaining the original plasmid or transferring the Tn1546 transposon to a resident plasmid or chromosomal DNA (de Niederhausern et al., 2011).

All 13 VRSA isolates reported in the U.S. belong to CC5 with only one exception, CC30, which is usually associated with community-acquired infections. Although the *vanA* gene cluster has been independently transferred to *S. aureus* on multiple occasions, why CC5 is predisposed to acquiring this gene cluster is still unknown (Kobayashi et al., 2012). In food-producing animals, VRSA has been isolated, but the *vanA* gene cluster was not present despite the isolates having phenotypic vancomycin resistance (Adegoke & Okoh, 2014; Bhattacharyya et al., 2016). More recently, VRSA isolates which carried *vanA* and *vanB* genes were detected in camel meat and abattoir workers (Al-Amery et al., 2019). However, this study failed to identify the original host of VRSA isolates and demonstrate the presence of VRSA in camels, since sampling was not conducted on the animals themselves. Although VRSA in livestock has scarcely been reported, there is a possibility of the emergence of animal adapted VRSA clones in the future in the presence of selection pressure. This fear is supported by a study determining that ST151, which is a bovine-specific hypervirulent clone, was hypersusceptible to the acquisition of vancomycin resistance *in vitro* through conjugation with *Enterococcus faecalis* due to a lack of R-M barriers (Sung &

Lindsay, 2007).

2.2 Antagonistic Interbacterial Interactions Involving *Staphylococcus aureus*

2.2.1 Competitive adhesion and biofilm-inhibiting bacteria

Attachment to biotic and/or abiotic surface is critical to bacterial pathogenicity during the infection process. Biofilms provide advantages to bacteria, and in fact, approximately 40 to 80% of cells on Earth reside in biofilms (Flemming & Wuertz, 2019). Compared to planktonic cells, life in a biofilm gives many advantages to *S. aureus* including easier access to nutrients, increased genetic exchange, and protection against predation and antimicrobial agents (Idrees et al., 2021). Several bacteria have abilities to limit *S. aureus* attachment to surfaces by inhibiting the initial attachment, competing for adhesion sites, and disrupting biofilm maturation (Figure 2.2).

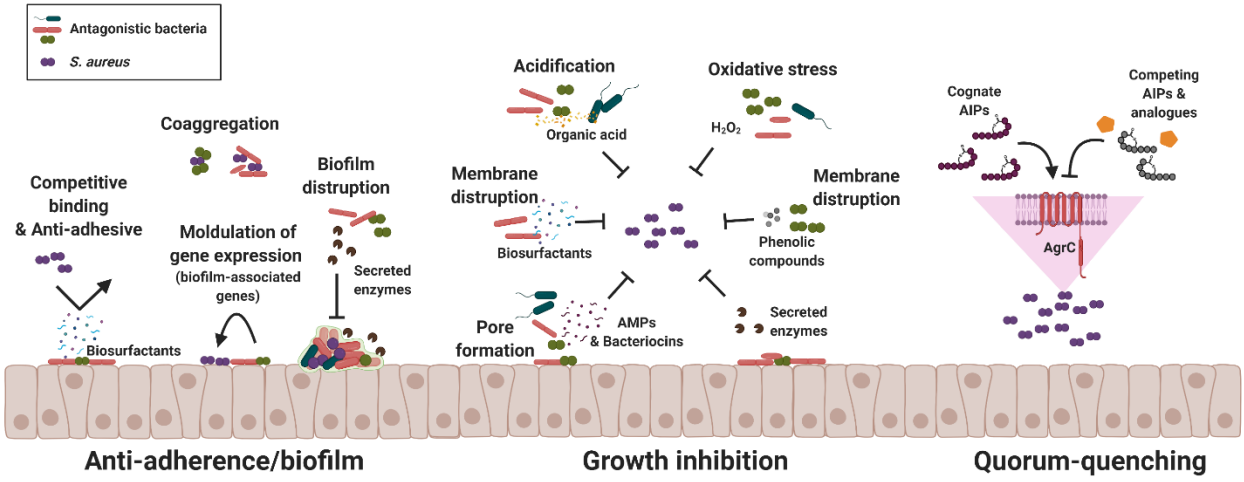

Figure 2.2 Antagonistic interactions towards *Staphylococcus aureus*. Many bacteria antagonize *S. aureus* in colonization, multiplication, and communication mainly by producing active compounds. Anti-adherence to any surface mediated by several negative interactions is a primary step to minimize *S. aureus* colonization. Inhibition and disruption of *S. aureus* biofilm formation is also an important stage to prevent *S. aureus* infection and persistence. Many bacterial metabolites and compounds exhibit growth-inhibiting activities towards *S. aureus*. Heterologous autoinducing peptides (AIPs) interrupt *agr* system in *S. aureus* and hinder virulence gene expressions that *S. aureus* needs in a timely manner to evade host immune response. This figure was created with BioRender.com.

Bacteria with strong adhesion forces coagulate *S. aureus* cells and consequently reduce *S. aureus* adhesion and colonization within the same niche. For example, *Lactobacillus* species display stronger adhesion force with *S. aureus* than *S. aureus* pairs, resulting in coaggregation (Ren et al., 2012; Younes et al., 2012). Bacteria with a strong binding affinity to host cells and matrices also outcompete and exclude *S. aureus* adherence (Mukherjee & Ramesh, 2015; Ren et al., 2012). Other biofilm-inhibiting bacteria may modulate the biosynthesis of polysaccharide intercellular adhesion, a process mediated by *icaADBC* operon and *icaR* (Gowrishankar et al., 2015; Melo et al., 2016). Alternatively, bacteria may produce biosurfactants and/or secrete enzymes such as proteases, which can impair *S. aureus* biofilms (Fang et al., 2018; Iwase et al., 2010; Sharma & Saharan, 2016).

S. aureus can be internalized by and survive in bovine mammary epithelial cells (bMECs), allowing *S. aureus* to escape the host immune response and antibiotic treatment (Almeida et al., 1996; Lammers et al., 1999; Rollin et al., 2017; Watkins & Unnikrishnan, 2020). To be internalized, *S. aureus* must successfully attach to bMEC and other extracellular components (Kerro Dego et al., 2002). Several bacteria, mostly LAB, have shown abilities to inhibit *S. aureus* invasion into bMECs through one or more mechanisms, including immunomodulation of bMECs, competition for attachment sites, and coaggregation (Assis et al., 2015; Bouchard et al., 2013; Souza et al., 2017). On the contrary, a recent study has shown a rapid internalization of *S. aureus* in bMECs subsequent to *Mycobacterium avium* subsp. *paratuberculosis* infection indicating the later species contribution to *S. aureus* persistence (Pena et al., 2020).

2.2.2 Growth-inhibiting bacteria

Antimicrobial agents produced by bacteria include antimicrobial peptides, biosurfactants, organic acid, H_2O_2, and active proteins (Figure 2.2). These agents are major bacterial weapons used to obtain living space and nutrients within a given environment. *S. aureus* produces bacteriocins to

compete with closely related bacteria in the same niche (Ceotto et al., 2009; Netz et al., 2002; Netz et al., 2001). *S. aureus* also uses a type VII protein secretion system (T7SS) to pass a large nuclease toxin into competing bacteria (Cao et al., 2016; Ulhuq et al., 2020).

Many anti-*S. aureus* bacteria produce antimicrobial peptides synthesized either ribosomally or non-ribosomally. Bacteriocins are ribosomally produced antimicrobial peptides capable of depolarizing cell membranes, disrupting membrane integrity, forming pore complexes, interfering with cell-wall synthesis, catalyzing cell-wall hydrolysis, and eventually causing cell death (Alvarez-Sieiro et al., 2016). Staphylococcins, bacteriocins produced by *Staphylococcus* species, appear to be important in fitness and competition among *Staphylococci* (de Freire Bastos et al., 2020; Newstead et al., 2020). LAB is another group of bacteria that produce diverse bacteriocins, including nisin, which is the most well-known bacteriocin produced by *L. lactis* (Alvarez-Sieiro et al., 2016; Millette et al., 2004). Unlike bacteriocins, non-ribosomally synthesized antimicrobial peptides (NRPs) are largely synthesized by enzymatic complexes of bacteria. Members of the genus *Bacillus* produce a broad spectrum of NRPs against pathogenetic microbes including *S. aureus* (Sumi et al., 2015).

Biosurfactant-producing bacteria exhibit multi-inhibitory effects on *S. aureus* including adhesion, biofilm, proliferation, and cellular communication. Biosurfactants are amphiphilic compounds which are either cell-bound or extracellular and can be classified into glycolipids, lipopeptides, fatty acids, polymeric biosurfactants, or particulates (Hajfarajollah et al., 2018; Sharma et al., 2021). LAB is a major group of bacteria producing biosurfactants that exert several inhibitory effects on *S. aureus* in growth, biofilm production, and QS (Hajfarajollah et al., 2018; Nataraj et al., 2021; Yan et al., 2019). Milkisin, a novel lipopeptide, produced by *Pseudomonas* sp. UCMA 17988 isolated from raw cow milk, also shows antagonistic activity against *S. aureus* and other bacteria (Schlusselhuber et al., 2018).

Bacterial organic acids such as lactic acid and acetic acid are generally recognized as safe antimicrobial agents (Coban, 2020). Organic acids diffuse across bacterial cell membranes and then become dissociated, resulting in acidification of the cytoplasm and disrupting enzymatic reactions and nutrient transportation (Cherrington et al., 1991). *S. aureus* has flexible and versatile responses to different acid stress, yet permeant organic acid stress induces slower transcriptional responses and more severe stress than strong inorganic acids (Rode et al., 2010). LAB are common organic acid producers and are often used in food processing to reduce pathogenic bacteria including *S. aureus* (Kostrzynska & Bachand, 2006).

Oxidation-inhibition and oxidative stress mediated by antagonistic bacteria can exert antimicrobial activity against *S. aureus*. For example, *P. aeruginosa* inhibits the normal growth of *S. aureus* by producing respiratory inhibitors such as HQNO and pyocyanin (Filkins et al., 2015; Voggu et al., 2006). Bacteria capable of producing H_2O_2 in aerobic conditions can cause damage to cell membranes and induce the SOS response, activating resident prophages (Painter et al., 2015; Selva et al., 2009). Such oxidative stress can induce the formation of *S. aureus* small-colony variants (SCVs) and enhance the production of catalase to neutralize the toxic effect of H_2O_2 (Regev-Yochay et al., 2006).

2.2.3 Quorum-quenching bacteria

Bacteria communicate with each other using their own signal molecules and regulate gene expression accordingly, and this mechanism is known as QS (Grandclement et al., 2016). *S. aureus* senses its cell density within an environment by producing, releasing, detecting, and responding to an autoinducing peptide (AIP) (Wang & Muir, 2016). At a concentration above threshold, the extracellular AIP cascades signal transduction which then regulates the expression of multiple virulence genes (Le & Otto, 2015). *S. aureus* QS is mediated by a global regulator, also known as

an accessory gene regulator, *agr*. *S. aureus agr* system is consisted of two divergent *agrBDCA* (RNAII) and RNAIII operons (Le & Otto, 2015). The *agrBDCA* operon is regulated by P1 and P2 promoters and encodes four proteins that are required for two-component signal transduction: AgrB (permease), AgrD (autoinducing peptide precursor), AgrC (AIP receptor and histidine kinase), and AgrA (response regulator) (Le & Otto, 2015). RNAIII operon, which is regulated by P3 promoter, encodes *hld* gene and also forms RNA duplexes by antisense base-pairing 5'-untranslated regions that can block the expression of multiple genes such as Protein A and Rot protein (Le & Otto, 2015).

QQ refers to any process that interrupts bacterial QS (Grandclement et al., 2016). During the progress of *S. aureus* infection, if the QQ lasts long enough for the recruitment of neutrophils, host immune cells can rapidly clear *S. aureus* from the infection site (Wright et al., 2005). Thus, QQ bacteria and their QQ compounds are promising therapeutics against *S. aureus*. Numerous synthetic and natural quorum-quenchers have been studied and appeared to be promising tools to prevent *S. aureus* infections in many fields such as medicine and agriculture (Gordon et al., 2013; Grandclement et al., 2016; Grunenwald et al., 2018).

Intra-species *agr* cross-inhibition in *S. aureus* is mediated by competitive binding of AIPs (AIP-I, II, III, and IV) to non-cognate receptors (Lyon et al., 2002; Wang & Muir, 2016). Inter-species QQ of *S. aureus* mediated by other CoNS is the most common, since both have similar QS systems and produce peptide analogous (Canovas et al., 2016; Mahmmod et al., 2018; Novick & Geisinger, 2008; Paharik et al., 2017). *S. epidermidis* and *S. caprae*, for example, produce heterologous AIPs and attenuate *S. aureus* virulence by competing with *S. aureus* AIP to AgrC (Otto et al., 2001; Paharik et al., 2017). Moreover, CoNS strains from the same niche show much broader inhibitory effects on *S. aureus* pathogenicity including *agr* crosstalk (Park et al., 2021; Peng et al., 2019; Toledo-Silva et al., 2021). This interaction between *S. aureus* and CoNS suggests that

CoNS species co-evolve with *S. aureus* in the same environment and thus antagonize *S. aureus* in both *agr*-dependent and *agr*-independent manners.

Other QQ bacteria also produce biomolecules that compete for the ligand-binding pocket of *S. aureus* AIPs on AgrC receptors (Mansson et al., 2011; Piewngam et al., 2018). *Bacillus* species produce fengycins, a family of lipopeptides, which interrupt AgrC-AIP binding and thus are able to eradicate *S. aureus* intestinal colonization in mice (Piewngam & Otto, 2020). A cyclic dipeptide producer, *L. reuteria* RC-14, inhibits all four AIP subgroups in the *S. aureus agr* system (Li et al., 2011). Another way to inhibit *S. aureus* QS is through membrane disruption. A long-chain AHL such as 3-oxo-C12-HSL produced by *Pseudomonas aeruginosa* has been shown to interact with *S. aureus* cytoplasmic membrane and exert a QS inhibitory activity, yet their relationship seems to be complicated and not fully understood (Qazi et al., 2006; Yung et al., 2021).

2.2.4 Probiotics as alternatives to antibiotics

Since ABR has become a global health crisis, bovine mastitis prevention and treatment strategies have been shifted towards antibiotic alternatives such as probiotics (Angelopoulou et al., 2019; El-Sayed & Kamel, 2021; Ghosh et al., 2019). The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) define probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2006). In agricultural sectors, live bacterial prophylactics have been gaining attention, and several commercial products are already available for poultry, pigs, and horses to prevent bacterial infections and promote productivity (Markowiak & Slizewska, 2018).

Despite the possible benefits of probiotics, the potential use of mammary probiotics in dairy cattle is still under debate because of the unexpected effects they may impose on the host and resident microbiome (Rainard, 2017; Rainard & Foucras, 2018). However, only short-term local

inflammatory responses have been observed, and probiotic intramammary infusions have shown to be effective at eliminating mastitis pathogens, with efficacy rates comparable to antibiotic treatments (Frola et al., 2012; Klostermann et al., 2008; Nagahata et al., 2020). This effectiveness is likely to be associated with the recruitment of neutrophils and lymphocytes to the mammary gland, which enhances innate immunity and subsequently clears the gland of infection (Crispie et al., 2008). Interestingly, therapeutic effects of probiotic species vary amongst mastitis pathogens, resulting in differing bacteriological cure rates. For example, treatment with *Lactococcus lactis* showed comparable cure rates on *S. aureus* infections to antibiotics, while *Bifidobacterium breve* infusion was more effective on CoNS infections (Klostermann et al., 2008; Nagahata et al., 2020). More documentation and field trials need to address concerns regarding the efficacy and safety of using mammary probiotics to treat bovine mastitis.

Mammary probiotics, topical probiotics, and probiotic-based treatments seem to be more practical as prophylactics than therapeutics. Intramammary inoculation with probiotic bacteria during dry period may be a useful strategy for preventing bovine mastitis and thus may be a promising replacement of traditional approaches (Berardo et al., 2020; Frola et al., 2013). Alternatively, intramammary infusion of inactivated bacteria such as *Enterococcus faecium* SF68 in drying-off cows can be used to enhance radical oxygen species generation, innate immunity, and mammary gland involution (Peng et al., 2013; Tiantong et al., 2015). Probiotic-based teat disinfectants and teat seals have been actively studied and have shown strong evidence to be another promising mastitis prevention method (Alawneh et al., 2020; Crispie et al., 2005; Paduch et al., 2020; Yu et al., 2017). Probiotics and their derivatives may be utilized to manage biofilm formation and maturation in dairy farm settings (Barzegari et al., 2020).

2.3 Bovine Udder Microbiota

2.3.1 Origin and diversity of bovine udder microbiota

Microbiome is defined as the collective genomes of microorganism inhabiting a particular environment or host from genomic-driven perspective, and a microbiota comprises all members forming the microbiome (Berg et al., 2020). Microbiome also refers to the ecological community of commensal, symbiotic, and pathogenic microorganisms that share the same niche. Host-microbe symbiotic relationships include mutualism, commensalism, and parasitism based on the effects on one or both species involved (Anhê et al., 2020). For example, commensal bacteria are beneficial to a host by supplying essential nutrients and barriers against pathogens while pathogenic bacteria are parasitic by producing harmful molecules and causing diseases. In dairy cattle, several studies have reported a broad range of complex bacterial community composition across the gastrointestinal tracks and udder (Derakhshani, Fehr, et al., 2018; O'Hara et al., 2020). However, details in symbiotic relationship between bovine and each member of bacteria found in and on bovine udder are still ongoing research area.

Mammalian colostrum and milk serve as a complete nutrient source for offspring as well as an excellent media for the offspring gut microbiome (Derakhshani, Fehr, et al., 2018). Until very recently, the raw milk emerging from the bovine teat and intramammary gland was thought to be sterile, and the microorganisms present in the raw milk were a result of external contamination (Rainard, 2017; Taponen et al., 2019). However, studies using next-generation sequencing (NGS) technology focusing on 16S rRNA gene have accumulated evidence of a diverse microbial community that resides in, on, and around the bovine udder (Kuehn et al., 2013; Oikonomou et al., 2012). Sophisticated aseptic sampling techniques using a needle and vacuum tube have supported this new paradigm, allowing milk to be collected directly from the mammary gland while minimizing contamination (Dahlberg et al., 2020; S. A. Metzger et al., 2018; Oliver et al., 2004).

More importantly, this new concept has prompted interest to define the role that the udder microbiota plays in milk quality and udder health, as well as potential applications of commensal bacteria.

The bovine udder is composed of four independent quarters. Each quarter contains a teat apex, canal, and cistern, as well as a gland cistern and duck. Though biochemical and structural characteristics of the teat canal naturally prevent the movement of incoming microbes, migration of external microorganisms through the teat canal is the main route of entry into the mammary gland (Derakhshani, Fehr, et al., 2018; Paulrud, 2005). In fact, reverse pressure during milking and suckling often permits microbial invasion of the teat canal (McDonald, 1970). An endogenous route or entero-mammary pathway in dairy cattle has also been proposed, yet the subject is still of debate (Oikonomou et al., 2020; Rainard, 2017). However, the detection of certain gut-associated bacteria in dairy milk as well as studies which report that a distinct milk microbiota exists compared to the udder skin and teat canal may support this endogenous hypothesis in ruminants (Derakhshani et al., 2020; Young et al., 2015).

The composition of udder microbiota varies depending on which part of the udder is sampled and can be affected by several host and environmental factors (Derakhshani, Fehr, et al., 2018; Porcellato et al., 2020). In different sampling locations, the udder microbiota is composed of both common and niche-specific microbes. When sampling milk, most of the microbiota is shared with the teat canal (Dahlberg et al., 2020; Derakhshani, Fehr, et al., 2018; Derakhshani et al., 2020). The fact that the udder microbiota is niche dependent suggests that the teat canal acts as a primary barrier for exogenous bacteria, and the mammary gland is potentially precluding the growth of certain environmental bacteria (Derakhshani et al., 2020). Internal factors such as breed, lactation cycle and stage, and health status have effects on the microbial composition of raw milk (Cremonesi

et al., 2018; Parente et al., 2020). The combination of bioactive components by cows, including immunoglobulin G, lactoferrin, and lysozyme, likely influence which organisms can thrive in the mammary gland at different stages of lactation (Krol et al., 2010; Lima et al., 2017). Several external factors such as cattle diet, housing, humidity, temperature, and geographic origin have shown to impact the microbial composition of raw milk (Doyle et al., 2017; Guo et al., 2021; Li et al., 2018; Zhang et al., 2015). This highly dynamic and seasonal milk microbiota observed challenges researchers to define a core milk microbiota (Guo et al., 2021; Li et al., 2018). Regardless, several studies have indicated that the bacterial community in raw milk is mainly dominated by *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *and Bacteroidetes*.

2.3.2 Bacterial risks of the bovine mammary gland

The microbiota contributes to host health by training and developing the immune system as well as limiting the growth of pathogenic microorganisms (Belkaid & Hand, 2014; Derakhshani, Fehr, et al., 2018). Microbiota in the teat canal seeds the emerging milk and provides initial inoculum for the offspring gut microbiota (Addis et al., 2016). Some beneficiary bacteria such as LAB produce bacteriocins, organic acids, and hydrogen peroxide to inhibit foodborne pathogens in raw milk (Reuben et al., 2020; Silva et al., 2018). The natural microbial community also develops the flavor of raw milk cheese (Yoon et al., 2016). Despite benefits of some members of the natural milk microflora, some commensal and infectious bacteria have detrimental effects on milk quantity and quality (Goncalves et al., 2020; Murphy et al., 2016).

Some native milk proteases and microbial enzymes are heat stable and can damage milk components such as milk fats and proteins (Ismail & Nielsen, 2010; Murphy et al., 2016). For instance, psychrotolerant bacteria, which produce proteolytic and lipolytic enzymes, are detrimental

to the shelf life of dairy products (Adams et al., 1975; de Oliveira et al., 2015). Mastitis also induces adverse effects on milk composition resulting from physiological and metabolic reactions, as well as local inflammatory responses (Wang et al., 2020). Certain mastitis pathogens such as *S. aureus* can decrease milk yield by invading into secretory epithelia and increasing mammary epithelial cell exfoliation (Goncalves et al., 2020; Nagasawa et al., 2018). More importantly, pathogenic bacteria and their toxins in raw milk and dairy products can cause zoonotic diseases and foodborne illnesses (Necidova et al., 2019; Sugrue et al., 2019).

In addition to food safety, the presence of pathogenic bacteria in the mammary gland and raw milk is also associated with biosecurity. Biosecurity refers to the strategic and integrated approach to analyze and manage relevant risk to animal, public, and environmental health (WHO, 2010). Studies on biosecurity in dairy farms are heavily weighted towards prevention of bovine mastitis rather than treatment to minimize the spread of disease and use of antibiotics. More recently, biosecurity has gained attention due to zoonotic transmission and AMR (Palma et al., 2020; Renault et al., 2021). The Canadian Food Inspection Agency and the Dairy Farmers of Canada proposed a national standard for biosecurity and producer planning guide for Canadian dairy farms, with the aim of implementing nation-wide biosecurity (CFIA, 2013a, 2013b). The documents aim to support producers to adopt farm-based biosecurity plans and strategies to implement nation-wide biosecurity practices in four control areas: animal health management, animal additions and movement, premises' management and sanitation, and personal, visitors, vehicles, and equipment. Premises' management and sanitation, for example, guide producers and farm workers how to manage manure, waste, deadstock, and pests. However, there are still gaps between the guidelines and the farm practices of dairy herds (Denis-Robichaud et al., 2019; Denis-Robichaud et al., 2020; Farrell et al., 2021).

2.3.3 Intramammary infections and bovine mastitis

Bacteria must combat physical, chemical, and biological barriers to colonize the mammary gland successfully. The teat opening is a way for bacteria to enter and reach the teat canal and cistern. Healthy teat skin is normally coated with fatty acids which slows bacterial growth, while in the teat canal keratin traps bacteria, hindering microbial migration into the teat cistern (Ezzat Alnakip et al., 2014). Though, some bacteria can travel through the teat cistern, and enter a microenvironment where they are challenged with iron and oxygen limitations, host antimicrobial compounds, and other native microorganisms (Ezzat Alnakip et al., 2014; Mayer et al., 2021). Some pathogens overcome or bypass this local stress. *S. aureus*, for example, resists lysozymes, produces staphyloferrin to scavenge iron, and forms biofilm to persist in the mammary gland, this can lead to a colonization and failure of medical intervention (Hammer & Skaar, 2011; Park & Ronholm, 2021; Pushkaran et al., 2015).

Lactating cows seems to be highly pro-inflammatory to new intramammary infections owing to the high IgG in both colostrum milk and mature milk (Butler et al., 2015; Hurley & Theil, 2011). Furthermore, the direct exposure of the mammary gland epithelium to microbes due to no mucosal layer increases its vulnerability to all bacterial infections (Rainard, 2017). Thus, any bacterium that reaches the mammary gland, even at the low cell density, can potentially cause inflammation. Indeed, experimental intramammary infusions have revealed that a small number of mastitis pathogens (< 100 CFU) can induce clinical mastitis (Bennett & Jasper, 1977; Chang et al., 2008; Oliver et al., 2012).

Microbial dysbiosis is characterized as a disruption to microbial homeostasis caused by internal and external factors. A reduction of beneficial microbes to the host commonly refers dysbiosis, thereby decreasing the resilience to pathogen invasion and colonization (Honda & Littman, 2012). Indeed, mastitis pathogens often outcompete commensal bacteria and reduce

species diversity leading to a single operational taxonomic unit (OTU) in the community (Andrews et al., 2019; Oikonomou et al., 2014). However, defining an imbalanced microbial composition is still challenging since there is no definition of normal or healthy mammary microbiota.

In several studies, significant differences in microbial profile and diversity have been observed between samples derived from clinically affected quarters and healthy quarters (Andrews et al., 2019; Hoque et al., 2019; Stephanie A. Metzger et al., 2018; Oikonomou et al., 2014). Each study independently demonstrates that the milk microbiome associated with mastitic quarters is less diverse and distinct from the microbiota in healthy quarters. During bovine mastitis, the infecting pathogen can affect the severity and duration of the disease, early immune response, and host metabolism. For instance, *E. coli* quickly elicits a strong inflammatory response in the udder and induces transcriptional changes in neighboring quarters, while *S. aureus* triggers unbalanced immune suppression and invades host cells (Griesbeck-Zilch et al., 2008; Gunther et al., 2017; Jensen et al., 2013). However, no study discusses if the immune response or virulence factors specific to the pathogen collectively affect disease severity, duration, and/or treatment efficacy.

Bovine mastitis appears to affect raw microbiota differently due to direct and indirect factors, which potentially lead to inter-study differences in the microbial changes in post-mastitic milk. Falentin et al., observed long-lasting effects in the microbiota of raw milk taken from quarters with a history of clinical mastitis, while Ganda et al., reported restoration of the microbiota 14 days after experimental infection (Falentin et al., 2016; Ganda et al., 2017). It remains unknown whether this discrepancy results from differences in infections (natural vs experimental), sampling (teat vs milk), etiological agents (non-specific vs *E. coli*), or other elements. In mastitis quarters, the use of antibiotics reduces the total bacterial load yet increases microbial complexity, while in healthy quarters, antibiotics have no significant effect on bacterial load nor diversity (Bonsaglia et al., 2017; Derakhshani, Plaizier, et al., 2018; Ganda et al., 2016; Ganda et al., 2017). Although it remains

unclear whether mammary dysbiosis is the cause or consequence of bovine mastitis, or if commensal mammary microbiota has a role during mastitis via direct and indirect interactions with the host or pathogens.

2.3.4 Future applications of bovine microbiota

Antibiotic treatment may not completely cure bovine mastitis. As a result, other therapeutic strategies such as bacteriophage therapy and nanoparticle-based therapy have been developed to reduce bovine mastitis. Though, these strategies have low efficacy, which has directed research towards the development of new prophylactics and therapeutics (Sharun et al., 2021). Early diagnosis of bovine mastitis and rapid identification of the responsible etiological agent followed by timely treatment is the most promising approach to reduce animal suffering, production loss, and unnecessary use of antibiotics. Microbial biomarkers are commonly used for early diagnosis of human diseases (Lun et al., 2019; Mangifesta et al., 2018; Rogers et al., 2011). Several studies on the milk microbiome have attempted to propose putative early microbial indicators of mastitis infections including *Staphylococcus*, *Microbacterium,* and *Sphingobacterium* (Derakhshani et al., 2020; Gryaznova et al., 2021). However, bacterial indicators do not seem to be applicable for early diagnosis of bovine mastitis due to the diversity of etiological agents, as well as the complex mammary microbial community impacted by geographical and seasonal factors.

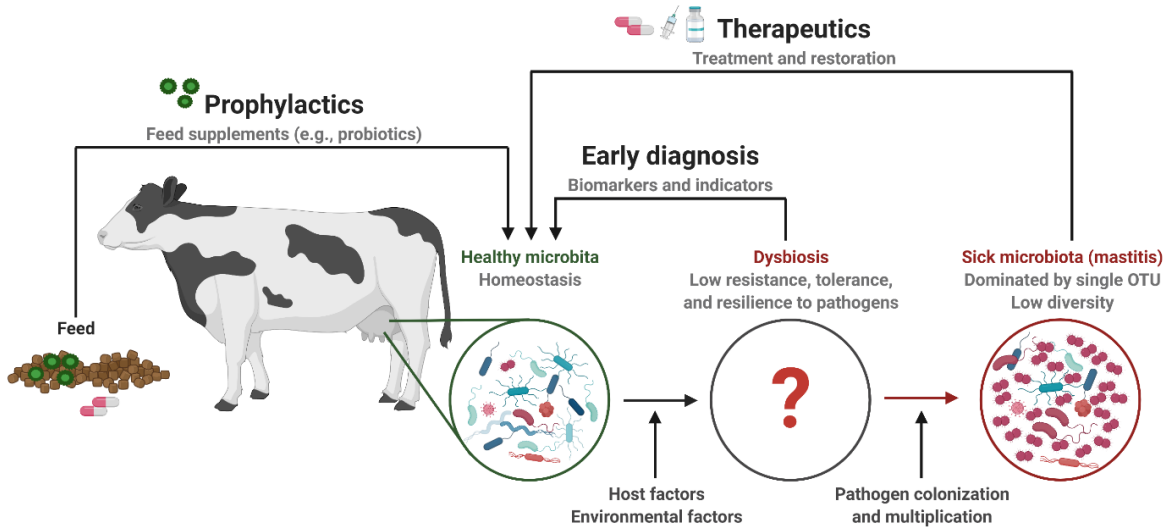

Figure 2.3 Maintenance strategy for healthy mammary microbiota in dairy cattle. Microbial dysbiosis in bovine mammary gland can lower the overall diversity of the udder microbiota, possibly increasing susceptibility to mastitis pathogens. Collective efforts on early diagnosis, therapeutics, and prophylactics may increase the prevention rate of bovine mastitis before the diseases occur by maintaining healthy microbial community in bovine mammary glands. This figure was created with BioRender.com.

Non-pathogenic mammary commensal bacteria play a major role in maintaining homeostasis of the mammary gland. Re-introducing healthy commensals into an unbalanced udder microbiota could restore diversity, and thus may be a promising approach of mitigating mastitis. Interestingly, rumen microbiota is an area more of interest to microbially modify than intramammary gland microbiota (Figure 2.3). Various studies have shown that gut microbiota is associated with udder health and milk production yield, yet the detailed mechanisms are still unclear (Tong et al., 2018; Wang et al., 2021; Xue et al., 2020; Zhong et al., 2018). This possible correlation is also supported by other studies which investigate the use of diets with supplements such as oral probiotics (Gao et al., 2020; Spaniol et al., 2015).

CONNECTING TEXT

In the previous chapter, a comprehensive review of related literature was conducted, introducing an overview of *S. aureus* and bovine mastitis, antagonistic interactions between *S. aureus* and other bacteria, and bovine udder microbiome. This review showcased the urgent need of alternatives to antibiotics to support the sustainability of the dairy industry. We also introduced the emergence of multi-drug resistant *S. aureus* and potential zoonosis and reverse zoonosis resulting from repetitive exposure and antibiotic use in dairy industry. Thus, as alternatives to antibiotics, we highlighted bacteria with antagonistic activity against *S. aureus* and microbiome modulation with resistance to the colonization of pathogenic bacteria. In Chapter 3, bovine-associated *S. aureus* isolates were compared to human-originated *S. aureus* to understand the genetic characteristics of bovine-adapted *S. aureus*. This chapter aims to describe the evolutionary relationship between bovine-associated *S. aureus* and human-originated *S. aureus* and further elucidate their host-specific genes and lineage-specific genes. We also investigated genetic barriers in *S. aureus* that limits the genetic material exchanges between *S. aureus* in different lineages.

CHAPTER 3. COMPARATIVE GENOMIC ANALYSIS OF *STAPHYLOCOCCUS AUREUS* ISOLATES ASSOCIATED WITH EITHER BOVINE INTRAMAMMARY INFECTIONS OR HUMAN INFECTIONS DEMONSTRATES THE IMPORTANCE OF RESTRICTION-MODIFICATION SYSTEMS IN HOST ADAPTATION

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4.1 Abstract

Staphylococcus aureus is a major etiological agent of clinical and subclinical bovine mastitis. The versatile and adaptative evolutionary strategies of this bacterium have challenged mastitis control and prevention globally, and the high incidence of *S. aureus* mastitis increases concerns about antimicrobial resistance (AMR) and zoonosis. This study aims to describe the evolutionary relationship between bovine intramammary infection (IMI)-associated *S. aureus* and human pathogenic *S. aureus* and further elucidate the specific genetic composition that leads to the emergence of successful bovine IMI-associated *S. aureus* lineages. We performed a phylogenomic analysis of 187 *S. aureus* isolates that originated from either dairy cattle or humans. Our results revealed that bovine IMI-associated *S. aureus* isolates showed distinct clades compared to human-originated *S. aureus* isolates. From a pan-genome analysis, 2,070 core genes were identified. Host-specific genes and clonal complex (CC)-specific genes were also identified in bovine *S. aureus* isolates, mostly located in mobile genetic elements (MGEs). Additionally, the genome sequences of three apparent human-adapted isolates (2 from CC97 and 1 from CC8), isolated from bovine mastitis samples, may provide a snapshot of the genomic characteristics in early host spillover events. Virulence and AMR genes were not conserved among bovine IMI-associated *S. aureus* isolates. Restriction-modification (R-M) genes in bovine IMI-associated *S. aureus* demonstrated that the Type I R-M system was lineage-specific and Type II R-M system was sequence type (ST)-specific. The distribution of exclusive, virulence, and AMR genes were closely correlated with the presence of R-M systems in *S. aureus*, suggesting that R-M systems may contribute to shaping clonal diversification by providing a genetic barrier to the horizontal gene transfer (HGT). Our findings indicate that the CC or ST lineage-specific R-M systems may limit genetic exchange between bovine-adapted *S. aureus* isolates from different lineages.

Keywords

Staphylococcus aureus, bovine mastitis, comparative genomics, restriction-modification system

Impact statement

S. aureus bovine mastitis is a costly disease in dairy cattle. Despite its clinical importance and overall burden in the dairy industry, studies of this bacterium isolated from cows have suffered due to a limited number of whole-genome sequences being available and a further limited subset of strains that can be genetically manipulated. Fully understanding *S. aureus* R-M systems may help explain HGT in this species and understand the dissemination of MGEs containing important virulence or AMR genes. Moreover, an understanding of *S. aureus* R-M systems may aid in designing strategies to bypass genetic barriers to make hyper recipients in a lineage of interest for genetic engineering applications. This approach may help facilitate studies on *S. aureus*, providing an improved understanding of its pathogenicity in a specific host.

Data summary

Short read data for bovine IMI-associated *S. aureus* isolates are available at NCBI-SRA under BioProject numbers PRJNA609123 and PRJNA622791.

Highly assembled human-originated *S. aureus* genomes used in this study are available at NCBI under the accession numbers listed in Appendix 1.

Metadata including source, collection year, geographical area, associated disease, and ST/CC, is summarized in Appendix 1 and 2.

3.2 Introduction

Staphylococcus aureus is an opportunistic pathogen that can infect humans as well as economically important livestock such as cows, sheep, and goats. Among livestock, cows are a common reservoir of *S. aureus,* and dairy cattle frequently experience clinical and subclinical mastitis due to *S. aureus* intramammary infections (IMIs) (Watts, 1988). *S. aureus* produces biofilms, survives in non-phagocytic and phagocytic host cells, and dynamically switches its phenotypes between wild-type and small colony variants (Atalla et al., 2010; Fox et al., 2005; Hebert et al., 2000). Each of these characteristics results in the persistence of *S. aureus* colonization in intramammary environments. This persistence within the mammary glands typically leads to treatment failure and recurrent bovine mastitis. Bovine mastitis control programs have been developed with the intention of infection prevention; however, knowledge gaps in clonal diversity, host immune response, and other elements that affect *S. aureus* IMIs hinder the development of effective prevention strategies (Rainard et al., 2018). Moreover, robust genetic defence mechanisms owing to restriction-modification (R-M) systems in *S. aureus* impede genetic manipulations, limiting researchers from understanding bovine IMI-associated *S. aureus* physiology, metabolism, and pathogenesis (Sadykov, 2016). Thus, *S. aureus* remains a primary etiological agent of bovine IMIs, causing significant challenges for researchers, dairy farmers, and veterinarians.

On a short evolutionary time-scale, *S. aureus* lineages are host-specific; although, on a longer time-scale, lineages often undergo zoonosis and zooanthroponosis. The word *lineage* is used in this study to refer to a group of isolates that have a commonality either through being from the same sequence type (ST) or clonal complex (CC). Bovine-adapted *S. aureus* lineages include CC97, CC133, and CC151 and human-adapted lineages include CC1, CC5, CC8, CC30, and CC45 (Park & Ronholm, 2021). *S. aureus* host spillover, followed by adaptation to a new host, is generally accompanied by loss of virulence and immune evasion genes from the previous host and acquisition

of a new set of genes specific for survival in the new host (Matuszewska et al., 2020). Whole-genome sequencing and a subsequent comparative genomic analysis can be used to understand the movement of virulence and other host adaptation genes between *S. aureus* isolates from different host species. Recent comparative genomic studies have shown that bovine-adapted strains rapidly lose genes involved in human infections, which likely increases their fitness in bovine hosts (Herron-Olson et al., 2007; Matuszewska et al., 2020). For instance, bovine-specific mobile genetic elements (MGEs) found in bovine adapted *S. aureus* lineages include the temperate phages φSaBov and φPV83, as well as pathogenicity islands SaPlbov1, SaPlbov2, and SaPlbov3 (Herron-Olson et al., 2007; Naushad et al., 2020; Park & Ronholm, 2021). These MGEs carry bovine-specific virulence factors such as Leukocidin LukMF' and von Willebrand factor-binding protein (vWbp) (Viana et al., 2010; Vrieling et al., 2015).

Cows are a source of antimicrobial-resistant (AMR) *S. aureus*, which may be transferred to persist in humans (Matuszewska et al., 2020). Specifically, livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) is believed, at least partially, to be responsible for community-associated MRSA due to the use of antimicrobials in veterinary medicine and modern agriculture (Cuny et al., 2015). Interestingly, the prevalence of AMR *S. aureus* among dairy cows differs significantly based on geography. In North America, for example, a low prevalence (less than 10%) of *blaZ* positive *S. aureus* has been reported, while China, Finland, Sweden, Iran, and Brazil have noted that 50% to 94% of bovine *S. aureus* isolates are penicillin-resistant (Bagcigil et al., 2012; Haran et al., 2012; Jamali et al., 2014; Marques et al., 2017; Saini et al., 2012; Yang et al., 2015). Most studies investigate only common STs or the prevalence of AMR genes, making it challenging to elucidate the links between genetic lineage, virulence, AMR, and adaptations to the bovine niche. Very few studies have attempted to correlate lineage and AMR to gain insight into this relationship. Among *S. aureus* CC97 isolated from cows, which includes several STs (ST97, ST115, and ST352), only a

few STs are reported to be positive for *blaZ* or *mecA* (Kappeli et al., 2019; Klibi et al., 2018; Schmidt et al., 2017). Some human-adapted lineages, such as ST5 (CC5), ST8 (CC8), and their variants have been isolated from cows and were positive for *blaZ* and *mecA* (Kappeli et al., 2019; Klibi et al., 2018; Schmidt et al., 2017). These studies support the idea that certain *S. aureus* lineages may be more prone to obtaining specific AMR genes, suggesting that lineage-specific factors might be involved in horizontal gene transfer (HGT). The existence of *S. aureus* lineage-specific R-M systems is a possible explanation for the unequal distribution of virulence/AMR genes in different lineages from the bovine niche.

Acquisition of foreign DNA is important in terms of bacterial evolution, fitness, adaptation, and clonal diversification. Many virulence, host-specific, and AMR genes are carried by *S. aureus* MGEs, and the movement of these MGEs contributes to strain differentiation. Indeed, 15% of any *S. aureus* genome consists of MGEs which play a prominent role in host adaptation and pathogenicity (Lindsay & Holden, 2004; Waldron & Lindsay, 2006). However, acquiring foreign DNA is not always advantageous due to the possibility of obtaining harmful, lethal, or superfluous genes. To control the retention of foreign DNA some bacteria including *S. aureus* have developed R-M systems. R-M systems are grouped into four types based on subunit architecture, the requirement for ATP/GTP, the level of sequence specificity, and DNA cleavage mechanism (Roberts, Belfort, et al., 2003). Lineage-specific R-M systems with the combination of Type I, II, III, and IV R-M genes control the spread of clinically important genes between *S. aureus* CCs, with Type I and II systems being the most common (Corvaglia et al., 2010; Roberts et al., 2013; Waldron & Lindsay, 2006). Type I R-M systems are multi-subunit complexes that consist of two M subunits, two R subunits, and one S subunit, encoded by *hsdM*, *hsdR*, and *hsdS*, respectively (Roberts, Belfort, et al., 2003). The S subunit is responsible for recognizing a specific DNA sequence, the R subunit cleaves DNA, and the M subunit catalyzes the methylation reaction (Roberts, Belfort, et al., 2003). Type I R-M

systems are located in νSaα and νSaβ as a part of the core genome in *S. aureus*. The Type I R-M system is the primary R-M system in *S. aureus*, the allele present are lineage-specific, and it constitutes a significant barrier to the movement of MGEs and intentional genetic manipulation (Lee et al., 2019). Type II R-M systems consist of a restriction endonuclease (*res*), that recognizes a specific DNA sequence and then introduces double-strand DNA breaks, and a methyltransferase that recognizes the same DNA sequence and methylates it (*mod*) (Bogdanova et al., 2008). Methylation modifies and thus protects target sequences from cleavage by hiding it from the restriction endonuclease (Bogdanova et al., 2008). Type II R-M systems are widely used in recombinant DNA technology and because of this application more than 3,500 have been discovered and characterized (Roberts, Vincze, et al., 2003). Type III R-M systems are also composed of two genes, *mod* and *res* that also function in DNA modification or restriction, respectively (Roberts, Belfort, et al., 2003). Type IV R-M systems are less well characterized, but are composed of one or two genes that encode proteins that cleave only modified DNA (Roberts et al., 2003). Understanding *S. aureus* lineage-specific genetic barriers can outline the HGT network and potential evolutionary directions, which would aid in understanding *S. aureus* and ultimately preventing *S. aureus* infections and dissemination of AMR genes. Furthermore, this knowledge enables us to improve our ability to manipulate non-transformable *S. aureus* for the purposes of future *S. aureus* studies.

In this study, we used a comparative genomics approach to investigate several aspects of bovine IMI-associated *S. aureus*. We attempted to further understand the evolutionary relationships between *S. aureus* isolated from humans and cows by identifying unequally distributed genes among the two hosts, as well as correlations between the presence of mastitis-associated virulence factors, AMR genes, and R-M system genes in bovine IMI-associated *S. aureus*.

3.3 Materials and Methods

3.3.1 Sequence genomes, assembly, and gene annotation

We previously reported whole-genome sequencing on bovine IMI-associated *S. aureus* isolates obtained from the Mastitis Pathogen Culture Collection (Appendix 1) (Demontier E, 2021; Dufour et al., 2019; Park et al., 2020). Each of these isolates was obtained from the cows in different health status (Appendix 2). The raw DNA sequences of bovine isolates (n = 63) were assembled following the same pipeline as previously described using the software pipeline ProkaryoteAssembly (v. 0.1.6) (https://pypi.org/project/ProkaryoteAssembly/) (Jung et al., 2021; Park et al., 2020). The quality of the genome assemblies (draft genomes) was evaluated using Qualimap (v. 2.2.2) (Okonechnikov et al., 2016). Complete genomes of human *S. aureus* isolates (n = 122) and two reference genomes, RF122 and Newbould 305 isolated from bovine milk samples, were obtained from the National Center for Biotechnology Information (NCBI) database (Appendix 1). All *S. aureus* genomes (n = 187) were then run through the annotation pipeline via Prokka (v. 1.14.5) with the genus/species option (Seemann, 2014). All draft and complete genomes were verified as *S. aureus* by confirming the presence of *crtOPQMN* operon and the binding site of *unc* universal primers (Becker et al., 2019; Tong et al., 2015).

3.3.2 Pan-genome and phylogenomic tree

A pan-genome of 187 *S. aureus* genomes was created using Roary (v. 3.13.0) with no paralog splitting and R plots options (Page et al., 2015). The pan-genome analysis was restricted to identifying the presence and absence of orthologs only; thus, paralogs copies were not taken into account during the analysis. The gene_presence_absence.csv file generated by Roary was used for pan-genome analysis. Core genes (core and soft core genes) and accessory genes (shell and cloud) were also identified using Roary.

The core gene alignment with 187 *S. aureus* genomes established by Roary was used to obtain phylogenetic estimates using IQ-TREE ModelFinder with 1000 replicate bootstraps (Kalyaanamoorthy et al., 2017). The best model was found to be GTR+F+R2, which was then used to construct a phylogenomic tree and later displayed by iTOL (https://itol.embl.de/) (Letunic & Bork, 2016). We analyzed seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi,* and *yqiL*) from each *S. aureus* genome to determine ST using mlst (https://github.com/tseemann/mlst) against the PubMLST database (Jolley & Maiden, 2010).

3.3.3 Exclusive gene analysis

The genes from bovine and human *S. aureus* genomes were compared using Venny (v. 2.1.0) using the list of genes from the gene_presence_absence.csv file generated by Roary (Oliveros, 2017). The genes only present in either bovine or human *S. aureus* were classified as exclusive genes. Within bovine *S. aureus*, lineage-specific genes were examined by comparing the genes present in each CC. The relative location of each exclusive gene or gene cluster was determined by aligning the draft genomes to the complete/reference genome of *S. aureus* RF122 (ET3-1) isolated from bovine using IslandViewer 4 (Bertelli et al., 2017; Herron-Olson et al., 2007). All exclusive or lineage-specific genes annotated as hypothetical or unknown functions from Roary were also searched on NCBI BLASTP using their amino acid sequences to identify potential functions (Johnson et al., 2008).

3.3.4 Identification of virulence/antimicrobial resistance/R-M genes

Virulence factors were analyzed using VFanalyzer (http://www.mgc.ac.cn/VFs/main.htm) and confirmed with gene_presence_absence.csv file produced by Roary (Liu et al., 2019). Antimicrobial resistance genes were analyzed using ABRicate (https://github.com/tseemann/abricate) through MEGARes database and Roary (Lakin et al., 2017; Page et al., 2015). R-M genes were searched on

Restriction-ModificationFinder 1.1 (https://cge.cbs.dtu.dk/services/Restriction-ModificationFinder/) and then analyzed with REBASE searching through amino acid sequences and then grouped with > 95% amino acid sequence homology (Roberts et al., 2015). We classified *agr* types based on the conserved regions of amino acid sequences in AgrD (AIP precursor) (Wang & Muir, 2016). The presence of the partially assembled or non-assembled genes was confirmed by Sanger sequencing followed by PCR with the target gene-specific primers (Appendix 3).

3.3.5 Mobile genetic elements (MGEs) identification

Plasmids were analyzed using NCBI BLAST initially with circular contigs from assembled sequences of bovine IMI-associated *S. aureus* isolates and then using ABRicate through the PlasmidFinder database (Carattoli et al., 2014). The verified plasmids were run through a local blast with the parameter of 97% identity and coverage against 65 bovine IMI-associated *S. aureus* genomes. Prophage and genomic islands sequences were identified using PHASTER and IslandViewer 4, respectively (Arndt et al., 2016; Bertelli et al., 2017). All bovine IMI-associated *S. aureus* draft genomes were aligned against the bovine-adapted *S. aureus* RF122 (ET3-1) genome in IslandViewer 4 (Bertelli et al., 2017; Herron-Olson et al., 2007).

3.4 Results

3.4.1 Phylogenomic tree and S. aureus pan-genome

Phylogenomic analysis was conducted using a core gene alignment and revealed that bovine IMI-associated *S. aureus* lineages included CC151, CC8, CC126, and CC97, and the majority of human isolates belonged to CC8 and CC5 (Figure 3.1A). Bovine IMI-associated *S. aureus* isolates were clustered into three clades and distinct from human isolates. Three suspicious isolates that show

evidence of having recently jumped between host species were CC8 (Sa1158c) and CC97 (ATCC BAA-39 and ATCC 6538).

All 489,086 coding sequences (CDS) from 187 *S. aureus* genomes were grouped into 6,182 gene clusters. Grouping of the CDS revealed that the core genome to be 2,070 genes (33.5%) shared by more than 95% of isolates and the accessory genome to be composed of 4,112 genes (66.5%) (Figure 3.1B). The pan-genome increased in size upon the addition of new genomes suggesting an open pan-genome (Figure 3.1C).

3.4.2 Unequally distributed genes between clonal complexes

Unique and exclusive genes were examined, with a concentration on identifying genes that were unique to bovine IMI-associated *S. aureus* isolates. From the total pan-genome, 326 and 2,653 elements were exclusive in either bovine IMI-associated or human *S. aureus* isolates, respectively. Most exclusive genes were either isolate-specific or CC lineage-specific. The most variable loci were found near *orfX* – the location of SCC*mec* integration in human isolates – and *hlb* (β-hemolysin) due to β-converting prophages and adjacent MGEs (Figure 3.2).

Among bovine IMI-associated *S. aureus*, > 90% (59/65) of isolates contained a pathogenicity island known as SaPIbov3, which encodes four common proteins: a class I SAM-dependent methyltransferase, a hypothetical protein, a multidrug transporter, and a CAAX protease immunity-related protein. These unique genes found in bovine IMI-associated isolates were absent in five CC126 isolates and in one CC8 isolate (Sa1158c). We also found *lukMF'*, bovine-specific virulence genes, in CC151 and ST352 (CC97). All isolates in CC151 encoded common lineage-specific genes at five different loci: an incomplete prophage close to φStauST398, *lukMF'* genes carried by φPV83, four genes in a non-MGE region, an enterotoxin gene cluster located in φSaBov, and two genes encoding Type II R-M subunits in a putative genomic island. The same Type II R-M

genes in CC151 were found in CC126. CC126 also had five unique genes, the functions of which were unclear. All ST2270 (CC126) isolates (n = 4) contained 20 unique genes within the lineage located in a putative genomic island near *hlb*. Sa1158c (ST8/CC8) possessed its own plasmid that encoded seven open reading frames (ORFs) with unknown functions. Among the CC97 isolates, 15 isolates mainly ST2187 (n = 13) carried bovine variants *vWbp* and *scn* located in a pathogenicity island. The *vWbp* and *scn* genes, associated with bovine immune evasion and commonly found in *S. aureus* isolated from cows, were rarely found in human isolates – only two human isolates (PCFH-226 and S58) encoded them. Additionally, 81.5% (31/38) of CC97 isolates carried the pCC97-1 plasmid, which encodes the *aurABCD*, *aurI*, *aurR,* and *aurT* genes for aureocin synthesis and transportation.

Additionally, exclusive genes were also found in human-originated *S. aureus* isolates. Three genes: *lytN*, *fmhC*, and *dprA* associated with cell division, cell wall protection, and DNA processing, respectively, were present in most human isolates (95.9%, 117/122) as a part of the core genome near *sucCD*, and these genes were found in only one bovine isolate Sa1158c (ST8), which was phylogenetically related to the human-adapted lineage. The majority of human isolates encoded an immune evasion cluster (80.3%, 98/122) at the *hlb* locus: *scn*, *chp*, and *sak*. The enterotoxin gene cluster found in CC151 was also present among human isolates (n = 45).

3.4.3 Genetic elements suggesting recent host spillover

Three isolates which might be part of recent host spillover events (ATCC 6538, ATCC BAA-39, and Sa1158c) were identified in the phylogenomic tree (Figure 3.1A). To identify potential genetic elements that might help *S. aureus* to enhance fitness or adaptation in a new host species, we further examined the presence of host-specific genes in these three isolates. One caveat to this analysis is

that human and bovine isolates were derived from different geographic regions, which may confound the analysis.

ATCC 6538 and ATCC BAA-39 were isolated from humans and belong to ST464 (CC97). The four genes commonly found in bovine isolates (n = 59) were absent in these two isolates. However, two bovine specific prophages similar to φPT1028 (incomplete) and φJS01 (intact) were found in these two isolates. A partial φPT1028 prophage that is frequently found in bovine IMI-associated *S. aureus* isolates (64.6%, 42/65) was found; however, the version of φPT1028 found in the ATCC6538 and ATCC BAA-39 human isolates contained an additional pathogenicity island encoding enterotoxin genes (*entK* and *entQ*) that is not present in the version of φPT1028 that is associated with bovine isolates. These two enterotoxin genes; however, were also present in 34.4% (42/122) of human-associated *S. aureus* isolates. Another prophage common to bovine isolates, φJS01, carried *scn* and *sak* and was inserted into *hlb* locus. The isolates ATCC 6538 and ATCC BAA-39 shared one *hsdS* with the bovine CC97 isolates, and showed defective Type I R-M genes, indicating a possible diverged evolutionary path via HGT (Figure 3.4).

Sa1158c (ST8), which was isolated from a bovine sample, encoded neither bovine-specific virulence genes nor the human immune evasion cluster carried by β-converting prophages. Sa1158c contained pSa1158c, a plasmid that encoded seven ORFs, but no known host adaptation genes. The pSa1158c plasmid was only found in the Sa1158c isolate, and did not have high sequence homology to any other plasmid. Type I R-M system genes were present, as with other ST8 isolates, although the presence of one of the *hsdM* genes was not confirmed due to incomplete genome assembly.

3.4.4 Distribution of R-M systems and clonal diversification

Using RESBASE database, four R-M systems (Type I, II, III and IV) were found among the 187 *S. aureus* genomes. Both human and bovine IMI-associated *S. aureus* isolates mainly possessed Type I

and/or II R-M systems and only few human isolates carried Type III/IV R-M genes (Figure 3.4). As a part of the core genome, the Type I R-M system is a primary barrier to free HGT, and the combination of two *hsdS* gene copies was found to be lineage-specific. In bovine IMI-associated *S. aureus* isolates, Type II R-M system seemed to be more ST-specific (Figure 3.3). In human *S. aureus* isolates, R-M systems in MGEs were either isolate-specific or ST-specific (Figure 3.4).

Bovine isolates had unique *hsdS* genes in two genomic islands (νSaα and νSaβ) and HsdS with > 95% amino acid sequence homology was only found in two ST80 human isolates (11819-97 and GR2). However, the combination of two Type 1 R-M system *hsdS* genes was unique in bovine-adapted STs. The two major bovine CCs (CC151 and CC97) carried unique *hsdS* alleles, which provide specificity to the Type I R-M enzymes. In CC151, the *hsdS* gene in νSaβ was truncated and additional *hsdS* genes were found in a prophage, likely to aid the primary Type I R-M system owing to high amino acid homology of this additional *hsdS* with the primary Type I *hsdS* found in other STs. All bovine CC97 isolates carried two sets of Type I R-M systems. Unlike the original Type I R-M genes, an additional *hsdRMS* locus was located near *orfX* (Figure 3.4). In ST2187 (CC97), due to the mutation in *hsdR* the primary Type I R-M was inactivated, yet the presence of additional *hsdRMS* suggested it replaced the original Type I R-M system in this ST.

All bovine isolates, except Sa1158c, possessed Type II R-M genes encoding two subunits (Mod and Res) in putative genomic islands. Although CC151 and CC126 shared the same Type II R-M genes, defective genes were found in SauRF122 Res subunit in all ST2270 isolates. Interestingly, Type II R-M genes in ST352 (CC97) were found right beside bovine-specific virulence genes *lukMF'*, suggesting that dissemination of these virulence genes is likely to be limited within ST352 and closely related STs.

3.4.5 Bovine IMI-associated S. aureus virulence factors and AMR genes in MGEs

To better understand the correlation between the distribution of virulence/AMR genes and *S. aureus* R-M systems, we examined virulence/AMR genes (Figure 3.3). Due to the lack of information regarding the chronological acquisition order of virulence/AMR and R-M genes, we only investigated their distribution. Their causal relationships were not taken into account in this analysis.

We identified a total of 103 virulence genes from 65 bovine IMI-associated *S. aureus* isolates, which included adhesions (n = 17), enzymes (n = 15), immune evasion elements (n = 23), secretion system (n = 12), and toxins (n = 36) (Appendix 4). Most virulence genes were conserved in all bovine isolates as a part of the core genome. It is noteworthy that the ST2187 isolate (CC97), in which the *hsdR* was inactivated, carried a bovine variant gene encoding for vWbp. Several toxin genes (n = 12) were located in MGEs and showed uneven distribution between STs. All CC151 isolates contained *selz* near *orfX* and an enterotoxin gene cluster (*seg*, *seln*, *selu*, *selk*, *selm*, and *selo*) in an intact prophage φSaBov closest to φIpla88. On the contrary, isolates from CC97 which had two *hsdRMS* sets were found to carry no enterotoxin gene cluster. We also found Sa3154 contained a pathogenicity island encoding three virulence factors (*tst*, *sec*, and *sell*) that was integrated adjacent to νSaα. Among CC97 isolates, only ST352 carried *lukMF'* in a putative prophage closest to φPV83, which contained Type II R-M genes for BcgI-like alpha/beta subunits serving as a self-restricted MGE. Interestingly, no CC lineage-specific virulence gene was found in CC126 although their Type I and II R-M genes were inactivated.

A total of 25 AMR genes were found using MEGARes database and Roary from 65 *S. aureus* isolated from bovine sources (Appendix 4). Of the 25 AMR genes, majority encoded efflux pumps (n = 10) and regulators (n = 7). A total of 14 genes were found in all bovine IMI-associated *S. aureus* isolates examined in this study: *aac*(3), *aph*(3), *arlR*, *arlS*, *pbuE*, *lmrS*, *mepA*, *mepB*, *slyA*,

mgrA, *norA*, *norB*, *rlmH* and *tet*(38). The remaining AMR genes (n = 11) in bovine isolates were located in MGEs, with the exception of *fosB*. Unlike the virulence genes identified, no AMR genes exclusive to bovine isolates were found. All CC126 isolates (n = 5) had *fosB*, and among them, only Sa2605 (CC126) carried *blaI*, *blaR*, and *blaZ*. In addition to the 14 core AMR genes, Sa1158c (CC8) contained eight more AMR genes: *fosB* in non-MGE, *mecR* and *mecA* at *orfX*, *blaI* and *blaZ* at the downstream of *SCCmec*, *aacA* in a genomic island, and *tetM* in another genomic island. Only Sa3 (CC151) among bovine IMI-associated isolates carried *tetK* in pSX10B1. Four isolates in ST352 (CC97) were found with *linA* carried by plasmids (Figure 3.3). The plasmids with *linA* were rarely present in human isolates (2.5 %, 3/122). Instead of carrying bovine-specific virulence genes, CC126 and CC8 isolated from bovine niche carried AMR genes which were highly restrained within the STs and not found in other STs. Unlike virulence genes, correlations between *S. aureus* R-M system and AMR genes were not found.

3.4.6 Gene content involved in bovine mastitis in non-MGEs

We additionally investigated *agr* genes and capsular biosynthesis genes that are associated with *S. aureus* quorum-sensing and pathogenesis (Figure 3.3). We investigated the autoinducing peptide (AIP) type in STs. Only AIP-I and II were found in bovine isolates, while all four known AIP types were found in human isolates; although only a minority of isolates (13.1%, 16/122) encoded either the AIP-III or AIP-IV precursor (Figure 3.5). All CC151 isolates encoded the AIP-I precursor, and others had *agrD* for the AIP-II precursor. The capsular biosynthesis gene cluster *capABCDEFGHIJKLMNOP* in CC151 was different from the other bovine isolates, specifically in four genes (*capHIJK*). CC151 encoded *cap8HIJK* while others carried *cap5HIJK*, as known that two capsular types are produced by bovine IMI-associated *S. aureus* isolates.

3.5 Discussion

In this study, we highlighted the pan-genome of 187 *S. aureus* isolates obtained from bovine and humans and compared their gene contents. Human *S. aureus* isolates (n = 122) contributed significantly to the pan-genome, which was mainly from the accessory genes. The origin of the isolates, collection period and area, and quality of the assembled genomes likely influence the pan-genome size (Bosi et al., 2016; Setubal et al., 2018). The accessory genomes of bovine and human isolates were composed of 1,375 and 3,771 genes, respectively. *S. aureus* genomes originated from humans used in this study were from various geographical areas over a broad range of collection years, while bovine *S. aureus* isolates were mainly collected for 2 years in Canada (Appendix 1). Moreover, the completeness of *S. aureus* genomes from humans was much better than bovine IMI-associated *S. aureus* genomes.

We examined host-specific genes of *S. aureus* isolated from both humans and cows. *S. aureus* host-specificity is closely correlated to genetic lineage – CC97, CC126, CC133, and CC151 are bovine adapted lineages, while human-adapted lineages include CC1, CC5, CC8, CC30, and CC45 (Park & Ronholm, 2021). The pathogenicity island known as SaPIbov3 carries four bovine-specific genes and has been reported to be exclusively found in bovine isolates (Kozytska et al., 2010). In this study, SaPIbov3 was not found in bovine isolates from either the CC126 or CC8 lineages. Similarly, human-specific genes such as *lytN, fmhC, dprA*, *scn*, *chp*, and *sak* were encoded within the majority of human STs, yet not all human isolates. The distribution of these host-specific genes suggests that there is no absolute or universal host-specific element, although they can increase an opportunity for the successful adaptation in a new host niche. Alternatively, losing human-specific MGEs and acquiring a single mutation may also confer a fitness advantage and alter host tropism (Resch et al., 2013; Viana et al., 2015).

From the phylogenomic tree, we found three suspicious host jumping isolates: ATCC 6538 and ATCC BAA-39 in CC97 and Sa1158c in CC8. Zoonotic and zooanthroponotic transfers of *S. aureus* between humans and cows in both CC97 and CC8 have been reported in other studies (Matuszewska et al., 2020; Schmidt et al., 2017; Spoor et al., 2013). It is still speculative whether these three *S. aureus* isolates truly result from the host-switching between humans and cows. However, the genetic elements in these isolates could provide an insight into the potential host jumping route or mechanism of other isolates in the same CC. The two human isolates (CC97), ATCC 6538 and ATCC BAA-39, carried two enterotoxin genes in a pathogenicity island and the immune evasion cluster located in β-converting prophage φJS01, which are highly associated with virulence and host-adaptation (Jia et al., 2015; McClure et al., 2018). Thus, these two CC97 isolates seem to be successfully adapted to the new host by acquiring this human immune evasion cluster. We previously observed that plasmid transformation from RN4220 (CC8) to CC97 more efficient than those from CC8 to CC151 under laboratory conditions (Nair et al., 2011; Park et al., 2021). This indicates CC97 is more prone to be accept genes from human-originated *S. aureus* than CC151, increasing its chance to survive in human niche. On the contrary, no host-specific gene was found in bovine-isolated Sa1158c (CC8). However, the loss of a beta-converting prophage has shown to be associated with human-to-bovine jump of *S. aureus*. The possibility of spillover and transmission of CC8 from human to bovine is still valid in this specific isolate and more zooanthroponotic transfer is possible via the same manner under the right selection pressure while repeated exposure of CC8 to bovine occurs.

Most genes exclusive to bovine-associated *S. aureus* were located within MGEs. These MGEs explain that clonal diversification of *S. aureus* may occur via HGT during the adaptation to the bovine niche. The differences between CC97 and CC151, two major bovine lineages, in MGEs were enterotoxin gene cluster in CC151 and *vWbp* for bovine-specific coagulase in CC97. Although

CC151 encoded more toxin genes than CC97, we found *tst* located in SaPIbov in RF122 and Sa3154, probably due to ST bias to ST151 that was previously reported not to carry SaPIbov (Wilson et al., 2018). We showed that all bovine-associated *S. aureus* had Type I R-M genes with a unique combination of *hsdS* genes in different lineages, and many bovine isolates also carried Type II R-M genes. This lineage- or ST-specific genetic barrier suggests that R-M systems in *S. aureus* are, at least in part, responsible for shaping clonal diversification. The original and additional Type I R-M systems are unlikely to form an interchangeable functional complex due to the low amino acid identity between the various subunits. This additional HsdRMS may play a critical role in ST2187 (CC97) due to a defective *hsdR* in the original complex and an overall enhanced genetic barrier in CC97. ST2270 (CC126) may be a restriction-defective ST with no known functional R-M system due to the inactivated HsdR (Type I R-M) and Res subunit (Type II R-M). However, restriction endonuclease deficient *S. aureus* strains are not necessarily hypersusceptible to gene transfer. We observed that ST2270 isolates did not carry more MGEs than other STs. It was previously demonstrated that the inactivation of Type I R-M system was insufficient to construct *S. aureus* mutants capable of efficiently accepting foreign DNA (Veiga & Pinho, 2009). *S. aureus* may naturally develop another barrier for gene transfer because lacking R-M system is more vulnerable to bacteriophage suggesting its detrimental effect over beneficial effect (Moller et al., 2019).

Of note, *S. aureus* R-M systems are not an absolute barrier for gene transfers. Under certain environmental pressure, increased dissemination of MGEs can occur within a lineage or across different lineages. Exposure to antibiotic pressure is one stimulator of genetic dissemination since antibiotic-induced SOS response promotes HGT of pathogenicity islands (Ubeda et al., 2005). This HGT network raises concerns regarding the dissemination of AMR genes from human to bovine hosts via host transmission we described in this study. Sa1158c (ST8) carrying *blaZ* and *mecA* is a potential donor of AMR genes to *S. aureus* in the bovine niche. AMR genes (*mecA* and *blaZ*) have

been identified in ST97 (CC97) and ST126 (CC126) in Brazil, suggesting that right selective pressure may overcome or bypass the genetic barrier to disseminate AMR genes in bovine-adapted lineages (Oliveira et al., 2016). *S. aureus* ST97 is a MRSA lineage extensively found in pigs and dairy cattle in Italy (Feltrin et al., 2016). Although ST97 shares the same Type I R-M genes with other STs of CC97 (Cormican & Keane, 2018), it does not carry Type II R-M genes that are present in ST352, making HGT from ST97 to ST352 more challenging than other STs of CC97. However, the HGT network of AMR genes in bovine-adapted lineage CC97 is already open under the right selective pressure.

Interestingly, we also observed antagonistic characteristics of CC151 and CC97 against each other. CC151 and CC97 encoded *agrD* for different AIP precursors involved in *S. aureus* quorum-sensing activity. AIP-I and II produced by *S. aureus* are known to exhibit cross-inhibition (Wang & Muir, 2016). Indeed, we previously confirmed that CC151 (*agr* type II) inhibited the quorum-sensing of CC97 (*agr* type I) in co-culture conditions (Park et al., 2021). The pCC97-1 plasmid, which is most homologous to pRJ80 plasmid with 99.78% identity, was found in CC97 isolates and encoded genes for aureocin 4181 products: aureocin peptides, known as heat-stable bacteriocins, bacteriocin immunity protein, bacteriocin regulatory protein, and bacteriocin exporter protein (Salustiano Marques-Bastos et al., 2020). Aureocin 4181 is known to exhibit strong antimicrobial activity against isolates of *Micrococcus luteus*, *Streptococcus agalactiase*, *S. aureus*, and other staphylococci (Salustiano Marques-Bastos et al., 2020). This bacteriocin may modify the microbial composition of the udder skin and teat canal thus disturbing the native microbiome. We also observed that CC97 carrying pCC97-1 plasmid inhibited the growth of CC151 *in vitro* (Park et al., 2021). The antagonistic relationship within bovine-adapted *S. aureus* lineages (CC97 and CC151) suggests that they have evolved independently and are unlikely to dominate the same host at the same time point.

The main limitation of this study was the lack of information on the chronological order of the acquisition of MGEs and R-M genes in *S. aureus*, leading to a failure to elucidate the causal relationships between them. Also, sequence recognition sites of each R-M enzyme commonly found in bovine IMI-associated *S. aureus* are unknown, so the presence of cognition sequences in MGEs could not be determined. An additional limitation of this study was from the incomplete draft genomes of bovine IMI-associated *S. aureus*. It is often recommended to use a draft genome with a "near finished" status (less than 1% missing fraction) in pan-genome computations (Setubal et al., 2018). Apart from common criteria such as GC%, N50, and a number of contigs, the genome size and exclusion of draft genomes smaller than 2.65 Mb, corresponding to < 97% of *S. aureus* RF122 genome size, was performed in this study. Some important genes in a few isolates were not assembled, yet the genes were still present. To confirm the presence of target genes, such as *hsdMS*, PCR amplification and Sanger sequencing needed to be performed. The presence of plasmids was also confirmed by plasmid DNA extraction and mapping to verify the size of the predicted plasmids. Lastly, 63 bovine IMI-associated *S. aureus* isolates used in this study give a bias to Canada while human isolates were from various continents. This strong geographical bias may result in misleading the data interpretation such as CC lineage-specific exclusive genes.

3.6 Conclusion

The genetic differences among bovine IMI-associated *S. aureus* lineages reveal that *S. aureus* in the bovine niche has evolved in multiple directions. Our results suggest that bovine-specific and exclusive genes, which are mainly located in MGEs, play an important role in clonal diversification and host adaptation. Moreover, R-M systems in *S. aureus* shape *S. aureus* clonal diversification and pathogenicity by discriminating MGEs. We highlight that *S. aureus* ST identification in dairy herds is important to assess the risk of transmission and intervention strategies due to the various potential

impacts of certain STs on dairy cows. We also bring attention to the possible MRSA transmission from humans to cows, suggesting the continued importance of farm biosecurity

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Author contributions

The responsibilities for conceptualization, supervision, project administration and funding for this paper were provided by J. Ronholm. and S. D.. F.D. provided the assembly software ProkaryoteAssembly. S. P., D. J., J. Ruffini, A. D., E. D., and J-F. L. performed whole-genome sequencing. The analysis tasks and tools were shared between S. P. and D. J. For data analysis, S. P performed the formal analysis, investigation, data curation and visualization and B. O. participated in R-M gene and MGEs analysis. The original draft of the manuscript was prepared by S. P. All authors were responsible for the review and editing of the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

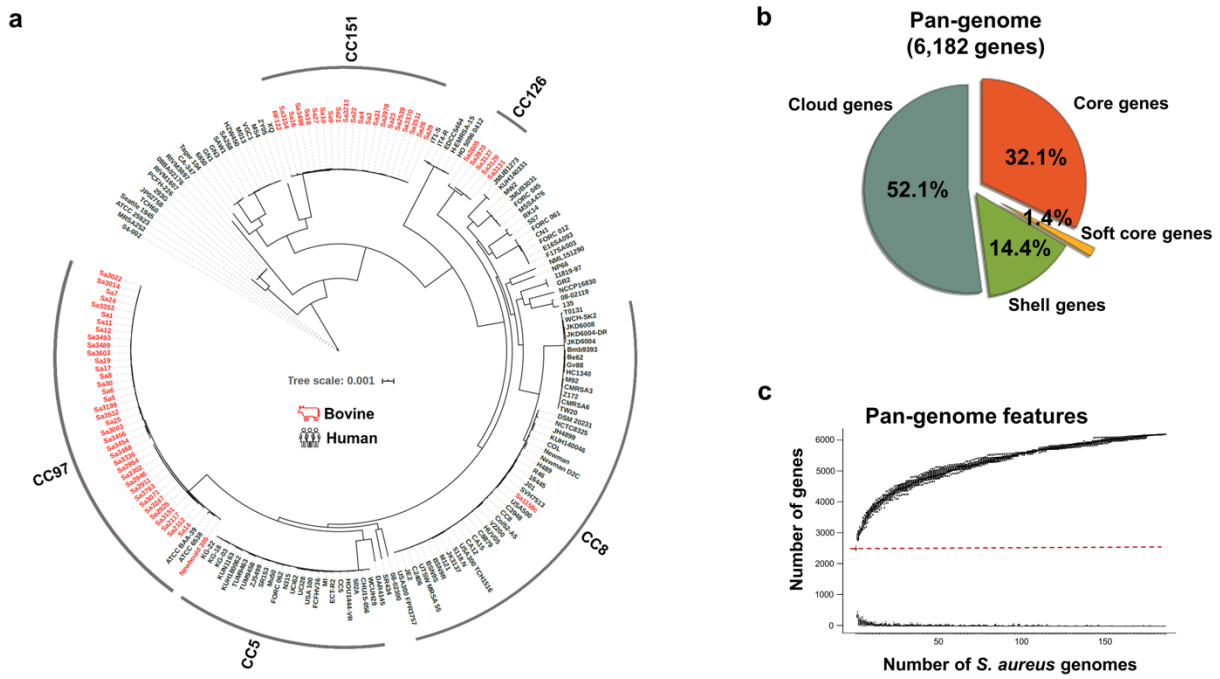

Figure 3.1 Phylogenomic tree and pan-genome of 187 *Staphylococcus aureus* from human and bovine origins. (A) All bovine IMI-associated *S. aureus* isolates except Sa1158c (CC8) were clustered into three main clades: CC151, CC126, and CC97. (B) The pan-genome of 187 *S. aureus* isolates were subdivided into four groups: core (genes present in 99% ≤ isolates ≤ 100%), soft core (95% ≤ isolates < 99%), shell (15% ≤ isolates < 95%), and cloud genes (0% ≤ isolates < 15%). (C) The red dotted line divides the graph into two features: total pan-genome in size (top) and the number of new genomes added to the total pan-genome (bottom) as new *S. aureus* genomes are added. The graph indicates an open state, and new genes are likely to be discovered continually as new genomes are added to the analysis.

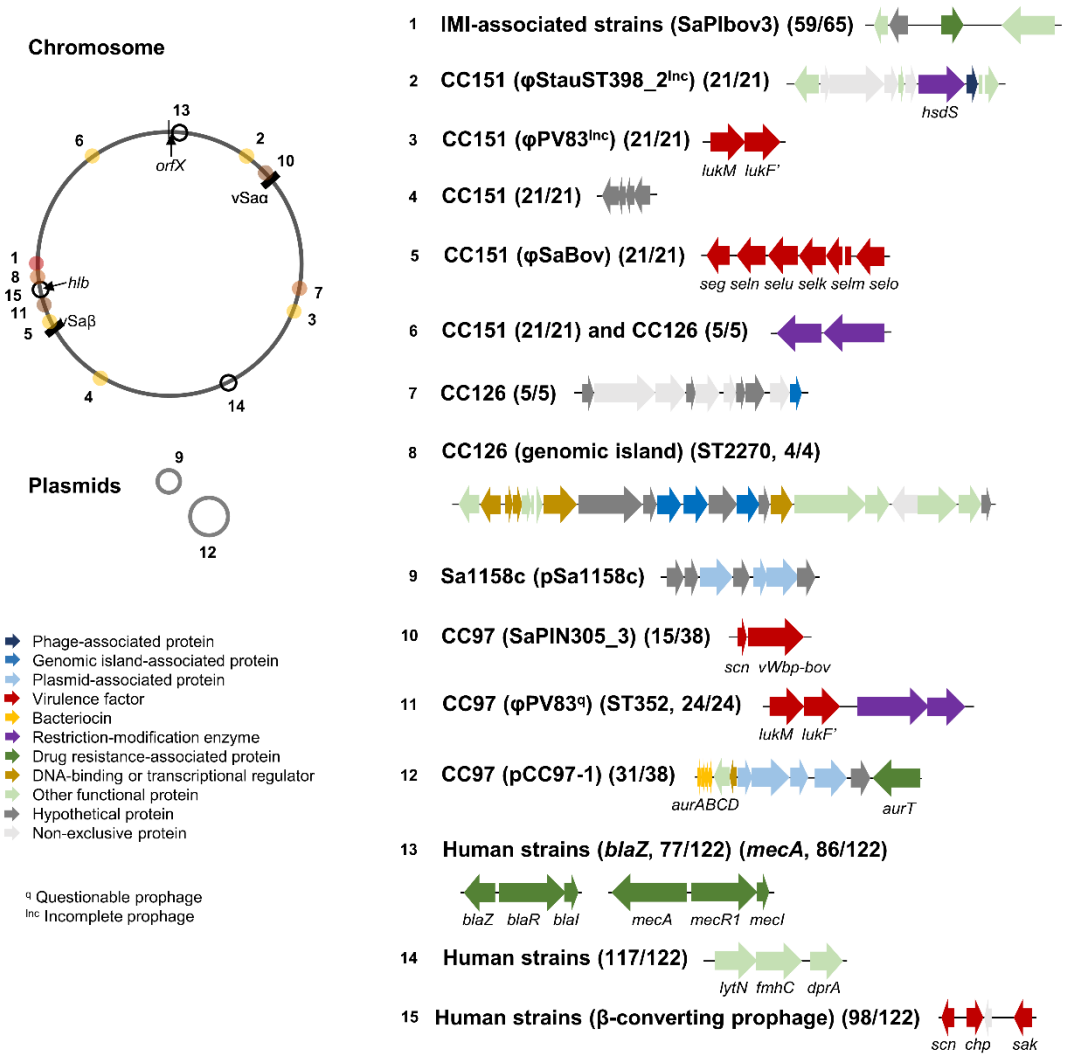

Figure 3.2 Distribution of lineage-specific genes in *Staphylococcus aureus*. This figure illustrates the genes present mainly in bovine IMI-associated *S. aureus* isolates. The unequally distributed genes were shown with their associated lineages, MGEs, and frequency. A reference genome, *S. aureus* RF122, was used to identify the relative location of these genes in the genome as indicated by the corresponding numbers (1-15).

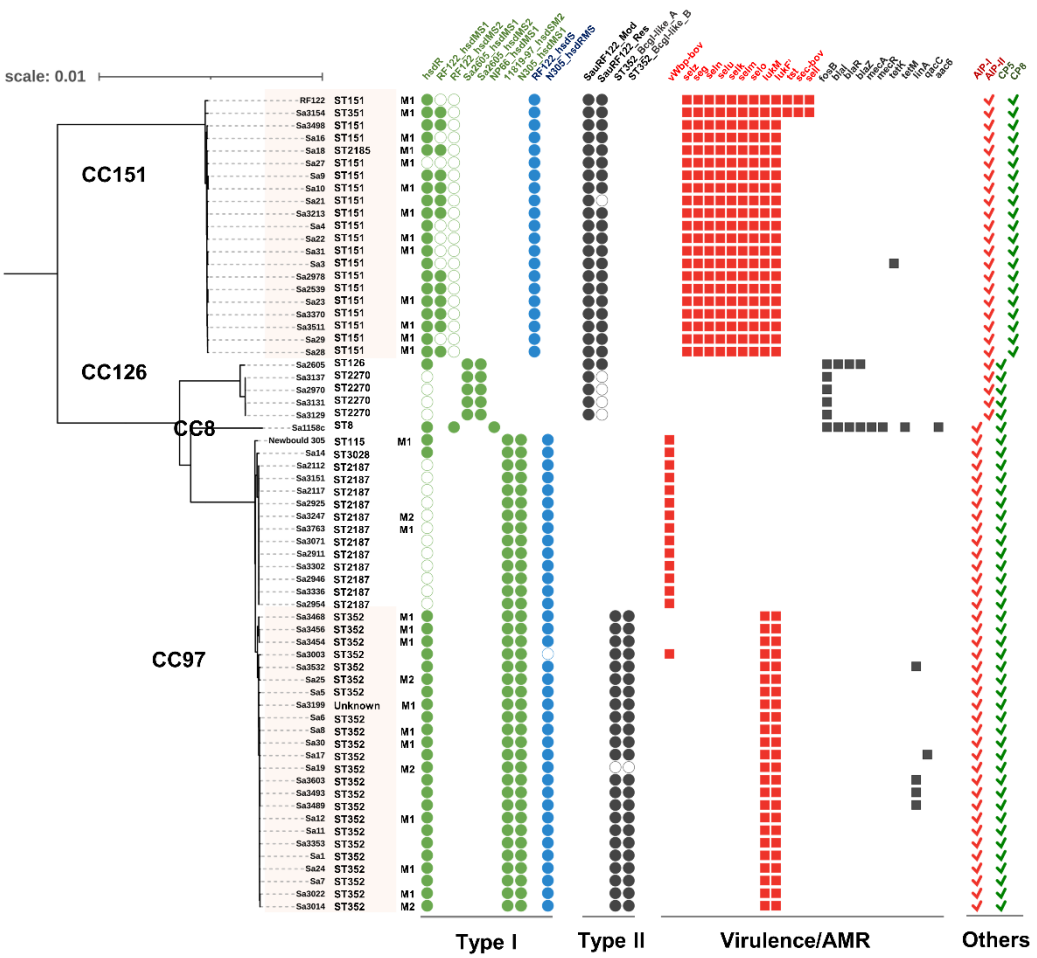

Figure 3.3 Distribution of restriction-modification genes and virulence/AMR genes in bovine IMI-associated *Staphylococcus aureus*. The phylogenomic tree of 65 bovine IMI-associated *S. aureus* and relevant genetic content show lineage-specific R-M genes. Virulence and AMR genes are either ST-specific or isolate-specific. In the Type I R-M system, the green circular boxes indicate the Type I R-M system genes (*hsdR* and *hsdMS*) that are located in νSaα and νSaβ and are part of the core genome. The blue circular boxes show additional Type I R-M *hsdRMS* genes which are not a part of the *S. aureus* core genome. The *hsdR* and *hsdM* genes are highly conserved within the *S. aureus* species; however, several *hsdS* alleles exist. While each of the *hsdMS* genes shown in green are interchangeable, part of the same R-M system, and should, in combination with the *hsdR*, form a functional complex, the Type I R-M system genes shown in blue would not be expected to be interchangeable, and would instead form a separate, and independent Type I R-M system. The black circular boxes represent Type II R-M genes that encode a pair of enzymes: a methyltransferase and a restriction endonuclease. The red and the black square boxes represent virulence and AMR genes, respectively. The open circular boxes in all shapes and colors indicate the presence of a pseudogene. The red and green checkmarks indicate signal molecule (AIP-I and AIP-II) and capsular polysaccharide (CP5 and CP8). The highlighted isolates were associated with clinical mastitis. M1 is used to indicate that the milk sample was collected on the day clinical mastitis was diagnosed, and M2 indicates the sample was collected 14 days after a diagnosis of clinical mastitis was made. An extended report of each of the R-M genes identified in all isolates included in this study can be found in Figure 3.4.

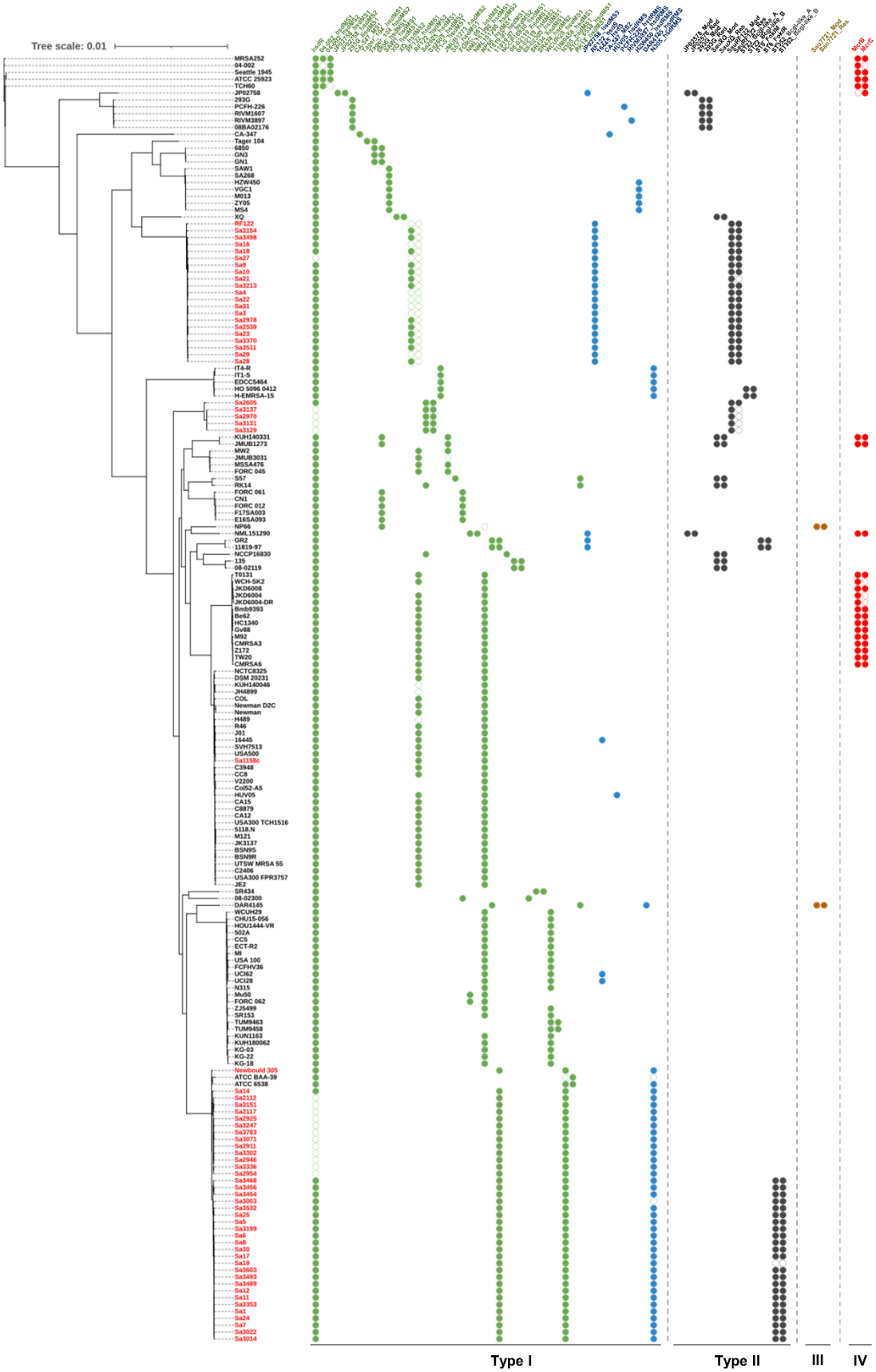
Tree scale: 0.01
Type I
Type II
III
IV

Figure 3.4 Distribution of restriction-modification genes in 187 *Staphylococcus aureus*. The phylogenomic tree of 187 *S. aureus* and aligned R-M genes show the lineage-specific distribution of the R-M genes. Both human and bovine IMI-associated *S. aureus* isolates mainly possessed TypeI/II R-M systems and only few human isolates carried TypeIII/IV R-M genes. The isolates in red in the phylogenomic tree were originated from bovine. The colored circles represent the presence of the R-M genes, and the open circles indicate the inactivated R-M genes.

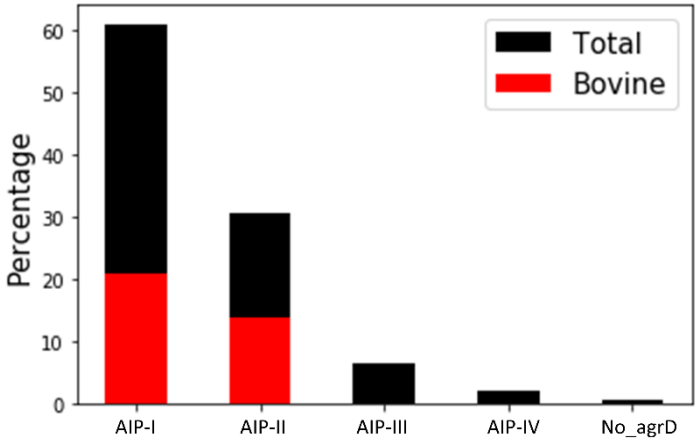

Figure 3.5 Distribution of AIPs in 187 *Staphylococcus aureus*. AIP-I precursor was the most prevalent in *S. aureus* in this study, followed by AIP-II. AIP-I and II were found in bovine isolates (n = 65), while all four known AIP types were found in human isolates (n = 122). Among human isolates, only minor population encoded AIP-III (n = 13) and AIP-IV (n = 4).

3.7 References

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., & Wishart, D. S. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res*, *44*(W1), W16-21. https://doi.org/10.1093/nar/gkw387

Atalla, H., Gyles, C., & Mallard, B. (2010). Persistence of a Staphylococcus aureus small colony variants (S. aureus SCV) within bovine mammary epithelial cells. *Vet Microbiol*, *143*(2-4), 319-328. https://doi.org/10.1016/j.vetmic.2009.11.030

Bagcigil, A. F., Taponen, S., Koort, J., Bengtsson, B., Myllyniemi, A. L., & Pyorala, S. (2012). Genetic basis of penicillin resistance of S. aureus isolated in bovine mastitis. *Acta Vet Scand*, *54*, 69. https://doi.org/10.1186/1751-0147-54-69

Becker, K., Schaumburg, F., Kearns, A., Larsen, A. R., Lindsay, J. A., Skov, R. L., & Westh, H. (2019). Implications of identifying the recently defined members of the Staphylococcus aureus complex S. argenteus and S. schweitzeri: a position paper of members of the ESCMID Study Group for Staphylococci and Staphylococcal Diseases (ESGS). *Clin Microbiol Infect*, *25*(9), 1064-1070. https://doi.org/10.1016/j.cmi.2019.02.028

Bertelli, C., Laird, M. R., Williams, K. P., Simon Fraser University Research Computing, G., Lau, B. Y., Hoad, G., Winsor, G. L., & Brinkman, F. S. L. (2017). IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Res*, *45*(W1), W30-W35. https://doi.org/10.1093/nar/gkx343

Bogdanova, E., Djordjevic, M., Papapanagiotou, I., Heyduk, T., Kneale, G., & Severinov, K. (2008).

Transcription regulation of the type II restriction-modification system AhdI. *Nucleic Acids Res*, *36*(5), 1429-1442. https://doi.org/10.1093/nar/gkm1116

Bosi, E., Monk, J. M., Aziz, R. K., Fondi, M., Nizet, V., & Palsson, B. O. (2016). Comparative genome-scale modelling of Staphylococcus aureus strains identifies strain-specific metabolic capabilities linked to pathogenicity. *Proc Natl Acad Sci U S A*, *113*(26), E3801-3809. https://doi.org/10.1073/pnas.1523199113

Carattoli, A., Zankari, E., Garcia-Fernandez, A., Voldby Larsen, M., Lund, O., Villa, L., Moller Aarestrup, F., & Hasman, H. (2014). In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother*, *58*(7), 3895-3903. https://doi.org/10.1128/AAC.02412-14

Cormican, P., & Keane, O. M. (2018). Complete Genome Sequences of Sequence Type 71 (ST71) and ST97 Staphylococcus aureus Isolates from Bovine Milk. *Microbiol Resour Announc*, *7*(5). https://doi.org/10.1128/MRA.00954-18

Corvaglia, A. R., Francois, P., Hernandez, D., Perron, K., Linder, P., & Schrenzel, J. (2010). A type III-like restriction endonuclease functions as a major barrier to horizontal gene transfer in clinical Staphylococcus aureus strains. *Proc Natl Acad Sci U S A*, *107*(26), 11954-11958. https://doi.org/10.1073/pnas.1000489107

Cuny, C., Wieler, L. H., & Witte, W. (2015). Livestock-Associated MRSA: The Impact on Humans. *Antibiotics (Basel)*, *4*(4), 521-543. https://doi.org/10.3390/antibiotics4040521

Demontier E, D.-D. A., Brouillette E, Larose A, Ster C, Lucier JF, Rodrigue S, Park S, Jung D, Ruffini J, Ronholm J, Dufour S, Roy JP, Ramanathan S, Malouin F. (2021). Relative virulence of Staphylococcus aureus bovine mastitis strains representing the main Canadian spa types and clonal complexes as determined using in vitro and in vivo mastitis models. *J Dairy Sci*.

Dufour, S., Labrie, J., & Jacques, M. (2019). The Mastitis Pathogens Culture Collection. *Microbiol Resour Announc*, *8*(15). https://doi.org/10.1128/MRA.00133-19

Feltrin, F., Alba, P., Kraushaar, B., Ianzano, A., Argudin, M. A., Di Matteo, P., Porrero, M. C., Aarestrup, F. M., Butaye, P., Franco, A., & Battisti, A. (2016). A Livestock-Associated, Multidrug-Resistant, Methicillin-Resistant Staphylococcus aureus Clonal Complex 97 Lineage Spreading in Dairy Cattle and Pigs in Italy. *Appl Environ Microbiol*, *82*(3), 816-821. https://doi.org/10.1128/AEM.02854-15

Fox, L. K., Zadoks, R. N., & Gaskins, C. T. (2005). Biofilm production by Staphylococcus aureus associated with intramammary infection. *Vet Microbiol*, *107*(3-4), 295-299. https://doi.org/10.1016/j.vetmic.2005.02.005

Haran, K. P., Godden, S. M., Boxrud, D., Jawahir, S., Bender, J. B., & Sreevatsan, S. (2012). Prevalence and characterization of Staphylococcus aureus, including methicillin-resistant Staphylococcus aureus, isolated from bulk tank milk from Minnesota dairy farms. *J Clin Microbiol*, *50*(3), 688-695. https://doi.org/10.1128/JCM.05214-11

Hebert, A., Sayasith, K., Senechal, S., Dubreuil, P., & Lagace, J. (2000). Demonstration of intracellular Staphylococcus aureus in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. *FEMS Microbiol Lett*, *193*(1), 57-62. https://doi.org/10.1111/j.1574-6968.2000.tb09402.x

Herron-Olson, L., Fitzgerald, J. R., Musser, J. M., & Kapur, V. (2007). Molecular correlates of host specialization in Staphylococcus aureus. *PLoS One*, *2*(10), e1120. https://doi.org/10.1371/journal.pone.0001120

Jamali, H., Radmehr, B., & Ismail, S. (2014). Short communication: prevalence and antibiotic resistance of Staphylococcus aureus isolated from bovine clinical mastitis. *J Dairy Sci*, *97*(4), 2226-2230. https://doi.org/10.3168/jds.2013-7509

Jia, H., Dong, W., Yuan, L., Ma, J., Bai, Q., Pan, Z., Lu, C., & Yao, H. (2015). Characterization and complete genome sequence analysis of Staphylococcus aureus bacteriophage JS01. *Virus Genes*, *50*(2), 345-348. https://doi.org/10.1007/s11262-015-1168-y

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., & Madden, T. L. (2008). NCBI BLAST: a better web interface. *Nucleic Acids Res*, *36*(Web Server issue), W5-9. https://doi.org/10.1093/nar/gkn201

Jolley, K. A., & Maiden, M. C. J. (2010). BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics*, *11*(1), 595. https://doi.org/10.1186/1471-2105-11-595

Jung, D., Park, S., Ruffini, J., Dussault, F., Dufour, S., & Ronholm, J. (2021). Comparative genomic analysis of Escherichia coli isolates from cases of bovine clinical mastitis identifies nine specific pathotype marker genes. *Microb Genom*, *7*(7). https://doi.org/10.1099/mgen.0.000597

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., & Jermiin, L. S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods*, *14*(6), 587-589. https://doi.org/10.1038/nmeth.4285

Kappeli, N., Morach, M., Corti, S., Eicher, C., Stephan, R., & Johler, S. (2019). Staphylococcus aureus related to bovine mastitis in Switzerland: Clonal diversity, virulence gene profiles, and antimicrobial resistance of isolates collected throughout 2017. *J Dairy Sci*, *102*(4), 3274-3281. https://doi.org/10.3168/jds.2018-15317

Klibi, A., Jouini, A., Gomez, P., Slimene, K., Ceballos, S., Torres, C., & Maaroufi, A. (2018). Molecular Characterization and Clonal Diversity of Methicillin-Resistant and -Susceptible Staphylococcus aureus Isolates of Milk of Cows with Clinical Mastitis in Tunisia. *Microb Drug Resist*, *24*(8), 1210-1216. https://doi.org/10.1089/mdr.2017.0278

Kozytska, S., Stauss, D., Pawlik, M. C., Hensen, S., Eckart, M., Ziebuhr, W., Witte, W., & Ohlsen, K. (2010). Identification of specific genes in Staphylococcus aureus strains associated with bovine mastitis. *Vet Microbiol*, *145*(3-4), 360-365. https://doi.org/10.1016/j.vetmic.2010.03.020

Lakin, S. M., Dean, C., Noyes, N. R., Dettenwanger, A., Ross, A. S., Doster, E., Rovira, P., Abdo, Z., Jones, K. L., Ruiz, J., Belk, K. E., Morley, P. S., & Boucher, C. (2017). MEGARes: an antimicrobial resistance database for high throughput sequencing. *Nucleic Acids Res*, *45*(D1), D574-D580. https://doi.org/10.1093/nar/gkw1009

Lee, J. Y. H., Carter, G. P., Pidot, S. J., Guerillot, R., Seemann, T., da Silva, A. G., Foster, T. J., Howden, B. P., Stinear, T. P., & Monk, I. R. (2019). Mining the Methylome Reveals Extensive Diversity in Staphylococcus epidermidis Restriction Modification. *mBio*, *10*(6). https://doi.org/ARTN e02451-19
10.1128/mBio.02451-19

Letunic, I., & Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res*, *44*(W1), W242-245. https://doi.org/10.1093/nar/gkw290

Lindsay, J. A., & Holden, M. T. (2004). Staphylococcus aureus: superbug, super genome? *Trends Microbiol*, *12*(8), 378-385. https://doi.org/10.1016/j.tim.2004.06.004

Liu, B., Zheng, D., Jin, Q., Chen, L., & Yang, J. (2019). VFDB 2019: a comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Res*, *47*(D1), D687-D692. https://doi.org/10.1093/nar/gky1080

Marques, V. F., Motta, C. C., Soares, B. D., Melo, D. A., Coelho, S. M., Coelho, I. D., Barbosa, H. S., & Souza, M. M. (2017). Biofilm production and beta-lactamic resistance in Brazilian

Staphylococcus aureus isolates from bovine mastitis. *Braz J Microbiol*, *48*(1), 118-124. https://doi.org/10.1016/j.bjm.2016.10.001

Matuszewska, M., Murray, G. G. R., Harrison, E. M., Holmes, M. A., & Weinert, L. A. (2020). The Evolutionary Genomics of Host Specificity in Staphylococcus aureus. *Trends Microbiol*, *28*(6), 465-477. https://doi.org/10.1016/j.tim.2019.12.007

McClure, J. M., Lakhundi, S., Kashif, A., Conly, J. M., & Zhang, K. (2018). Genomic Comparison of Highly Virulent, Moderately Virulent, and Avirulent Strains From a Genetically Closely-Related MRSA ST239 Sub-lineage Provides Insights Into Pathogenesis. *Front Microbiol*, *9*, 1531. https://doi.org/10.3389/fmicb.2018.01531

Moller, A. G., Lindsay, J. A., & Read, T. D. (2019). Determinants of Phage Host Range in Staphylococcus Species. *Appl Environ Microbiol*, *85*(11). https://doi.org/10.1128/AEM.00209-19

Nair, D., Memmi, G., Hernandez, D., Bard, J., Beaume, M., Gill, S., Francois, P., & Cheung, A. L. (2011). Whole-genome sequencing of Staphylococcus aureus strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. *J Bacteriol*, *193*(9), 2332-2335. https://doi.org/10.1128/JB.00027-11

Naushad, S., Nobrega, D. B., Naqvi, S. A., Barkema, H. W., & De Buck, J. (2020). Genomic Analysis of Bovine Staphylococcus aureus Isolates from Milk To Elucidate Diversity and Determine the Distributions of Antimicrobial and Virulence Genes and Their Association with Mastitis. *mSystems*, *5*(4). https://doi.org/10.1128/mSystems.00063-20

Okonechnikov, K., Conesa, A., & Garcia-Alcalde, F. (2016). Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics*, *32*(2), 292-294. https://doi.org/10.1093/bioinformatics/btv566

Oliveira, C. J., Tiao, N., de Sousa, F. G., de Moura, J. F., Santos Filho, L., & Gebreyes, W. A. (2016). Methicillin-Resistant Staphylococcus aureus from Brazilian Dairy Farms and Identification of Novel Sequence Types. *Zoonoses Public Health*, *63*(2), 97-105. https://doi.org/10.1111/zph.12209

Oliveros, J. (2017). 2007–2015. Venny. An interactive tool for comparing lists with Venn's diagrams. In.

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T., Fookes, M., Falush, D., Keane, J. A., & Parkhill, J. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics*, *31*(22), 3691-3693. https://doi.org/10.1093/bioinformatics/btv421

Park, S., Classen, A., Gohou, H. M., Maldonado, R., Kretschmann, E., Duvernay, C., Kim, G. J., & Ronholm, J. (2021). A new, reliable, and high-throughput strategy to screen bacteria for antagonistic activity against Staphylococcus aureus. *BMC Microbiol*, *21*(1), 189. https://doi.org/10.1186/s12866-021-02265-4

Park, S., Jung, D., Dufour, S., & Ronholm, J. (2020). Draft Genome Sequences of 27 Staphylococcus aureus Strains and 3 Staphylococcus Species Strains Isolated from Bovine Intramammary Infections. *Microbiol Resour Announc*, *9*(19). https://doi.org/10.1128/MRA.00300-20

Park, S., & Ronholm, J. (2021). Staphylococcus aureus in Agriculture: Lessons in Evolution from a Multispecies Pathogen. *Clin Microbiol Rev*, *34*(2). https://doi.org/10.1128/CMR.00182-20

Rainard, P., Foucras, G., Fitzgerald, J. R., Watts, J. L., Koop, G., & Middleton, J. R. (2018). Knowledge gaps and research priorities in Staphylococcus aureus mastitis control. *Transbound Emerg Dis*, *65 Suppl 1*, 149-165. https://doi.org/10.1111/tbed.12698

Resch, G., Francois, P., Morisset, D., Stojanov, M., Bonetti, E. J., Schrenzel, J., Sakwinska, O., & Moreillon, P. (2013). Human-to-bovine jump of Staphylococcus aureus CC8 is associated

with the loss of a beta-hemolysin converting prophage and the acquisition of a new staphylococcal cassette chromosome. *PLoS One*, *8*(3), e58187. https://doi.org/10.1371/journal.pone.0058187

Roberts, G. A., Houston, P. J., White, J. H., Chen, K., Stephanou, A. S., Cooper, L. P., Dryden, D. T., & Lindsay, J. A. (2013). Impact of target site distribution for Type I restriction enzymes on the evolution of methicillin-resistant Staphylococcus aureus (MRSA) populations. *Nucleic Acids Res*, *41*(15), 7472-7484. https://doi.org/10.1093/nar/gkt535

Roberts, R. J., Belfort, M., Bestor, T., Bhagwat, A. S., Bickle, T. A., Bitinaite, J., Blumenthal, R. M., Degtyarev, S., Dryden, D. T., Dybvig, K., Firman, K., Gromova, E. S., Gumport, R. I., Halford, S. E., Hattman, S., Heitman, J., Hornby, D. P., Janulaitis, A., Jeltsch, A., . . . Xu, S. Y. (2003). A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res*, *31*(7), 1805-1812. https://doi.org/10.1093/nar/gkg274

Roberts, R. J., Vincze, T., Posfai, J., & Macelis, D. (2003). REBASE: restriction enzymes and methyltransferases. *Nucleic Acids Res*, *31*(1), 418-420. https://doi.org/10.1093/nar/gkg069

Roberts, R. J., Vincze, T., Posfai, J., & Macelis, D. (2015). REBASE--a database for DNA restriction and modification: enzymes, genes and genomes. *Nucleic Acids Res*, *43*(Database issue), D298-299. https://doi.org/10.1093/nar/gku1046

Sadykov, M. R. (2016). Restriction-Modification Systems as a Barrier for Genetic Manipulation of Staphylococcus aureus. *Methods Mol Biol*, *1373*, 9-23. https://doi.org/10.1007/7651_2014_180

Saini, V., McClure, J. T., Scholl, D. T., DeVries, T. J., & Barkema, H. W. (2012). Herd-level association between antimicrobial use and antimicrobial resistance in bovine mastitis Staphylococcus aureus isolates on Canadian dairy farms. *J Dairy Sci*, *95*(4), 1921-1929. https://doi.org/10.3168/jds.2011-5065

Salustiano Marques-Bastos, S. L., Varella Coelho, M. L., Ceotto-Vigoder, H., Carlin Fagundes, P., Silva Almeida, G., Brede, D. A., Nes, I. F., Vasconcelos de Paiva Brito, M. A., & de Freire Bastos, M. D. C. (2020). Molecular characterization of aureocin 4181: a natural N-formylated aureocin A70 variant with a broad spectrum of activity. *Braz J Microbiol*, *51*(4), 1527-1538. https://doi.org/10.1007/s42770-020-00315-z

Schmidt, T., Kock, M. M., & Ehlers, M. M. (2017). Molecular Characterization of Staphylococcus aureus Isolated from Bovine Mastitis and Close Human Contacts in South African Dairy Herds: Genetic Diversity and Inter-Species Host Transmission. *Front Microbiol*, *8*, 511. https://doi.org/10.3389/fmicb.2017.00511

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics*, *30*(14), 2068-2069. https://doi.org/10.1093/bioinformatics/btu153

Setubal, J. C., Almeida, N. F., & Wattam, A. R. (2018). Comparative Genomics for Prokaryotes. *Methods Mol Biol*, *1704*, 55-78. https://doi.org/10.1007/978-1-4939-7463-4_3

Spoor, L. E., McAdam, P. R., Weinert, L. A., Rambaut, A., Hasman, H., Aarestrup, F. M., Kearns, A. M., Larsen, A. R., Skov, R. L., & Fitzgerald, J. R. (2013). Livestock origin for a human pandemic clone of community-associated methicillin-resistant Staphylococcus aureus. *mBio*, *4*(4). https://doi.org/10.1128/mBio.00356-13

Tong, S. Y. C., Schaumburg, F., Ellington, M. J., Corander, J., Pichon, B., Leendertz, F., Bentley, S. D., Parkhill, J., Holt, D. C., Peters, G., & Giffard, P. M. (2015). Novel staphylococcal species that form part of a Staphylococcus aureus-related complex: the non-pigmented Staphylococcus argenteus sp. nov. and the non-human primate-associated Staphylococcus schweitzeri sp. nov. *Int J Syst Evol Microbiol*, *65*(Pt 1), 15-22.

https://doi.org/10.1099/ijs.0.062752-0
Ubeda, C., Maiques, E., Knecht, E., Lasa, I., Novick, R. P., & Penades, J. R. (2005). Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. *Mol Microbiol*, *56*(3), 836-844. https://doi.org/10.1111/j.1365-2958.2005.04584.x
Veiga, H., & Pinho, M. G. (2009). Inactivation of the SauI type I restriction-modification system is not sufficient to generate Staphylococcus aureus strains capable of efficiently accepting foreign DNA. *Appl Environ Microbiol*, *75*(10), 3034-3038. https://doi.org/10.1128/AEM.01862-08
Viana, D., Blanco, J., Tormo-Mas, M. A., Selva, L., Guinane, C. M., Baselga, R., Corpa, J., Lasa, I., Novick, R. P., Fitzgerald, J. R., & Penades, J. R. (2010). Adaptation of Staphylococcus aureus to ruminant and equine hosts involves SaPI-carried variants of von Willebrand factor-binding protein. *Mol Microbiol*, *77*(6), 1583-1594. https://doi.org/10.1111/j.1365-2958.2010.07312.x
Viana, D., Comos, M., McAdam, P. R., Ward, M. J., Selva, L., Guinane, C. M., Gonzalez-Munoz, B. M., Tristan, A., Foster, S. J., Fitzgerald, J. R., & Penades, J. R. (2015). A single natural nucleotide mutation alters bacterial pathogen host tropism. *Nat Genet*, *47*(4), 361-366. https://doi.org/10.1038/ng.3219
Vrieling, M., Koymans, K. J., Heesterbeek, D. A., Aerts, P. C., Rutten, V. P., de Haas, C. J., van Kessel, K. P., Koets, A. P., Nijland, R., & van Strijp, J. A. (2015). Bovine Staphylococcus aureus Secretes the Leukocidin LukMF' To Kill Migrating Neutrophils through CCR1. *mBio*, *6*(3), e00335. https://doi.org/10.1128/mBio.00335-15
Waldron, D. E., & Lindsay, J. A. (2006). Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into Staphylococcus aureus and between S. aureus isolates of different lineages. *J Bacteriol*, *188*(15), 5578-5585. https://doi.org/10.1128/JB.00418-06
Wang, B., & Muir, T. W. (2016). Regulation of Virulence in Staphylococcus aureus: Molecular Mechanisms and Remaining Puzzles. *Cell Chem Biol*, *23*(2), 214-224. https://doi.org/10.1016/j.chembiol.2016.01.004
Watts, J. L. (1988). Etiological agents of bovine mastitis. *Vet Microbiol*, *16*(1), 41-66. https://doi.org/10.1016/0378-1135(88)90126-5
Wilson, G. J., Tuffs, S. W., Wee, B. A., Seo, K. S., Park, N., Connelley, T., Guinane, C. M., Morrison, W. I., & Fitzgerald, J. R. (2018). Bovine Staphylococcus aureus Superantigens Stimulate the Entire T Cell Repertoire of Cattle. *Infect Immun*, *86*(11). https://doi.org/10.1128/IAI.00505-18
Yang, F., Wang, Q., Wang, X. R., Wang, L., Xiao, M., Li, X. P., Luo, J. Y., Zhang, S. D., & Li, H. S. (2015). Prevalence of blaZ gene and other virulence genes in penicillin-resistant Staphylococcus aureus isolated from bovine mastitis cases in Gansu, China. *Turkish Journal of Veterinary & Animal Sciences*, *39*(5), 634-636. https://doi.org/10.3906/vet-1504-81

CONNECTING TEXT

In previous chapter, we showed distinct clades of bovine-associated *S. aureus* could be differentiated from human-originated *S. aureus* in phylogenomic tree. *S. aureus* CC151 and CC97 were two major CCs found in Canadian dairy farms. Host-specific genes and CC-specific genes were mainly located in MGEs. We specifically investigated virulence genes and AMR genes in bovine-associated *S. aureus* showing correlation with the presence of R-M genes. Our findings indicate that R-M systems may contribute to shaping clonal diversification by providing a genetic barrier to HGT and limiting genetic exchange between bovine-adapted *S. aureus* isolates and lineages that are adapted to different host species lineages. With the newfound knowledge from the previous chapter, we focused on developing a new plasmid-based strategy to screen bacteria with antagonistic activity towards *S. aureus* strains belonging to CC151 and CC97 (the most common bovine adapted lineages in Canada). The objective was to create a highly stable recombinant plasmids (pQS series) which carry a reporter gene under the control of a *S. aureus* quorum-sensing (QS) promoter (*agr*P3) to monitor *S. aureus* growth and QS simultaneously in co-culture condition. We also screened commensal bacteria isolated from dairy milk with antagonistic activity towards *S. aureus* using the pQS-based screening methods.

CHAPTER 4. A NEW RELIABLE HIGH-THROUGHPUT PLASMID-BASED STRATEGY TO SCREEN BACTERIA WITH ANTAGONISTIC ACTIVITY AGAINST *STAPHYLOCOCCUS AUREUS*

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4.1 Abstract

Background: Antibiotic-resistant *Staphylococcus aureus* clones have emerged globally over the last few decades. Probiotics have been actively studied as an alternative to antibiotics to prevent and treat *S. aureus* infections, but identifying new probiotic bacteria, that have antagonistic activity against *S. aureus*, is difficult since traditional screening strategies are time-consuming and expensive. Here, we describe a new plasmid-based method which uses highly stable plasmids to screen bacteria with antagonistic activity against *S. aureus*.

Results: We have created two recombinant plasmids (pQS1 and pQS3) which carry either gfp_{bk} or *mCherry* under the control of a *S. aureus* quorum-sensing (QS) promoter (*agr*P3). Using this recombinant plasmid pair, we tested 81 bacteria isolated from Holstein dairy milk to identify bacteria that had growth-inhibiting activity against *S. aureus* and suggest potential explanations for the growth inhibition. The stability test illustrated that pQS1 and pQS3 remained highly stable for at least 24 hours in batch culture conditions without selection pressure from antibiotics. This allowed co-culturing of *S. aureus* with other bacteria. Using the newly developed pQS plasmids, we found commensal bacteria, isolated from raw bovine milk, which had growth-inhibiting activity (n = 13) and quorum-quenching (QQ) activity (n = 13) towards both *S. aureus* Sa25 (CC97) and Sa27 (CC151). The pQS-based method is efficient and effective for simultaneously screening growth-inhibiting and QQ bacteria against *S. aureus* on agar media.

Conclusion: It was shown that growth-inhibiting and QQ activity toward pQS plasmid transformants of *S. aureus* can be simultaneously monitored by observing the zone of growth inhibition and reporter protein inhibition on agar plates. Newly identified antagonistic bacteria and their functional biomolecules are promising candidates for future development of probiotic drugs and prophylactics/therapeutics for bacterial infections including *S. aureus*. Furthermore, this new

approach can be a useful method to find bacteria that can be used to prevent and treat S. aureus infections in both humans and animals.

Keywords

Staphylococcus aureus, Quorum-sensing, Quorum-Quenching, pKK30

4.2 Background

Staphylococcus aureus is a common bacterial pathogen that has potential to cause serious infections in humans and several species of wild and agricultural animals (Park & Ronholm, 2021). *S. aureus* isolates have a remarkable level of variation in terms of metabolic potential, virulence, and antibiotic resistance (ABR) (Balasubramanian et al., 2017). Unfortunately, several multi-drug resistant *S. aureus* lineages have emerged in hospitals, community settings, and livestock operations globally over the last few decades (Park & Ronholm, 2021). Alternatives to antibiotics for the treatment and prevention of *S. aureus* infections in both human and veterinary medicine are needed. Probiotics have been suggested as a possible alternative to antibiotics, and specific probiotics that are able to prevent *S. aureus* colonization and growth, such as lactic acid bacteria, are of great interest (Karska-Wysocki et al., 2010; Sikorska & Smoragiewicz, 2013). For instance, probiotic *Bacillus* bacteria abolish *S. aureus* colonization, eliminates it, and inhibits its signaling system (Piewngam & Otto, 2020; Piewngam et al., 2018; York, 2018).

Quorum-quenching (QQ) is a means of disrupting *S. aureus* quorum-sensing (QS) ability which has shown the potential to reduce *S. aureus* pathogenicity (Paharik et al., 2017; Scoffone et al., 2019). In *S. aureus* several genes, including virulence factors, are under the control of the accessory gene regulator (*agr*) QS system. There are four different *S. aureus agr* groups, and each *agr* group is associated with different *S. aureus* phylogenetic lineages. Members of the same *agr*

group produce the same autoinducing peptides (AIP) and specific receptors for the designated AIP (Wang & Muir, 2016). Subtypes of AIPs have been shown to be inhibitory towards heterologous *agr* systems via interfering in the interactions between cognate AIP and their receptors (Wang & Muir, 2016). Non-pathogenic bacteria with *S. aureus* QQ activity have the potential to be further developed as probiotics. *S. epidermidis* and *S. caprae*, for example, produce heterologous AIPs and attenuate *S. aureus* virulence by interfering its *agr*-mediated QS (Otto, Echner, Voelter, & Gotz, 2001; Paharik et al., 2017). Other bacteria capable of perturbing *S. aureus* membrane and inhibiting RNA III have a potent to suppress the virulence phenotype of *S. aureus* (Mansson et al., 2011; Qazi et al., 2006). Synthetic and natural quorum-quenchers have been studied in multiple papers and appeared to be effective in drug development (Gordon et al., 2013; Grunenwald et al., 2018).

In several instances probiotics that have successfully antagonized and reduced the growth of human bacterial pathogens have been originally isolated from the microbiome of healthy individuals (Huidrom & Sharma, 2018; Liu et al., 2020). However, traditional methods to identify isolates with potential antimicrobial or QQ activity are time-consuming and laborious, and separate experimental pipelines are required to detect growth inhibition and QQ ability. In antimicrobial activity tests, co-culturing *S. aureus* with other bacteria in liquid media requires a prolonged enumeration step such as plate counting on selective agar media (Acai et al., 2019). More sophisticated techniques are available to test QQ such as beta-galactosidase assay (Peng et al., 2019), fluorescent reporter assay (Paharik et al., 2017), and mRNA quantification (Urbano et al., 2018). However, few genetically engineered *S. aureus* strains are available, and this limits options for rapidly testing potential antagonists against a broad range of *S. aureus* lineages.

Manipulation of bacterial plasmids is easier and safer than manipulation of chromosomal DNA. Plasmid-based genetic tools are commonly used to introduce reporter genes to bacterial cells, but a plasmid-based system is not always the best option in co-culture conditions due to the

requirement to include antibiotics in the media to retain plasmids. This limitation results from two elements: the potential metabolic changes in the presence of antibiotics, and the susceptibility of putative antagonistic bacteria to the antibiotics used. However, Krute et al. generated a highly stable plasmid in the absence of antibiotics (pKK30), and Rodriguez et al. modified this plasmid by inserting reporter genes to visualize *S. aureus* cells *in vitro* and *in vivo* (Krute et al., 2016; Rodriguez et al., 2017). These studies demonstrated that the stability of pKK30 and its recombinant plasmids was remarkably well maintained for more than 100 generations.

In this study, our aim was to develop a new high-throughput plasmid-based strategy to screen bacterial isolates for antagonistic activity against *S. aureus* while minimizing costs and labour. We evaluated the stability and performance of the pQS series of plasmids and then applied this new system to screen a bacterial culture collection of bovine mammary commensals to identify isolates those with antagonistic activity against two lineages of *S. aureus* which commonly cause mastitis in dairy cattle (CC151 and CC97) (Park & Ronholm, 2021). Using our new system, we were able to simultaneously identify which isolates were able to inhibit *S. aureus* growth as well as determine which were accomplishing growth inhibition through QQ. Our results highlight the benefits of this novel screening approach.

4.3 Materials and Methods

4.3.1 Bacterial strains, plasmids, and media

Escherichia coli DH5αλpir (NR-50350, BEI resources) served as the host for plasmid constructions and grew in lysogeny broth (LB) medium supplemented with ampicillin (100 µg/mL) (Sigma-Aldrich) or tryptic soy broth (TSB) medium with trimethoprim (10 µg/mL) (Sigma-Aldrich). *S. aureus* strains were cultured in TSB with or without trimethoprim (10 µg/mL). All engineered plasmids in *E. coli* DH5αλpir were transferred into *S. aureus* RN4220 (BEI resources, NR-45946)

as an intermediate strain, and then introduced to other *S. aureus* isolates, which were collected from Holstein dairy milk (Table 4.1). This study used various bacterial strains, plasmids, and oligonucleotides (Table 4.1). We purchased *Lactococcus lactis* subsp. *Lactis* (ATCC 11454) and *S. epidermis* (ATCC 14990) from the American Type Culture Collection (Manassas, VA, USA) and obtained non-aureus Staphylococci strains (n = 17) isolated from dairy cows from the mastitis pathogens culture collection (Appendix 5) (Dufour et al., 2019). A total of 64 bacterial isolates from raw milk taken from animals with no signs of clinical mastitis was obtained from the Macdonald Campus Farm, McGill University, Ste-Anne-de-Bellevue, Québec were used for the antagonistic test (Appendix 5).

4.3.2 Molecular genetic techniques

Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs (NEB). Hot Start Taq Master Mix was purchased from Qiagen (Hilden, Germany), and PCR was performed with a Veriti™ 96-Well Thermal Cycler (Applied Biosystems). Oligonucleotides, including primers and *agr*P3-*mCherry,* were synthesized by IDT DNA Technologies (Coralville, IA). The sequence of codon-optimized *mCherry* was obtained from NCBI under accession number LC088724 (Kato et al., 2017). All amplicons and digested DNA were purified using Monarch® PCR & DNA Cleanup Kit (NEB). Plasmid DNA was purified using Monarch® Plasmid Miniprep Kit (NEB) after pre-treatment of *S. aureus* cells with 20 µg of lysostaphin (Sigma-Aldrich) for 30 minutes at 37 °C.

4.3.3 Construction of QS reporter vectors

From the genomic DNA of *S. aureus* 31210331, the QS promoter (*agr*P3) was amplified by PCR using the AGRP3-F3 and AGRP3-R1 primers (Table 4.1). The *agr*P3 amplicons and pBGR1 plasmid, containing bidirectional reporter genes (*dsRed* and gfp_{bk}), were digested with *Eco*RI and

*Bam*HI and then ligated together to create pBGR1-agrP3. Next, the *agr*P3-*gfp*$_{bk}$ module from pBGR1-agrP3 was amplified using the AGRP3-F4 and GFP-R1 primers (Table 4.1) and then digested with *Nhe*I and *Eco*RV. The plasmid pKK30 was digested with *Nhe*I and *Sma*I and ligated together with the digested *agr*P3-*gfp*$_{bk}$ amplicon to generate pQS1. The synthetic module *agr*P3-*mCherry* from pBGR1-agrP3 was amplified using AGRP3-F4 and mCherry-R2 (Table 4.1). The *agr*P3-*mCherry* amplicons and pKK30 were digested with *Nhe*I and *Bam*HI and then ligated together to create pQS3.

Heat-shock was used to transform recombinant into calcium competent *E. coli* DH5αλpir. Electrocompetent *S. aureus* cells were prepared as described previously with minor modifications (Grosser & Richardson, 2016). Approximately 0.1 µg of plasmid DNA and 70 µL of electro-competent *S. aureus* cells were combined and then pulsed at 2.3 kV, 100 Ω, and 25 µF in 0.1 cm cuvette using the Gene Pulser Electroporation System (Bio-Rad Laboratories). The pulsed cells were transferred into 1 mL of BHI broth and incubated for 1 hour at 37 ℃ at 200 rpm. The cell suspensions were grown on TSA with trimethoprim (10 µg/mL) and incubated overnight at 37 ℃. Only transformants harbouring either pQS1 or pQS3 were recovered.

4.3.4 Cell culture and evaluation of pQS series

We prepared bacterial cultures by inoculating a single colony in 5 mL of TSB with trimethoprim (10 µg/mL) and incubated at 37 ℃ at 200 rpm up to an OD_{600} of 1.5-2.0. Next, 1 mL of the culture broth was centrifuged; the supernatant was removed, and then the pellet was resuspended by adding 0.5 mL of TSB. The density of the cell cultures was determined, and the resuspensions were diluted up to an OD_{600} of 0.01 in the required volume of TSB. Next a series of tests to assess plasmid stability, plasmid compatibility, cell growth, and the expression of reporter proteins were performed to evaluate pQS plasmids.

To assess the stability of the pQS series in four *S. aureus* strains, a batch culture test was performed from the early exponential phase to the stationary phase. Briefly, the resuspended cells were diluted to an OD_{600} of 0.01 in 20 mL of TSB (0 hour), and then incubated in three independent 5 mL aliquots at 37 ℃ at 200 rpm. Immediately after the first inoculation a sample was collected, diluted to 10^{-3}, and 0.1 mL of the 10^{-3} dilution was spread on TSA. TSA plates were incubated for 24 hours at 37 ℃. At 18 and 24 hours of incubation, 0.5 mL sample of each culture was serially diluted to 10^{-6}, and 0.1 mL of the final dilution was spread on TSA plates. After 24-hour incubation, plates were observed under a UV lamp, and the total number of colonies and the number of non-fluorescent colonies were counted.

To examine plasmid compatibility, we performed a rapid method previously described (Palomares & Perea, 1980). From the initial culture cells of *S. aureus* Sa25 and its transformants, we diluted the resuspended cells up to an OD_{600} of 0.01 in 5 mL of TSB either with or without trimethoprim (10 μg/mL). After incubation at 37 ℃ up to an OD_{600} of 1.5, plasmid DNA was extracted using 3 mL of culture cells from each test tubes. The thickness of plasmid bands was compared on a DNA agarose gel after gel electrophoresis.

The resuspended cells were diluted to an OD_{600} of 0.01 in 1 mL of TSB to monitor the growth rate and the expression of GFP and mCherry. From this dilution, 0.2 mL of diluted cells were transferred into a black 96-well plate in triplicate. The plate was incubated in Synergy HTX (BioTek) for 48 hours at 37 ℃, 205 cpm (5 mm) in continuous shaking mode, and the OD_{600} and relative fluorescence units (RFU) of GFP and mCherry were measured every hour. GFP was excited at 485 nm, and the emission was detected at 528 nm. mCherry was excited at 575 nm, and the emission was detected at 620 nm.

4.3.5 Antagonistic activity test

Testing for antagonistic activity between commensal isolates from milk and *S. aureus* were carried out on TSA, *S. aureus* transformants containing either pQS1 or pQS3 were used to make a bacterial lawn. The pre-culture of transformants was prepared as described above. *S. aureus* cells resuspended to an OD_{600} of 0.01 in 25 mL of soft TSA media at approximately 45 ℃ and placed in a petri dish. After the agar solidified, the commensal bacteria isolated from dairy milk were streaked on the plates and then incubated for 16-24 hours at 37 ℃. The growth inhibition and the QQ zone were observed under white light and UV light, respectively.

4.3.6 Statistical analysis

The results were subjected to one-way analysis of variance at a significance level of $p < 0.05$, to compare tested samples. The average of each triplicate was used for the statistical analysis.

4.4 Results

The recombinant plasmids pQS1 and pQS3 which encode the fluorescent genes gfp_{bk} and *mCherry*, respectively, under *S. aureus* QS promoter (*agr*P3) were generated using a variety of restriction enzymes and T4 DNA ligase (Figure 4.1A). The sequences of pQS1 (accession number MW344079) and pQS3 (accession number MW344080) have been deposited in GenBank at the NCBI (GenBank, https://www.ncbi.nlm.nih.gov/genbank/). *S. aureus* harbouring both pQS1 and pQS3 expressed GFP and mCherry, yet the expression of the reporter proteins was not detected in *S. aureus* RN4220 (*agr* defective strain) (Figure 4.2). We then applied the pQS series to establish a new screening strategy to monitor *S. aureus* QS in agar plates. The workflow of this newly proposed method is less demanding and more time efficient compared to traditional methods due to the simultaneous screening (Figure 4.1B).

4.4.1 Stability of the pQS series in S. aureus

The stability test revealed that both pQS1 and pQS3 were highly stable in four *S. aureus* strains (Sa2, Sa25, Sa27, and Sa30) for at least 24 hours in batch culture conditions without antibiotic selection pressure (Table 4.2). We also examined the compatibility of pQS plasmids with a naive pCC97-1 in *S. aureus* Sa25 by performing plasmid prep and DNA gel electrophoresis. The similarity in band thickness between pQS series and pCC97-1 in wild type and transformants supported the co-existence of two different plasmids in *S. aureus* Sa25 (Figure 4.3).

4.4.2 Growth curve and expression of reporter proteins

To determine if the newly introduced pQS series influences *S. aureus* growth, the growth rate of four *S. aureus* strains and their transformants was examined. Similar growth rates were observed comparing transformants with the wild-type strains, suggesting no detectable adverse effects of the pQS series on the cells for 48 hours (ANOVA p-value = 0.43 (Sa2), 0.51 (Sa27), 0.80 (Sa30)) (Figure 4.4A). The Sa25 transformant of pQS1 showed a relatively lower optical density than wild type and other transformants (pKK30 and pQS3) (ANOVA p-value = 0.04), yet this was not found to be significant during 24 hours of incubation (ANOVA p-value = 0.60). The kinetic patterns of reporter proteins in two lineages (CC97 and CC151) were different (Figure 4.4B and 4.4C). The signals from CC151 strains plateaued once the cell reached the stationary phase, while the signals from CC97 strains increased continuously in a linear fashion. The intensity of fluorescent signals from each strain varied. The QS of Sa25 activated 2-5 hours earlier than other strains, resulting in strong signals on agar plates.

4.4.3 Screening antagonistic bacteria against S. aureus

We confirmed the growth-inhibiting activity of *L. lactis* subsp. *lactis* ATCC 11454 against *S. aureus* and variable QQ activities of *S. epidermidis* ATCC 14990 toward *S. aureus* Sa25 (Appendix 5). From a culture collection containing 81 bacterial isolates from raw milk taken from healthy cows, we identified 13 isolates which were able to inhibit the growth of *S. aureus*, and 13 that have QQ activity toward both Sa25 (CC97) and Sa27 (CC151). All growth-inhibiting bacteria, which were various in phenotypes, were active toward both Sa25 and Sa27 and showed different activities. The growth-inhibiting activity of test 32 was similar to those of *L. lactis* subsp. lactis (test 1) and others (test 11, 63, and 65), while some isolates belonging to *B. pumilus* showed a relatively large zone of growth inhibition. The Sa25 strain had strong QS abilities, and bright fluorescent lawns could be observed after 18 hours of incubation (Figure 4.5). The expression of mCherry in plates prepared with pQS3 transformant of Sa25 was visible by the naked eye in white light. We observed the growth inhibition zone around the test bacteria in white light and QQ zone under the UV lamp, which allowed us to screen positive bacteria. Growth-inhibiting bacteria were all *Bacillus* species except for *Aerococcus viridans*, and all QQ bacteria belonged to the *Staphylococcus* genus (Appendix 5).

4.5 Discussion

The newly constructed plasmids (pQS series) are retained in transformed *S. aureus* without antibiotic selection pressure in batch conditions for at least 24 hours. Taking advantage of this high stability, we developed a new method to screen bacteria for both growth-inhibiting and QQ activity towards *S. aureus*. Ultimately, we were able to identify 13 commensal isolates that were able to inhibit the growth of *S. aureus*, and a further 13 that exhibited QQ activity. These bacteria were identified previously through 16S rRNA sequencing and MALDI-TOF. This is a relatively high rate

of growth-inhibiting activity (13/81) and might be explained by the natural evolution of *S. aureus* and commensal bacteria that have been adapted in the same niche.

At the species level *S. aureus* can cause infection in humans and several species of birds and mammals, however, on a short evolutionary time span certain CCs of *S. aureus* are specialized to single or very few host species (Matuszewska et al., 2020; Park & Ronholm, 2021). Bovine mastitis is often a result of *S. aureus* infections, and the *S. aureus* lineages most commonly associated with bovine mastitis are CC8, CC20 CC97, CC151, and C188 (Matuszewska et al., 2020; Park & Ronholm, 2021). We monitored QS kinetics in two different *S. aureus* lineages commonly associated with clinical bovine mastitis, CC97 and CC151. The isolates Sa25 and Sa30 belong to CC97 and show an exponential up-regulation in the expression of reporter proteins, which is a typical pattern of QS kinetics (Novick & Geisinger, 2008). Unlike the two CC97 isolates, the two CC151 isolates (Sa2 and Sa27) showed continuous QS activity even after reaching the stationary phase. This kinetic pattern of QS is not a common pattern of QS-regulation (Podbielski & Kreikemeyer, 2004). It is unclear if the QS regulation in CC151 provides any selective advantage in bovine intramammary glands, but this could be a possible explanation of this odd pattern of QS-regulation.

Members of the *Bacillus* genus produce a broad spectrum of non-ribosomally synthesized antimicrobial peptides which have antagonistic activity against pathogenic microbes including *S. aureus* (Sumi et al., 2015). We observed that *B. pumilus* inhibited *S. aureus* growth and it was significantly stronger than a known nisin-producer *L. lactis*. *B. pumilus* and *B. subtilis* have been known to exhibit anti-Staphylococcus activity by producing various antimicrobial peptides (Terekhov et al., 2020; Wu et al., 2005; Zidour et al., 2019). The *agr* cross-interfering of *S. aureus* mediated by other staphylococci species is the most common as they have similar QS systems and produce peptide analogues (Canovas et al., 2016; Mahmmod et al., 2018; Paharik et al., 2017). *S.*

epidermidis and *S. caprae*, for example, produce heterologous AIPs and attenuate *S. aureus* virulence by interfering its *agr* mediated QS (Otto, Echner, Voelter, & Götz, 2001; Paharik et al., 2017). Interestingly, the QS of Sa25 was more frequently and strongly affected by other *Staphylococcus* species than Sa27, indicating *agr* type I may be more vulnerable to the QQ. Another study revealed that cross-talk between *S. epidermidis* and *S. aureus* tends to favour *S. epidermidis*, and other *Staphylococcus* species modulate *S. aureus* colonization through the *agr* cross-talk (Otto, Echner, Voelter, & Gotz, 2001; Peng et al., 2019). In this study, we observed no QQ activity of *S. epidermidis* ATCC 14990 toward either AIP-I and AIP-II producers, presumably due to subinhibitory concentration of AIP_{ep}-I in co-culture condition (Appendix 5). Although antagonistic bacteria have been suggested as an alternative solution to reduce antibiotics use in dairy farming, intramammary probiotics or intramammary infusion seems to be considered carefully due to the inflammation of mammary glands (Klostermann et al., 2008; Mignacca et al., 2017; Rainard & Foucras, 2018). Indeed, *A. viridans* and non-aureus staphylococci screened in this study are also known clinical and subclinical mastitis pathogens, invalidate their further development as probiotics. However, antagonistic bacteria and their active biomolecules capable of inhibiting *S. aureus* are still promising candidates for therapeutics.

An important aspect of this new screening method is the ability to monitor true interactions between co-cultured bacteria. Several studies have reported the cross-inhibition of AIPs produced by *S. aureus* (Wang & Muir, 2016). A previous study examined the interactions between receptors and cognate AIPs, explaining critical aspects of the QS mechanisms in *S. aureus* (Lyon et al., 2002). Previous studies used different *S. aureus* strains with different *agr* systems to examine the interference of *S. aureus* QS mediated by non-cognate AIP in culture supernatant or purified non-cognate AIP of another *S. aureus* strain (Jarraud et al., 2000; Ji et al., 1997). However, these conditions cause bias towards the non-cognate AIP producer due to counter inhibition being omitted

from the assays. In this study, we observed no QQ activity of Sa25 (*agr* type I) toward Sa27 (*agr* type II) in co-culture conditions, whereas Sa27 inhibited the QS of Sa25. This result suggests that AIP-dependent cross-inhibition might not reflect the natural QS interferences when *S. aureus* strains with different *agr* systems co-colonize and interact bi-directionally in the same niche. A superior *agr* type over another might exist between *S. aureus* during co-colonization resulting from the timing of QS activation and strength. More importantly, antagonistic molecules produced by bacteria are not primary metabolites, so without survival advantages under certain conditions, bacteria rarely accumulate these compounds enough to kill or interrupt others. Many studies have supported the idea that interspecies interactions stimulate bacterial metabolism and induce the expression of silence genes (Bertrand et al., 2014; Moody, 2014; Netzker et al., 2015; Traxler et al., 2013). This metabolic response in bacteria should be considered in the development of antibacterial therapy and probiotic drugs.

A limitation of this new pQS series mainly stems from the possibility of losing pQS plasmids from *S. aureus* host cells leading to false positives. The stationary phase during co-culture can affect the plasmid stability due to fitness costs in unfavourable conditions such as nutrient exhaustion, accumulated inhibitory metabolites, and lack of physical space. Antagonistic compounds produced by co-cultured bacteria increase fitness cost affecting plasmid stability in *S. aureus*. Despite this limitation, pQS plasmids can broaden the range of target *S. aureus* strains in screening antagonistic bacteria in the simplest way. The fluorescent reporter genes in the pQS plasmids can be readily replaced by other reporter genes such as *lacZ,* providing flexibilities in detection. Although the pQS series favours qualitative assay, quantitative assay in QS of *S. aureus* is feasible in co-culture conditions. The pQS plasmids are applicable to other research fields in *S. aureus* studies such as image dynamics and bio-sensors specific for *S. aureus* QS.

4.6 Conclusion

We demonstrated the high stability of the newly constructed pQS plasmids without antibiotic selection pressure. Taking advantage of the highly stable plasmids and the QS-dependent expression of reporter proteins, we were able to co-culture *S. aureus* with other bacteria and examine *S. aureus* growth and QS simultaneously on agar media. Our results demonstrate that this high-throughput screening strategy significantly reduces workload and processing time in finding antagonistic bacteria against *S. aureus*. Newly found antagonistic bacteria and their bioactive compounds can be used to develop promising probiotic drugs and prophylactics/therapeutics capable of preventing and treating *S. aureus* infections.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The sequences of pQS1 (accession number MW344079) and pQS3 (accession number MW344080) are available in GenBank at the NCBI (GenBank, https://www.ncbi.nlm.nih.gov/genbank/). The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SP designed the study, wrote the manuscript, and performed experimentation. JR and GK provided critical expertise for the experimental design and the manuscript. HG and RM participated in the plasmid stability test and AC participated in the antagonistic activity test. EK and CD isolated commensal bacteria from dairy milk. All authors read and approved the final manuscript.

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Table 4.1 Bacterial strains, plasmids, and oligonucleotides

Bacteria, plasmid, oligonucleotides	Relevant characteristic(s) or sequence	Source
Bacterial strains		
E. coli DH5αλpir	Plasmid cloning strain	(Krute et al., 2016)
S. aureus RN4220	Restriction deficient strain, partially defective AgrA	(Nair et al., 2011)
S. aureus 10400326 or Sa2	ST351, AIP-II producer	(Park et al., 2020)
S. aureus 10303344 or Sa25	ST352, AIP-I producer	(Park et al., 2020)
S. aureus 41000044 or Sa27	ST151, AIP-II producer	(Park et al., 2020)
S. aureus 21000024 or Sa30	ST352, AIP-I producer	(Park et al., 2020)
L. lactis subsp. *lactis* ATCC 11454	Nisin producer	(Millette et al., 2004)
S. epidermidis ATCC 14990	AIP_{ep}-I producer	(Otto et al., 1998)
Plasmids		
pBGR1	Promoter trap vector	(Han et al., 2008)
pKK30	Highly stable plasmid containing P_{sarAP1}-*dfrA*	(Krute et al., 2016)
pQS1	pKK30 containing P_{agrP3}-gfp_{bk}	This study
pQS3	pKK30 containing P_{agrP3}-*mCherry*	This study
Primers		
AGRP3-F3 (*Eco*RI)	AAAGAATTCGTAATTTGTATTTAATATTTTAAC	This study
AGRP3-F4 (*Nhe*I)	AAAGCTAGCGTAATTTGTATTTAATATTTTAAC	This study
AGRP3-R1 (*Bam*HI)	AAAGGATCCCAACTATTTTCCATCAC	This study
GFP-R1 (*Eco*RV)	AAAGATATCTTATTTGTAGAGCTC	This study
mCherry-R1 (*Bam*HI)	AAAGGATCCCTACTTGTACAGCTC	This study

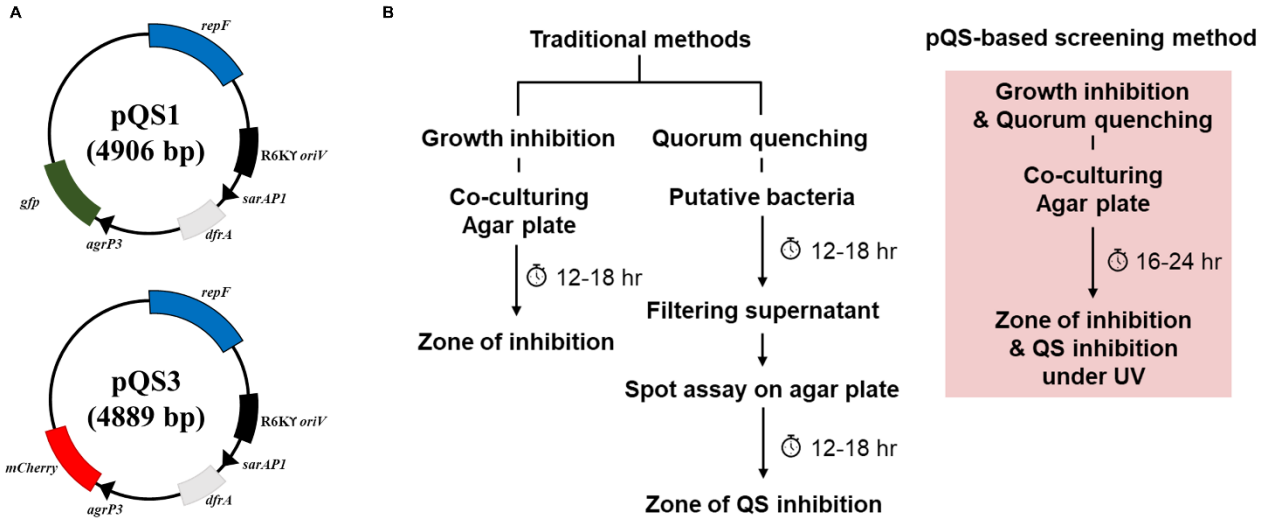

Figure 4.1 Diagrams of pQS series and a schematic representation of the pQS-based screening method. (A) Two newly engineered plasmids contain a trimethoprim-resistant gene (*dfrA*), two replication origins for *E. coli* and *S. aureus*, and a fluorescent gene (gfp_{bk} or *mCherry*) that is controlled by *agr*P3. (B) Compared to the traditional methods with two different pipelines, the new pQS-based screening method shows the combined workflow for growth inhibition and QQ and reduces workload and processing time.

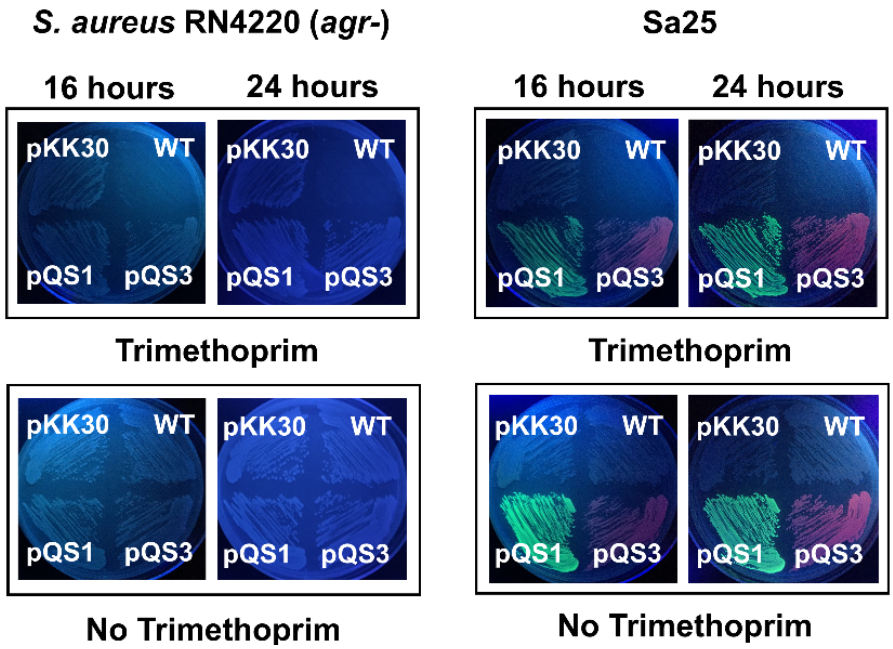

Figure 4.2 Expression of the reporter protein in *agr* defective and positive strains. Two plasmids pQS1 and pQS3 were transformed to *S. aureus* RN4220 (*agr*-) and Sa25. After 24 hours of incubation either with or without trimethoprim, no fluorescent colony was observed from the transformants of *S. aureus* RN4220, while the transformants of *S. aureus* Sa25 exhibited clear fluorescent phenotypes.

Table 4.2 Stability of pQS series in batch culture

Strain and plasmid	% of colonies with plasmid		
	0 hour	18 hours	24 hours
S. aureus Sa2			
pQS1	99.97 ± 0.05	99.88 ± 0.24	99.64 ± 0.64
pQS3	100.0 ± 0.00	99.74 ± 0.38	98.24 ± 3.26
S. aureus Sa25			
pQS1	99.62 ± 0.22	99.37 ± 0.55	99.40 ± 0.46
pQS3	99.65 ± 0.09	99.30 ± 0.57	99.13 ± 0.64
S. aureus Sa27			
pQS1	99.93 ± 0.12	99.43 ± 0.51	99.61 ± 0.52
pQS3	99.88 ± 0.15	99.55 ± 0.93	97.96 ± 3.48
S. aureus Sa30			
pQS1	99.63 ± 0.19	99.42 ± 0.58	99.41 ± 0.46
pQS3	99.65 ± 0.09	99.30 ± 0.57	99.13 ± 0.64

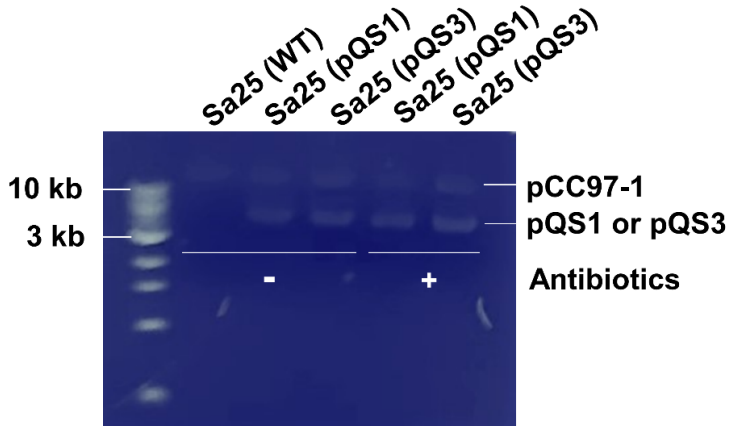

Figure 4.3 Agarose gel electrophoresis of plasmid DNA. The first lane contains a single DNA band of pCC97-1. Other lands contain two DNA bands corresponding to pCC97-1 and pQS plasmids indicating the co-existence of two plasmids in the same *S. aureus* cells.

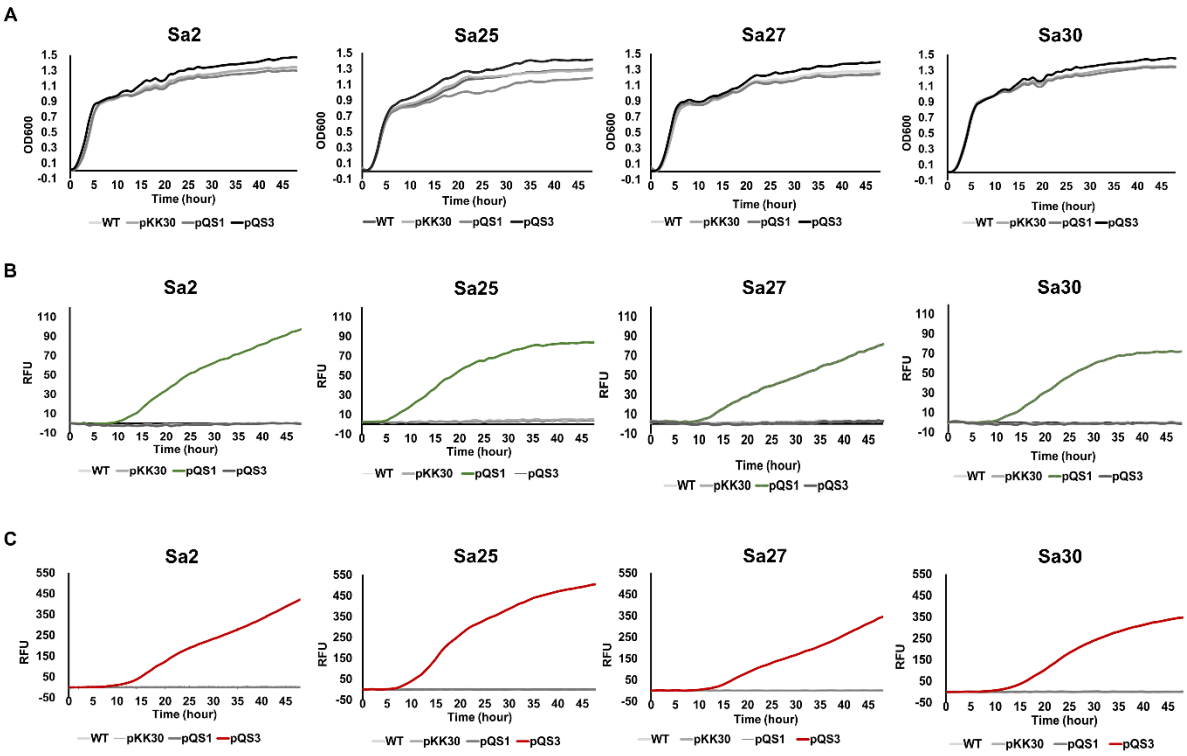

Figure 4.4 Growth curve and expression of reporter proteins in four *Staphylococcus aureus* strains and their transformations with pKK30, pQS1, and pQS3. (A) Growth curves all transformants show no significant difference compared to the wild type of each strain for at least 24 hours (ANOVA *p*-value = 0.86 (Sa2), 0.60 (Sa25), 0.51 (Sa27), and 0.99 (Sa30)). The expression of GFP (B) and mCherry (C) in CC151 (Sa2 and Sa27) and CC97 (Sa25 and Sa30) show different patterns but are similar within the same CC.

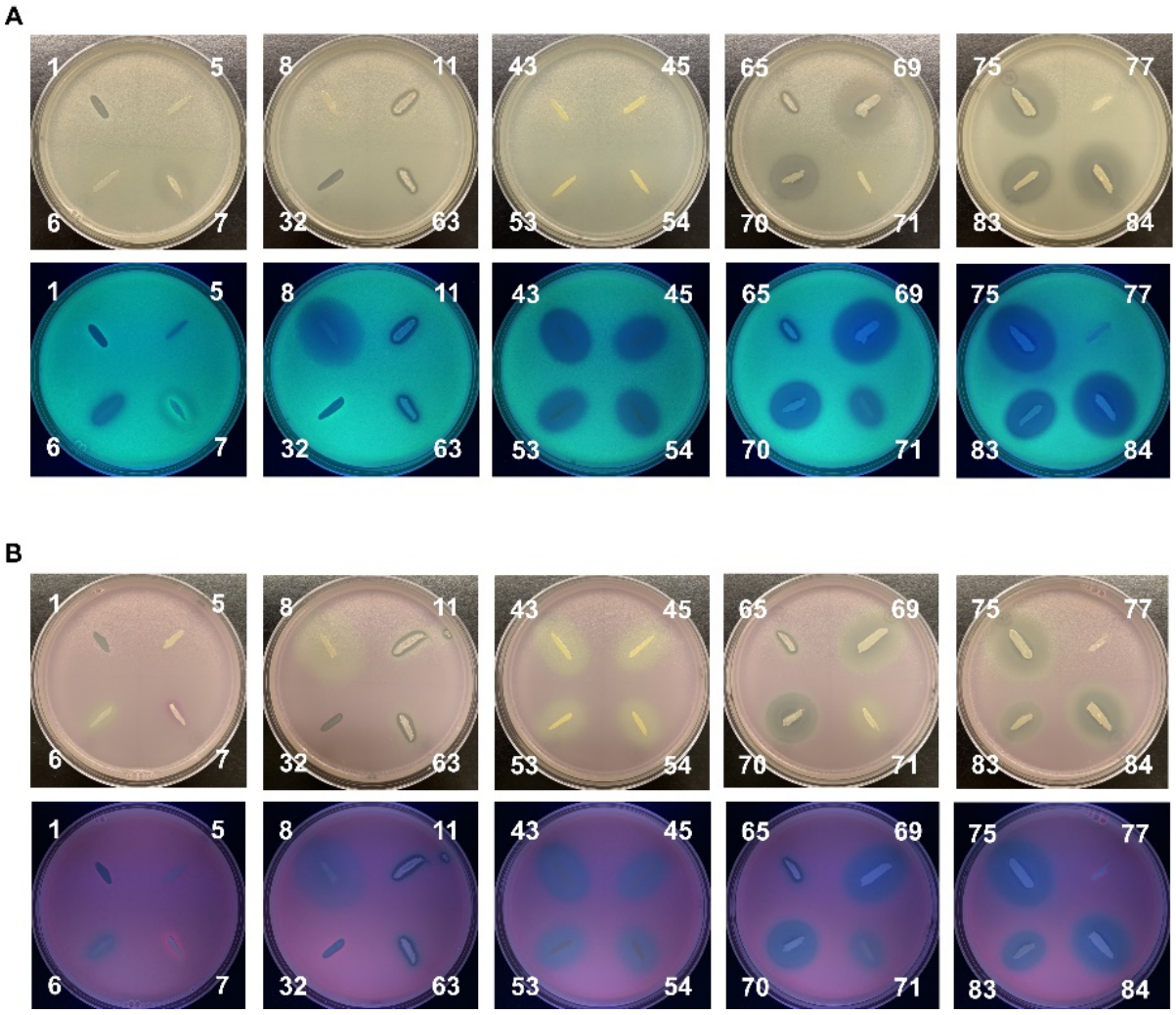

Figure 4.5 Screening antagonistic bacteria against *Staphylococcus aureus* Sa25 using pQS series. Bacterial lawn prepared with pQS1 (A) and pQS3 (B) transformants of *S. aureus* Sa25 exhibits the zone of growth inhibition and QQ in the presence of antagonistic bacteria. Plates show glowing fluorescence under UV light (bottom plates of (A) and (B)). Test bacteria streaked on *S. aureus* Sa25 lawn include *L. lactis* (test 1)*, S. aureus* Sa25 (test 5), *S. aureus* Sa27 (test 6), *Bacillus pumilus* (test 7, 69, 70, 75, 77, 84), *B.altitudinis* (test 8), *B. subtilis* (test 11 and 63), *Aerococcus viridans* (test 32), *S. chromogenes* (test 43, 53, and 54), *S. saprophyticus* (test 45), *Bacillus* species (test 65), *S. pasteuri* (test 71), and unknown species (test 83).

4.7 References

Acai, P., Medved'ova, A., Mancuskova, T., & Valik, L. (2019). Growth prediction of two bacterial populations in co-culture with lactic acid bacteria. *Food Sci Technol Int*, *25*(8), 692-700. https://doi.org/10.1177/1082013219860360

Balasubramanian, D., Harper, L., Shopsin, B., & Torres, V. J. (2017). Staphylococcus aureus pathogenesis in diverse host environments. *Pathog Dis*, *75*(1). https://doi.org/10.1093/femspd/ftx005

Bertrand, S., Bohni, N., Schnee, S., Schumpp, O., Gindro, K., & Wolfender, J. L. (2014). Metabolite induction via microorganism co-culture: a potential way to enhance chemical diversity for drug discovery. *Biotechnol Adv*, *32*(6), 1180-1204. https://doi.org/10.1016/j.biotechadv.2014.03.001

Canovas, J., Baldry, M., Bojer, M. S., Andersen, P. S., Grzeskowiak, P. K., Stegger, M., Damborg, P., Olsen, C. A., & Ingmer, H. (2016). Cross-Talk between Staphylococcus aureus and Other Staphylococcal Species via the agr Quorum Sensing System. *Front Microbiol*, *7*, 1733. https://doi.org/10.3389/fmicb.2016.01733

Dufour, S., Labrie, J., & Jacques, M. (2019). The Mastitis Pathogens Culture Collection. *Microbiol Resour Announc*, *8*(15). https://doi.org/10.1128/MRA.00133-19

Gordon, C. P., Williams, P., & Chan, W. C. (2013). Attenuating Staphylococcus aureus virulence gene regulation: a medicinal chemistry perspective. *J Med Chem*, *56*(4), 1389-1404. https://doi.org/10.1021/jm3014635

Grosser, M. R., & Richardson, A. R. (2016). Method for Preparation and Electroporation of S. aureus and S. epidermidis. *Methods Mol Biol*, *1373*, 51-57. https://doi.org/10.1007/7651_2014_183

Grunenwald, C. M., Bennett, M. R., & Skaar, E. P. (2018). Nonconventional Therapeutics against Staphylococcus aureus. *Microbiology spectrum*, *6*(6), 10.1128/microbiolspec.GPP1123-0047-2018. https://doi.org/10.1128/microbiolspec.GPP3-0047-2018

Han, S. S., Lee, J. Y., Kim, W. H., Shin, H. J., & Kim, G. J. (2008). Screening of promoters from metagenomic DNA and their use for the construction of expression vectors. *J Microbiol Biotechnol*, *18*(10), 1634-1640. https://www.ncbi.nlm.nih.gov/pubmed/18955811

Huidrom, S., & Sharma, N. (2018). Isolation and screening of novel isolates of Bifidobacteria from human milk as potential probiotic with antidiarrheal activity. *Ann Appl Microbiol Biotechnol J*, *2*(1), 1007-1015.

Jarraud, S., Lyon, G. J., Figueiredo, A. M., Lina, G., Vandenesch, F., Etienne, J., Muir, T. W., & Novick, R. P. (2000). Exfoliatin-producing strains define a fourth agr specificity group in Staphylococcus aureus. *J Bacteriol*, *182*(22), 6517-6522. https://doi.org/10.1128/jb.182.22.6517-6522.2000

Ji, G., Beavis, R., & Novick, R. P. (1997). Bacterial interference caused by autoinducing peptide variants. *Science*, *276*(5321), 2027-2030. https://doi.org/10.1126/science.276.5321.2027

Karska-Wysocki, B., Bazo, M., & Smoragiewicz, W. (2010). Antibacterial activity of Lactobacillus acidophilus and Lactobacillus casei against methicillin-resistant Staphylococcus aureus (MRSA). *Microbiol Res*, *165*(8), 674-686. https://doi.org/10.1016/j.micres.2009.11.008

Kato, F., Nakamura, M., & Sugai, M. (2017). The development of fluorescent protein tracing vectors for multicolor imaging of clinically isolated Staphylococcus aureus. *Sci Rep*, *7*(1), 2865. https://doi.org/10.1038/s41598-017-02930-7

Klostermann, K., Crispie, F., Flynn, J., Ross, R. P., Hill, C., & Meaney, W. (2008). Intramammary infusion of a live culture of Lactococcus lactis for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. *J Dairy Res*, *75*(3), 365-373. https://doi.org/10.1017/S0022029908003373

Krute, C. N., Krausz, K. L., Markiewicz, M. A., Joyner, J. A., Pokhrel, S., Hall, P. R., & Bose, J. L. (2016). Generation of a Stable Plasmid for In Vitro and In Vivo Studies of Staphylococcus Species. *Appl Environ Microbiol*, *82*(23), 6859-6869. https://doi.org/10.1128/AEM.02370-16

Liu, W., Chen, M., Duo, L., Wang, J., Guo, S., Sun, H., Menghe, B., & Zhang, H. (2020). Characterization of potentially probiotic lactic acid bacteria and bifidobacteria isolated from human colostrum. *J Dairy Sci*, *103*(5), 4013-4025. https://doi.org/10.3168/jds.2019-17602

Lyon, G. J., Wright, J. S., Muir, T. W., & Novick, R. P. (2002). Key determinants of receptor

activation in the agr autoinducing peptides of Staphylococcus aureus. *Biochemistry*, *41*(31), 10095-10104. https://doi.org/10.1021/bi026049u

Mahmmod, Y. S., Klaas, I. C., Svennesen, L., Pedersen, K., & Ingmer, H. (2018). Communications of Staphylococcus aureus and non-aureus Staphylococcus species from bovine intramammary infections and teat apex colonization. *J Dairy Sci*, *101*(8), 7322-7333. https://doi.org/10.3168/jds.2017-14311

Mansson, M., Nielsen, A., Kjaerulff, L., Gotfredsen, C. H., Wietz, M., Ingmer, H., Gram, L., & Larsen, T. O. (2011). Inhibition of virulence gene expression in Staphylococcus aureus by novel depsipeptides from a marine photobacterium. *Mar Drugs*, *9*(12), 2537-2552. https://doi.org/10.3390/md9122537

Matuszewska, M., Murray, G. G. R., Harrison, E. M., Holmes, M. A., & Weinert, L. A. (2020). The Evolutionary Genomics of Host Specificity in Staphylococcus aureus. *Trends Microbiol*, *28*(6), 465-477. https://doi.org/10.1016/j.tim.2019.12.007

Mignacca, S. A., Dore, S., Spuria, L., Zanghi, P., Amato, B., Dupre, I., Armas, F., Biasibetti, E., Camperio, C., Lollai, S. A., Capucchio, M. T., Cannas, E. A., Di Marco Lo Presti, V., & Marianelli, C. (2017). Intramammary infusion of a live culture of Lactococcus lactis in ewes to treat staphylococcal mastitis. *J Med Microbiol*, *66*(12), 1798-1810. https://doi.org/10.1099/jmm.0.000641

Millette, M., Smoragiewicz, W., & Lacroix, M. (2004). Antimicrobial potential of immobilized Lactococcus lactis subsp. lactis ATCC 11454 against selected bacteria. *J Food Prot*, *67*(6), 1184-1189. https://doi.org/10.4315/0362-028x-67.6.1184

Moody, S. C. (2014). Microbial co-culture: harnessing intermicrobial signaling for the production of novel antimicrobials. *Future Microbiol*, *9*(5), 575-578. https://doi.org/10.2217/fmb.14.25

Nair, D., Memmi, G., Hernandez, D., Bard, J., Beaume, M., Gill, S., Francois, P., & Cheung, A. L. (2011). Whole-genome sequencing of Staphylococcus aureus strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. *J Bacteriol*, *193*(9), 2332-2335. https://doi.org/10.1128/JB.00027-11

Netzker, T., Fischer, J., Weber, J., Mattern, D. J., Konig, C. C., Valiante, V., Schroeckh, V., & Brakhage, A. A. (2015). Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. *Front Microbiol*, *6*, 299. https://doi.org/10.3389/fmicb.2015.00299

Novick, R. P., & Geisinger, E. (2008). Quorum sensing in staphylococci. *Annu Rev Genet*, *42*, 541-564. https://doi.org/10.1146/annurev.genet.42.110807.091640

Otto, M., Echner, H., Voelter, W., & Gotz, F. (2001). Pheromone cross-inhibition between Staphylococcus aureus and Staphylococcus epidermidis. *Infect Immun*, *69*(3), 1957-1960. https://doi.org/10.1128/IAI.69.3.1957-1960.2001

Otto, M., Echner, H., Voelter, W., & Götz, F. (2001). Pheromone cross-inhibition between Staphylococcus aureus and Staphylococcus epidermidis. *Infection and immunity*, *69*(3), 1957-1960. https://doi.org/10.1128/IAI.69.3.1957-1960.2001

Otto, M., Sussmuth, R., Jung, G., & Gotz, F. (1998). Structure of the pheromone peptide of the Staphylococcus epidermidis agr system. *FEBS Lett*, *424*(1-2), 89-94. https://doi.org/10.1016/s0014-5793(98)00145-8

Paharik, A. E., Parlet, C. P., Chung, N., Todd, D. A., Rodriguez, E. I., Van Dyke, M. J., Cech, N. B., & Horswill, A. R. (2017). Coagulase-Negative Staphylococcal Strain Prevents Staphylococcus aureus Colonization and Skin Infection by Blocking Quorum Sensing. *Cell Host Microbe*, *22*(6), 746-756 e745. https://doi.org/10.1016/j.chom.2017.11.001

Palomares, J. C., & Perea, E. J. (1980). A rapid method of determining the incompatibility group of R plasmid. *Plasmid*, *4*(3), 352-353. https://doi.org/10.1016/0147-619x(80)90073-6
Park, S., Jung, D., Dufour, S., & Ronholm, J. (2020). Draft Genome Sequences of 27 Staphylococcus aureus Strains and 3 Staphylococcus Species Strains Isolated from Bovine Intramammary Infections. *Microbiol Resour Announc*, *9*(19). https://doi.org/10.1128/MRA.00300-20
Park, S., & Ronholm, J. (2021). Staphylococcus aureus in Agriculture: Lessons in Evolution from a Multispecies Pathogen. *Clin Microbiol Rev*, *34*(2). https://doi.org/10.1128/CMR.00182-20
Peng, P., Baldry, M., Gless, B. H., Bojer, M. S., Espinosa-Gongora, C., Baig, S. J., Andersen, P. S., Olsen, C. A., & Ingmer, H. (2019). Effect of Co-inhabiting Coagulase Negative Staphylococci on S. aureus agr Quorum Sensing, Host Factor Binding, and Biofilm Formation. *Front Microbiol*, *10*, 2212. https://doi.org/10.3389/fmicb.2019.02212
Piewngam, P., & Otto, M. (2020). Probiotics to prevent Staphylococcus aureus disease? *Gut Microbes*, *11*(1), 94-101. https://doi.org/10.1080/19490976.2019.1591137
Piewngam, P., Zheng, Y., Nguyen, T. H., Dickey, S. W., Joo, H.-S., Villaruz, A. E., Glose, K. A., Fisher, E. L., Hunt, R. L., Li, B., Chiou, J., Pharkjaksu, S., Khongthong, S., Cheung, G. Y. C., Kiratisin, P., & Otto, M. (2018). Pathogen elimination by probiotic Bacillus via signalling interference. *Nature*, *562*(7728), 532-537. https://doi.org/10.1038/s41586-018-0616-y
Podbielski, A., & Kreikemeyer, B. (2004). Cell density--dependent regulation: basic principles and effects on the virulence of Gram-positive cocci. *Int J Infect Dis*, *8*(2), 81-95. https://doi.org/10.1016/j.ijid.2003.04.003
Qazi, S., Middleton, B., Muharram, S. H., Cockayne, A., Hill, P., O'Shea, P., Chhabra, S. R., Camara, M., & Williams, P. (2006). N-acylhomoserine lactones antagonize virulence gene expression and quorum sensing in Staphylococcus aureus. *Infect Immun*, *74*(2), 910-919. https://doi.org/10.1128/IAI.74.2.910-919.2006
Rainard, P., & Foucras, G. (2018). A Critical Appraisal of Probiotics for Mastitis Control. *Front Vet Sci*, *5*, 251. https://doi.org/10.3389/fvets.2018.00251
Rodriguez, M. D., Paul, Z., Wood, C. E., Rice, K. C., & Triplett, E. W. (2017). Construction of Stable Fluorescent Reporter Plasmids for Use in Staphylococcus aureus. *Front Microbiol*, *8*, 2491. https://doi.org/10.3389/fmicb.2017.02491
Scoffone, V. C., Trespidi, G., Chiarelli, L. R., Barbieri, G., & Buroni, S. (2019). Quorum Sensing as Antivirulence Target in Cystic Fibrosis Pathogens. *Int J Mol Sci*, *20*(8). https://doi.org/10.3390/ijms20081838
Sikorska, H., & Smoragiewicz, W. (2013). Role of probiotics in the prevention and treatment of meticillin-resistant Staphylococcus aureus infections. *Int J Antimicrob Agents*, *42*(6), 475-481. https://doi.org/10.1016/j.ijantimicag.2013.08.003
Sumi, C. D., Yang, B. W., Yeo, I. C., & Hahm, Y. T. (2015). Antimicrobial peptides of the genus Bacillus: a new era for antibiotics. *Can J Microbiol*, *61*(2), 93-103. https://doi.org/10.1139/cjm-2014-0613
Terekhov, S. S., Nazarov, A. S., Mokrushina, Y. A., Baranova, M. N., Potapova, N. A., Malakhova, M. V., Ilina, E. N., Smirnov, I. V., & Gabibov, A. G. (2020). Deep Functional Profiling Facilitates the Evaluation of the Antibacterial Potential of the Antibiotic Amicoumacin. *Antibiotics (Basel)*, *9*(4). https://doi.org/10.3390/antibiotics9040157
Traxler, M. F., Watrous, J. D., Alexandrov, T., Dorrestein, P. C., & Kolter, R. (2013). Interspecies interactions stimulate diversification of the Streptomyces coelicolor secreted metabolome. *mBio*, *4*(4). https://doi.org/10.1128/mBio.00459-13
Urbano, R., Karlinsey, J. E., Libby, S. J., Doulias, P. T., Ischiropoulos, H., Warheit-Niemi, H. I., Liggitt, D. H., Horswill, A. R., & Fang, F. C. (2018). Host Nitric Oxide Disrupts Microbial

Cell-to-Cell Communication to Inhibit Staphylococcal Virulence. *Cell Host Microbe*, *23*(5), 594-606 e597. https://doi.org/10.1016/j.chom.2018.04.001

Wang, B., & Muir, T. W. (2016). Regulation of Virulence in Staphylococcus aureus: Molecular Mechanisms and Remaining Puzzles. *Cell Chem Biol*, *23*(2), 214-224. https://doi.org/10.1016/j.chembiol.2016.01.004

Wu, S., Jia, S., Sun, D., Chen, M., Chen, X., Zhong, J., & Huan, L. (2005). Purification and characterization of two novel antimicrobial peptides Subpeptin JM4-A and Subpeptin JM4-B produced by Bacillus subtilis JM4. *Curr Microbiol*, *51*(5), 292-296. https://doi.org/10.1007/s00284-005-0004-3

York, A. (2018). Silencing Staphylococcus aureus with probiotics. *Nat Rev Microbiol*, *16*(12), 715. https://doi.org/10.1038/s41579-018-0111-3

Zidour, M., Belguesmia, Y., Cudennec, B., Grard, T., Flahaut, C., Souissi, S., & Drider, D. (2019). Genome Sequencing and Analysis of Bacillus pumilus ICVB403 Isolated from Acartia tonsa Copepod Eggs Revealed Surfactin and Bacteriocin Production: Insights on Anti-Staphylococcus Activity. *Probiotics Antimicrob Proteins*, *11*(3), 990-998. https://doi.org/10.1007/s12602-018-9461-4

CONNECTING TEXT

In the chapter 4, we proposed a new strategy to find antagonistic bacteria and screened commensal bacteria isolated from raw dairy milk exhibiting either growth-inhibiting or quorum-quenching activity towards *S. aureus*. This new method was more effective and efficient than traditional screening strategies owing to the co-culturing approach. The antagonistic bacteria found in this study and their active biomolecules have the potential to be used as alternatives to antibiotics to prevent and treat *S. aureus* clinical mastitis. To broaden the scope of knowledge with regards to bacterial interactions at the community level, in this chapter, we conducted a longitudinal cohort study on the raw milk microbiome associated with *S. aureus* clinical mastitis. The objective of this chapter was to characterize and compare milk microbiome in healthy and sick quarters before, during, and after *S. aureus* clinical mastitis (CM) and to identify bacterial genera and species that are negatively correlated with *Staphylococcus*. We collected quarter-level milk samples throughout the lactation from 698 Holstein dairy cows and diagnosed natural *S. aureus* CM cases (n = 11). With all milk samples from the cows diagnosed with *S. aureus* CM regardless of healthy status, we investigated microbial changes over time, microbial network, and correlation between the inflammation level and the relative abundance of each genus found in the milk sample.

CHAPTER 5. THE EFFECT OF MILK MICROBIOME COMPOSITION ON HOST SUSCEPTIBILITY TO *STAPHYLOCOCCUS AUREUS* CLINICAL MASTITIS IN DAIRY CATTLE

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5.1 Abstract

Background: *Staphylococcus aureus* is a common cause of clinical mastitis (CM) in dairy cattle. Optimizing the bovine mammary gland microbiota to resist *S. aureus* colonization is a growing area of research. However, the details of the interbacterial interactions between *S. aureus* and commensal bacteria, which would be required to manipulate the microbiome to resist infection, are still unknown. This study aims to characterize changes in the bovine milk microbial community before, during, and after *S. aureus* CM and to compare milk microbial communities between infected and healthy quarters.

Methods: We collected quarter-level milk samples from 698 Holstein dairy cows during the entire lactation. A total of 11 quarters from 10 cows were affected by *S. aureus* CM and milk samples from these 10 cows (n = 583) regardless of health status were analyzed by performing 16S rRNA gene amplicon sequencing.

Results: The milk microbiota of healthy quarters was distinguishable from that of *S. aureus* CM quarters two weeks before CM diagnosis via visual inspection. Microbial network analysis showed that 11 OTUs had negative associations with OTU0001 (*Staphylococcus*). A low diversity or dysbiotic milk microbiome did not necessarily correlate with increased inflammation. Specifically, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, and *Aerococcus urinaeequi* were each abundant in milk from the quarters with low levels of inflammation.

Conclusion: Our results show that the udder microbiome is highly dynamic, yet a certain change in group of bacteria can be a potential indicator of *S. aureus* CM. This study has identified potential prophylactic bacterial species that could act as a barrier against *S. aureus* colonization and prevent future instances of *S. aureus* CM.

Keywords

Staphylococcus aureus, bovine mastitis, microbiome, bacterial interactions

5.2 Introduction

Bovine mastitis, a mammary gland inflammation mainly caused by bacterial infection, is one of the most prevalent and costly diseases in dairy cattle and has a significant impact on the profitability of the dairy industry, animal welfare, antimicrobial use, and public health. Bovine mastitis costs the dairy industry approximately $2 billion in the USA, about CAD$794-million in Canada, and £168 million in the UK annually (Aghamohammadi et al., 2018; Bradley, 2002; Donovan et al., 2005). *Staphylococcus aureus* is a common etiological agent of bovine mastitis that can be responsible for either subclinical mastitis (SCM) or clinical mastitis (CM); although, knowledge gaps persist and influence diagnosis, treatment, and prevention (Levison et al., 2016; Olde Riekerink et al., 2008; Rainard et al., 2018). Bovine specific pathoadaptive clonal lineages of *S. aureus* have emerged and spread alongside the use of antimicrobials in the dairy industry – increasing the prevalence of antimicrobial resistance in these lineages (Park & Ronholm, 2021). More recently, antibiotics, especially high priority category I and II antibiotics, have been banned or highly regulated in agriculture to reduce the dissemination of antibiotics resistant genes into human pathogens (Park & Ronholm, 2021; Scott et al., 2019; WHO, 2011). The withdrawal of antimicrobials raises other concerns such as farm productivity and the prevalence of infectious diseases in livestock. Thus, alternatives to antimicrobials are required to support sustainable agriculture (Sharma et al., 2017).

Microbiome plays a fundamental role in maintaining host health by metabolizing indigestible nutrients, biosynthesizing vitamins, educating the immune system, and providing microbial defences to the outgrowth of pathogens (Derakhshani et al., 2018; Thomas et al., 2017). It is now well accepted that commensal and symbiotic bacteria inhabiting the host have a potential role in resilience to exogenous perturbances. Thus, targeting and modulating the microbiome have been suggested as a promising alternative for mastitis prevention and treatment (El-Sayed & Kamel,

2021; Hu et al., 2019). Several studies on the oral administration of probiotics in breastfeeding women have shown the efficacy of probiotics for human mastitis prevention and treatment while its effectiveness on bovine mastitis is still unclear (Arroyo et al., 2010; Fernandez et al., 2016; Gao et al., 2020). Intramammary probiotics or their infusion to dairy cows also remains questionable due to pro-inflammatory effects (Frola et al., 2012; Klostermann et al., 2008; Mignacca et al., 2017; Rainard & Foucras, 2018). Despite the absence of evidence supporting the effectiveness of probiotics to prevent or treat bovine mastitis, the use of probiotics and their active biomolecules remains an area of interest for the development of alternative prophylactics and therapeutics (Angelopoulou et al., 2019; El-Sayed & Kamel, 2021).

Studies examining the microbiota of the bovine udder and raw milk have shown the presence of a diverse and dynamic microbial community (Andrews et al., 2019; Derakhshani et al., 2018; Derakhshani et al., 2020). Ganda *et al*. (2016) showed reduced species diversity in raw milk collected from quarters with *Escherichia coli* CM infection compared to those from healthy quarters in 40 cows (Ganda et al., 2016). Another study showed that infected quarters (n = 28) were frequently dominated by a single operational taxonomic unit (OTU) (Andrews et al., 2019). There have been inter-study differences in the microbial changes in post-mastitic milk. Falentin *et al*. observed long-lasting microbiome perturbations in quarters with a history of clinical mastitis in the previous lactations while Ganda *et al*. reported the restoration of the microbiota 14 days after diagnosis of mastitis (Falentin et al., 2016; Ganda et al., 2017). However, it is unknown if the disruption in the microbial diversity occurs because of a CM infection, or if the microbial changes can be detected prior to the infection and play a role in susceptibility to CM.

In this study, we hypothesize that the composition of the microbiota prior to CM may be predictive of which quarters will develop *S. aureus* CM, and that the presence of certain members of the microbiota may have a protective effect against *S. aureus* colonization. We aimed to understand

the microbial changes in both healthy and sick quarters before, during, and after *S. aureus* CM and to identify specific bacterial taxa that are negatively correlated with colonization by *S. aureus* and may, therefore, have antagonistic relationships with this important pathogen.

5.3 Materials and Methods

5.3.1 Milk sample collection

A total of 698 Holstein dairy cows from Canadian dairy herds located in the province of Quebec, in proximity to the Faculty of Veterinary Medicine of Université de Montréal (Saint-Hyacinthe) were enrolled in the project. Quarter level milk samples were collected every other week from the recruited cows before dry-off and following parturition as well as during lactation, between December 2018 and February 2020, from five different dairy herds. All milk samples were collected aseptically according to the recommended instruction by the Mastitis Network (http://www.reseaumammite.org/tactic/fr/echantillonnage/). More than 27,000 individual milk samples were collected by the research staff during this study period and kept between -10°C and -20°C due to the limited cold storage space; although, rapid freezing at -80°C would have been ideal (Bharti & Grimm, 2019). Producers (daily) and research staff (during every other week sampling visit) identified CM via visual inspection of the milk and udder. Somatic cell count (SCC) was measured on most non-clinical milk samples. Microbiological culture of all milk samples was conducted by spreading 10 µL of raw milk on 5% sheep blood agar (National Mastitis Council, 2016). After a 24 to 48-hour incubation period at 35°C, the number of different bacterial phenotypes observed on the agar were enumerated. Milk samples harboring three or more dissimilar colony types on blood agar were considered contaminated according to national mastitis council recommendation (Hogan et al., 1999). On non-contaminated samples, colonies were enumerated, and a colony representative of each phenotype (1 or 2 phenotypes) was analyzed using matrix-

assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to identify the etiological agents (Cameron et al., 2017). Mammary quarters with CM and from which *S. aureus* was isolated in pure or mixed culture, including samples that produced >3 types of colony morphology on blood agar, were considered to be infected by *S. aureus*. Among the 166 quarters from 135 cows diagnosed with CM during our study period, 11 quarters from 10 cows were diagnosed with *S. aureus* CM. From those 10 cows, a total of 599 milk samples were collected from all 40 quarters (infected and not infected) every two weeks preceding and following *S. aureus* CM as well as on the CM diagnosis day (Figure 5.1). The naming convention used, for example H1C120, is indicative of heard number (H) and cow number (C). The naming conventions for each milk sample included: collection date (YYMMDD), the assigned cow number (C) and a quarter (Q).

5.3.2 DNA extraction

Milk samples were thawed on ice and mixed thoroughly by inverting the tubes. A 1.0 mL aliquot of milk was used for DNA extraction. Each milk sample was centrifuged at 16,000 x g for 10 min and then the supernatant was discarded. For 16S rRNA gene amplicon sequencing, bacterial DNA was extracted from the remaining pallet via bead beating using DNeasy® PowerFood® Microbial Kit (QIAGEN, Germany) in combination with the QIACube instrument (QIAGEN, Germany) following the manufacturer's instructions. Bacterial DNA from milk samples with Good's coverage < 99.0% after sequencing were re-extracted and re-sequenced. An independent negative extraction control, which included extracting DNA from DNA/RNA free water using each of the reagents present in the extraction kit, was performed for each kit used in this study. A positive extraction control, which included total DNA extracted from a generous donor (GD) bovine rumen sample, was also performed using each DNA extraction kit used in this study, and results from each kit were compared to verify consistency in the study. For shotgun metagenomic sequencing, bacterial DNA

was extracted using the same kit with 1.0 to 6.0 mL of milk, and the extracted DNA was then cleaned up using DNeasy® PowerClean® Pro Cleanup Kit (QIAGEN, Germany). The concentration and purity of DNA were evaluated using Invitrogen™ Quant-iT™ dsDNA Assay Kit (Thermo Fisher Scientific, USA) and a Nanodrop 2000 (Thermo Scientific, USA).

5.3.3 PCR amplification, library preparation, and high-throughput 16S rRNA gene amplicon sequencing

Milk samples (n = 593) were analyzed by 16S rRNA gene amplicon sequencing (Appendix 6). Illumina MiSeq paired-end (2×250 bp) sequencing was used to determine the bacterial community of each milk sample. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the F548 and R806 primer pair (Kozich et al., 2013). The PCR was performed with denaturation at 95°C from 5 minutes, 35 cycles of amplification (95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute), and one final extension cycle at 72°C for 10 minutes using HotStartTaq® *Plus* Master Mix Kit (QIAGEN, Germany). An independent negative PCR control, which consisted of an attempt to amplify DNA/RNA free water, was included for each 96-well PCR reaction performed as part of this study and subjected to sequencing. The amplicons were purified using Agencourt AMPure® XP (Beckman Coulter, Brea, CA, USA) and quantified with Invitrogen™ Quant-iT™ dsDNA Assay Kit. The DNA was pooled at equimolar concentration prior to the sequencing and then the pooled library was sequenced using the MiSeq and the MiSeq reagent kit V2 (Illumina Inc., USA) for 500 cycles (251 x 2).

5.3.4 Shotgun metagenomic library preparation and high-throughput sequencing

Milk samples (n = 3) identified as being of interest for additional analysis, based on the results of 16S rRNA gene amplicon sequencing, because of a microbiota highly dominated by a single

taxonomic group with the low SCC (< 200,000 cells/mL). These samples were further analyzed via shotgun metagenomics. Sequencing libraries were prepared with Nextera XT DNA Flex Library Preparation Kit (Illumina Inc., USA) and Nextera XT Index Kit (Illumina Inc., USA) according to the manufacturer's instructions. Paired-end sequencing (2×150 bp) was performed on a NovaSeq 6000 machine (Illumina Inc., USA) at Genome Quebec (Montreal, Canada).

5.3.5 16S rRNA gene amplicon sequencing data analysis

The FASTQ files obtained from the MiSeq sequencer were analyzed using Mothur (v. 1.42.3) (Schloss et al., 2009). OTU picking was performed using the SILVA v138.1 database (Quast et al., 2013). Good's coverage was calculated and performed using MicrobiomeAnalyst (Dhariwal et al., 2017). Sequences were rarefied (vegan::rarefy.perm) repeatedly 1,000 times to minimum number of sequences (n = 3,068) to obtain the average rarefied OTU table with vegan R package (v. 2.6-2) (Cameron et al., 2021; Oksanen, 2013; Weiss et al., 2017), which was then used for further analysis. Alpha-diversity (Chao1, Shannon, and Simpson) was calculated and Mann-Whitney test (no paired) was performed with vegan R package. Beta-diversity (Bray-Curtis index) was calculated (vegan::vegdist), permutational multivariate analysis of variance (PERMANOVA) was performed (vegan::adonis2), and nonmetric multidimensional scaling (NMDS) ordination was used (vegan:: metaMDS) to plot the data with vegan R package. Linear discriminant analysis effect size (LEfSe) was performed using Mothur (Segata et al., 2011). Relative abundance (%) as well as alpha-diversity (Shannon index) was also subjected to the correlation with $\log_{10}$(SCC). Spearman correlation and a standard regression model was calculated in R software (Team, 2013).

5.3.6 Microbial change analysis

In this study, we focused on the first *S. aureus* CM event from each cow in a new lactation cycle. To compare milk microbial changes in quarters with *S. aureus* CM and healthy quarters, we selected only

one healthy quarter from each cow with low SCC (< 200,000 cells/mL) over the whole lactation as controls, except for the one quarter (Q3) of H4C88 due to the overall high SCC in all four quarters in the same cow (Appendix 6). One cow (H2C7) was excluded in this specific analysis due to no sequencing result on the first week (Week 0) of *S. aureus* CM. We then compared the milk from mammary quarters that experienced *S. aureus* CM (n = 10) to that of healthy (control) quarters (n = 9). Microbial changes at five time points up to 6 weeks before and 2 weeks after *S. aureus* CM (Week -6, Week -4, Week -2, Week 0, and Week 2) were then analyzed by comparing the milk microbial composition of heathy quarters and *S. aureus* CM quarters. Individual mammary quarters were considered the experimental units used for alpha-diversity, beta-diversity, and LEfSe analysis. We divided *S. aureus* CM cases into two groups (Group I and II) based on the relative abundance (16S rRNA gene amplicon sequencing) of the *Staphylococcus* genus in sick quarters in the first week of the *S. aureus* CM (Figure 5.2). In Group I (14 quarters from 7 cows), the same number of healthy and mastitic milk samples were used in each week: 6 milk samples at both Week -6 and Week -4, 12 samples at Week -2, 14 samples at Week 0, and 8 samples at Week 2. In Group II (6 quarters from 3 cows), we included 6 samples in each week except for Week 2 (4 samples).

5.3.7 Microbial network analysis

The average rarefied OTU table of all milk samples (n = 583) was used to perform microbial network analysis in R software (Team, 2013). Only the 293 OTUs detected at least 10% of the milk samples were included in the analysis to reduce the complexity of the network. The microbial network was analyzed by calculating co-occurrence via Spearman correlation between the OTUs and corroborated with two OTU linear models (GLM), one GLM that included only environmental independent variables and one that included independent variables and relative abundance of each other OTUs (Altshuler et al., 2019). Quasipoisson distribution on the 16S rRNA abundance data for each OTU-

OTU combination was used for GLM analysis. Sample source (cow and quarter) was considered as an independent variable. Correlations between two OTUs were filtered by *p*-value (< 0.01) in both analyses (Altshuler et al., 2019; Barberán et al., 2012). Potential false positive or negative interactions indicated by non-corroborated results from the Spearman analysis and the GLM analysis were excluded in further analysis. The interactions where the Spearman's ρ was ≥ 0.2 or ≤ -0.2 were included, which was then visualized using Cytoscape (v. 3.8.2) by the β (Shannon et al., 2003).

5.3.8 Shotgun metagenomic sequencing data analysis

The resulting FASTQ files were processed to trim low-quality bases for a cut-off value of 20 and adaptors and host-specific reads were removed using the ReadQC module of metaWRAP (v. 1.2.1) (Uritskiy et al., 2018). Bos taurus 3.1 (UMD 3.1, https://bovinegenome.elsiklab.missouri.edu/downloads/UMD_3.1) was used as a reference genome to remove the host-specific reads. The cleaned reads were then used to analyze microbial community at species using Kraken2 with miniKraken database (Wood & Salzberg, 2014). The cleaned reads were also assembled with the metaSPAdes (v. 3.15.2) and then classified into taxonomic bins using CONCOCT (v.1.0.0), MaxBIN2 (v. 2.2.6), and metaBAT2 (v. 2.2.15) (Alneberg et al., 2014; Kang et al., 2019; Nurk et al., 2017; Wu et al., 2016). The classified bins were processed to reduce contamination through RefineM (v. 0.1.2), and the refined bins were then evaluated with CheckM (v. 1.1.3) (Parks et al., 2015; Parks et al., 2017). Following the bin refinement, metagenome-assembled genomes (MAGs) were processed in Prokka (v. 1.14.5) to annotate the encoded genes (Seemann, 2014). Virulence factor was analyzed using ABRicate (https://github.com/tseemann/abricate) through the VFDB database. BAGEL4, AntiSMASH, and KEGG were used to find bacteriocin, secondary metabolites, and metabolic pathways (Blin et al., 2021; Ogata et al., 1999; van Heel et al., 2018).

5.4 Results

In this study, 19.3% (135/698) of the dairy cattle were affected by CM, of these infections, only 7.4% (10/135) were caused by *S. aureus*; the other infections were caused by various etiological agents such as non-aureus Staphylococci (NAS), *Escherichia coli*, or *Klebsiella pneumoniae*. The first *S. aureus* CM cases from each cow occurred between 8 and 203 days of milk (DIM): three in transition (1–21 DIM), two in early lactation (22–100 DIM), four in mid lactation (101–200 DIM), and one in late lactation (> 201 DIM). A total of 599 milk samples from all four quarters were collected from 10 Holstein dairy cows diagnosed with *S. aureus* CM during the 15-month study period. Among those, sequencing failed on six samples due to low bacterial DNA concentration. A total of 13,854,684 sequence reads passed filter with an average count of 22,418 sequence reads per sample, including milk samples (n = 593) and controls (n = 25). During rarefication, ten milk samples were removed due to low library size, leaving 583 milk samples for further analysis. None of the negative controls included in PCR reactions resulted in visible PCR bands on gel electrophoresis and cross-contamination of the negative and positive controls were not recognized. Thus, OTUs derived from controls were not removed from the sample dataset.

5.4.1 Overall microbiota across cows during sampling period

Taxonomic profile analysis with all milk samples showed bacterial phyla with different relative abundance were shared by the five herds. *Firmicutes* was predominant with an average relative abundance of 65.7% followed by *Bacteroidota*, *Proteobacteria*, and *Actinobacteriota* (Figure 5.3A). *Firmicutes* was highly prevalent across cows during the sampling period. Notably, the average relative abundance of *Aerococcus* was higher than *Staphylococcus* in H4C88 and H4C419 while *Actinobacteriota*, mainly *Glutamicibacter*, was more abundant in H2C7 and H2C42 (Figure 5.3B).

Within H4C88 and H4C419, differences in the relative abundance of *Aerococcus* were observed at the quarter level with higher abundance in one or two quarters (Figure 5.3D). Similarly, *Glutamicibacter* was more abundant in one quarter compared to adjacent quarters in H2C7 and H2C42. The variations in the relative abundances of major phyla and genera in each cow contributed to a cow/quarter-specific microbial community.

5.4.2 Microbial changes before, during, and after S. aureus CM

S. aureus CM cases, where *S. aureus* was isolated from the milk collected from the quarters in the first week of CM (Week 0), showed differences in the relative abundance of the *Staphylococcus* genus. This led us to divide *S. aureus* CM cases into two groups. Group I was composed of seven cows (63.6%, 7/11) with the relatively high *Staphylococcus* at Week 0 of infection. Group II was consisted of three cows where the relative abundance of *Staphylococcus* was extremely low (< 10%) at the diagnosis of infection.

In Group I animals (n = 7), up to four weeks prior to *S. aureus* CM (Week -4), both alpha-diversity and beta-diversity in the healthy quarters and the future CM quarters were not significantly different (Figure 5.4). LEfSe analysis was also unable to identify any specific OTU correlated with either the healthy or the future CM quarters at Week -6 and Week -4. Differences in the microbial profiles were observed starting two weeks before *S. aureus* CM (Week -2). The alpha-diversity in the future CM quarters at Week -2 was significantly different between healthy and future CM quarters (Shannon $p < 0.05$; Mann-Whitney statistic 32) although Chao1 and Simpson indices showed no significant difference (Appendix 7). PERMANOVA analysis of the Bray-Curtis dissimilarities revealed that the beta-diversity at Week -2 was highly dissimilar between healthy and future CM quarters (PERMANOVA $p < 0.05$, $F = 2.32$). LEfSe identified 4 OTUs that were highly associated with healthy quarters at Week -2. In the first week of *S. aureus* CM (Week 0), both

alpha- and beta-diversity in the sick quarters were significantly distinguished from the healthy quarters (Shannon $p < 0.05$; Mann-Whitney statistic 47; PERMANOVA $p < 0.05$, $F = 7.07$). LEfSe identified 12 differentially abundant OTUs, yet none of them was overlapped with those found at Week -2. OTU0002 (LDA score = -4.47, $p = 0.03$) and OTU0001 (LDA score = 5.58, $p = 0.002$) were highly associated with healthy and mastitic quarters, respectively. Two weeks after *S. aureus* CM (Week 2), both alpha- and beta-diversity in the infected quarters were indistinguishable from the healthy quarters suggesting the re-establishment of the microbiota. However, five OTUs were still significantly more abundant in the healthy quarters and OTU0009 corresponding to *Ruminococcaceae* unclassified was found in healthy quarters at Week 0 (LDA score = -3.93, $p = 0.008$) and Week 2 (LDA score = -4.17, $p = 0.04$) consecutively.

Group II animals consisted of cows (n = 3) where the relative abundance of *Staphylococcus* was barely detected at Week 0 in CM milk samples (191119C120Q2, 190805C419Q4, and 191118C184Q4). Those milk samples were initially diagnosed with *S. aureus* CM because *S. aureus* was isolated from mastitic milk samples using microbiological culture. From those CM milk samples in Group II, we found that other bacteria were sometimes isolated concurrently with *S. aureus* (Appendix 8). Two more phenotypically different colonies with no hemolytic activity on blood agar were isolated with *S. aureus* (1 CFU/0.01 mL of milk) from 191119C120Q2. *Corynebacterium bovis* (4 CFU/0.01 mL of milk) and *S. aureus* (1 CFU/0.01 mL of milk) were isolated from 190805C419Q4, yet the abundance of *Corynebacterium* was not detected in 190805C419Q4. *Aerococcus viridans* (10 CFU/0.01 mL of milk) and *S. aureus* (10 CFU/0.01 mL of milk) were isolated from 191118C184Q4, and we confirmed that the relative abundance of *Aerococcus* was higher than 34% in 191118C184Q4. To note, the sick quarters of H4C419 and H5C184 experienced CM caused by other etiological agents at Week -2. Interestingly, the alpha- and beta-diversity of three cows in Group II was not significantly dissimilar between healthy and

sick quarters at all time-points from Week -6 to Week 2 (Appendix 7). LEfSe identified that 14 OTUs highly associated with either healthy or mastitic quarters before and during *S. aureus* CM. Among them, OTU0001 (*Staphylococcus*) was significantly associated with healthy quarters (LDA score = -5.28, $p = 0.05$).

5.4.3 Network analysis of the microbial community

Out of 85,837 possible species interactions (293^2 - 12, self-interactions excluded), only 5,561 interactions involving 278 OTUs were left after filtering (Appendix 9). Among them, two OTUs (OTU0001 and OTU0012) corresponding to *Staphylococcus* were involved in 25 interactions with 14 OTUs (Figure 5.5A). All interactions between OTU0001 and other OTUs were bi-directional except for OTU0021 and the interactions between OTU0012 and two other OTUs were one-direction. The β in GLM analysis showed that all 11 OTUs had stronger negative impacts on OTU0001 (*Staphylococcus*) than the reciprocal effects. However, the relationships between OTU0001 and 11 OTUs were negligible (Spearman's $\rho > -0.2$). Only two OTUs corresponding to UCG-005 and *Aerococcus* had moderate (Spearman's $\rho > -0.4$) and weak (Spearman's $\rho > -0.3$) interactions with OTU0001, respectively. For a stricter analysis we excluded all samples (n = 45) where colonies with more than three phenotypes were isolated on blood agar. This analysis also identified the same OTUs as having a negative impact on OTU0001, except for OTU0022 (*Ruminobacter*) which was identified in the first analysis but was not identified in the second more stringent analysis. We further compared the relative abundance of 11 OTUs collectively with OTU0001 (Figure 5.5B). The relative abundance of 11 OTUs in healthy quarters was consistently higher than OTU0001 before, during, and after *S. aureus* CM dramatically varied in CM quarters. The relative abundance of 11 genera as a group was higher than OTU0002 in *S. aureus* CM quarters at Week -6 and Week -4, became lower than OTU0001 at Week -2 and Week 0. This difference in

the relative abundance between 11 OTUs and OTU0001 became more obvious during consecutive weeks while *S. aureus* CM continued for few more weeks (Week0_during), and then decreased as before *S. aureus* CM at Week2.

5.4.4 Relationship between SCC and milk microbiota

The range of SCC in this study was from 4,000 cells/mL to 35,891,000 cells/mL in non-CM milk (n = 385). The correlation between the relative abundance of each OTU (n = 5,068) and $\log_{10}$(SCC) was either negligible or weak. Interestingly, OTU0001 (*Staphylococcus*) and OTU0002 (*Aerococcus*) were observed at relatively high abundance in several milk samples at low SCC (< 200,000 cells/mL) (Figure 5.6A). For instance, high abundance of single OTU was found in milk samples: 190507C7Q2 (OTU0001, 100%), 190923C74Q1 (OTU0001, 86.5%) and 190204C88Q3 (OTU0002, 37%). The correlation between these two OTUs and $\log_{10}$(SCC) were weak and the directions were opposite. To examine the relationship between bacterial diversity and inflammation, we further analyzed the correlation between Shannon index and $\log_{10}$(SCC). Shannon index was negatively correlated with $\log_{10}$(SCC), but it was weak (Spearman's $\rho > -0.3$) (Figure 5.6B). This week correlation was also observed in the analysis that excluded milk samples that produced >3 types of colony morphology on blood agar (n = 45).

5.4.5 Staphylococcus spp. and Aerococcus spp. in healthy milk

To further explore what species of OTU0001 (*Staphylococcus*) and OTU0002 (*Aerococcus*) were present in healthy milk samples, we performed shotgun metagenomic sequencing on three milk samples: 190507C7Q2 (SCC = 59,000 cells/mL), 190923C74Q1 (SCC = 143,000 cells/mL), and 190204C88Q3 (SCC = 43,000 cells/mL). Among them, sample 190204C88Q3 produced >3 different phenotypes on blood agar. Kraken2 analysis showed the majority portion of bacterial

species in these milk samples were *S. xylosus* (95%, 190507C7Q2), *S. epidermidis* (62%, 190923C74Q1), and *A. urinaeequi* (55%, 190204C88Q3) (Figure 5.6C). Of these, we were able to reconstruct two MAGs from the taxonomic bins with good quality (> 85% completeness, < 2% contamination). These MAGs were identified as *S. xylosus* and *A. urinaeequi* with the genome size of 2.3 Mb (CDS 2,234) and 1.5 Mb (CDS 1,373), respectively. No known virulence genes were found from either *S. xylosus* MAG or *A. urinaeequi* MAG. AntiSMASH of *S. xylosus* MAG showed five gene clusters associated with secondary metabolites including staphyloferrin A, staphyloxanthin, and squalene. However, *A. urinaeequi* MAG showed no predicted gene cluster in the final bin; although, a gene cluster for lycopene biosynthesis was found in pre-refined bins.

5.5 Discussion

In this study, we investigated the milk microbiota before, during, and after *S. aureus* CM by tracking the health status of all four quarters in 10 dairy cows that developed *S. aureus* mastitis during the 15-month study period. We performed 16S rRNA gene amplicon sequencing on all samples and shotgun metagenomics on three samples from healthy control quarters. This longitudinal cohort study on the milk microbiota allows us to study microbial changes associated with cows experiencing a natural *S. aureus* CM – rather than cows infected in an artificial challenge model.

The composition of the host microbiome is different across body sites, time, and health status. In dairy cattle, prior microbiota studies have focused on the microbial profiles of different niches, the differences between CM quarters and healthy quarters at the same time point, and microbial alteration in response to mastitis treatments (Andrews et al., 2019; Ganda et al., 2016; Oikonomou et al., 2014). However, farm-to-farm variation in the microbial composition could lead to discrepancies between milk microbiome studies. Several studies have shown that each farm is a

particular niche with its own persistent microbiota (Porcellato et al., 2021; Skeie et al., 2019). In this study, we observed that composition of milk microbiota varied at the cow level as well as the quarter level. These variations were likely due to the environment or infections, yet we failed to prove the herd-specificity due to a limited and uneven number of cows from each herd included in this study. Cow/quarter-specific microbiota and its variations challenge the milk quality control and the development of the early microbial detection method for bovine mastitis using microbial indicators. Indeed, the relative abundance of all biomarkers (OTUs) in this study identified by LEfSe analysis was inconsistent during the study period, suggesting no single taxon able to represent microbial health in bovine intramammary glands. However, the relative abundance of 11 OTUs as a group was detectable over the lactation (Figure 5.7).

In this study, we only considered the first *S. aureus* CM in each quarter during a new lactation cycle to investigate the microbial changes before, during, and after *S. aureus* CM. Andrews *et al*. previously reported that the milk microbiome of infected quarters was frequently dominated by a single OTU among milk sampled collected from 28 infected quarters (Andrews et al., 2019). In the first week of the *S. aureus* CM, OTU0001 was predominant with the relative abundance of higher than 80% in five milk samples. We also observed the relative abundance of OTU0001 was high (> 80%) in three milk samples two weeks prior to the *S. aureus* CM being noted by the producer or research staff via visual inspection. SCC of the milk samples two weeks prior to *S. aureus* CM being diagnosed was between 57,000 cells/mL and 12,107,000 cells/mL, indicating largely different stages of the intramammary infections. This difference may result from the pathogenicity of *S. aureus*, the resistance/tolerance of the resident microbiota to *S. aureus*, or the immune response mounted by a particular animal (Benjamin et al., 2015; Demontier et al., 2021; Krismer et al., 2017). It may be simply because of different time gaps between *S. aureus* colonization and subsequent inflammation of the mammary glands (Petzl et al., 2008). However,

due to the unavailability of data on the exact starting date of *S. aureus* CM, the time gaps between two weeks prior and the actual onset of CM could not be determined. We also observed that the microbiota in sick quarters recovered and resembled the microbiota of healthy quarters within 2 weeks after *S. aureus* CM. This result agrees with the previous studies conducted by Ganda et al. showing the re-establishment of milk microbiome of the CM quarters within 14 days via natural infection and experimental infection of Gram-negative pathogens (Ganda et al., 2016; Ganda et al., 2017).

Unexpectedly, we also found *S. aureus* CM cases where *Staphylococcus* was barely detected at Week 0 although *S. aureus* was isolated from the same milk samples. This discordance has been rarely reported previously probably due to the insufficient sequencing depth unable to detect rare members of the microbiota (DiGiulio et al., 2008; Feazel et al., 2012; Price et al., 2009). This may result from other intrinsic factors and extrinsic factors we could not detect or notice. It is worth emphasizing that there was another CM infection in the same quarter right before *S. aureus* CM in Group II, which might overshadow *Staphylococcus*. At Week0 in Group II, more interestingly, *Staphylococcus* (OTU0001) was significantly associated with the healthy quarters (Appendix 7). Considering the low SCC (15,000 cells/mL to 282,000 cells/mL), *Staphylococcus* in these healthy quarters was likely to be NAS. This finding suggests that high alpha-diversity neither represent microbial resilience and susceptibility against pathogenic bacteria nor is associated with healthy outcome in bovine intramammary glands.

From network analysis, we observed that 11 OTUs had negative impacts on the relative abundance of OTU0001 (*Staphylococcus*). Each of 11 OTUs comprised a minor population at different time points, yet they were commonly found in milk samples we analyzed (32% to 91%). Beside the interactions between these 11 OTUs with OTU0001, they had positive impacts on each other and many other OTUs (n = 202). This intertwined microbiota could provide the microbial

resilience to pathogen colonization collectively and allow variations of an individual group in the udder. This result suggests that bacteria in bovine mammary glands may collaborate and serve as healthy microbiota and offer cross-protection against mastitis pathogens in different manners directly and indirectly.

We also investigated the relationship between SCC and bacterial abundance. Although there was no specific taxon strongly correlated with $\log_{10}$(SCC) in this study, we found two OTUs either *Staphylococcus* or *Aerococcus* was highly predominant in some milk samples from the healthy quarters with low SCC (< 200,000 cells/mL), which indicates no pro-inflammatory activity caused by these OTUs present in those specific milk samples. This finding is interesting because the significantly decreased microbial diversity generally implies an imbalanced microbiota, which tends to be more vulnerable to incoming or pathogenic bacteria (Maity & Ambatipudi, 2020; Mallon et al., 2015). However, in this study, we observed a single taxon was predominant without triggering host immune response. If this specific group of bacteria are also equipped with antagonizing ability toward mastitis pathogens, they could be promising candidates for bovine intramammary probiotics. Shotgun metagenomic sequencing revealed that *S. xylosus*, *S. epidermidis*, and *A. urinaeequi* were highly predominant in three different healthy milk samples and represented 95%, 62%, and 55% of the bacterial population in each sample. Subclinical or milk clinical mastitis can be caused by NAS, such as *S. xylosus* and *S. epidermidis*, and they are often isolated from quarters with low SCC as well as high SCC (Condas et al., 2017; De Buck et al., 2021). *S. xylosus* is known to interfere with the *S. aureus agr* quorum-sensing system and inhibit the biofilm formation ability of *S. aureus* (Leroy et al., 2020; Mahmmod et al., 2018). *S. epidermidis* is a well-known antagonistic bacterium against *S. aureus* in biofilm formation, growth, and quorum-sensing (Iwase et al., 2010; Otto et al., 2001; Sandiford & Upton, 2012). Thus, NAS which are not associated with strong intramammary inflammation, such as those observed in samples 190507C7Q2 and 190923C74Q1, have the

potential to be developed into anti-*S. aureus* probiotics. Unlike NAS, *A. urinaeequi* has been scarcely studied and its antimicrobial activity has been previously identified against only Gram-negative bacteria (Sung & Jo, 2020). However, our group has previously reported that *A. urinaeequi* strain isolated from dairy milk was able to inhibit intramammary gland infection-associated *S. aureus* strains in co-culture conditions (Park et al., 2021), making this genus also of interest for the development of anti-*S. aureus* probiotics.

In this microbiome study, we characterized the microbial community and its changes before, during, and after *S. aureus* CM in dairy cows and identified bacterial interaction that may play an important role in udder health. We also identified bacterial interactions where 11 OTUs or possibly more were negatively involved with *Staphylococcus* (OTU0001) and may be associated with the susceptibility to *S. aureus* CM. We also provide evidence that unbalanced milk microbiota caused by a certain group of bacteria was not always associated with disease or inflammation. Our findings are suggestive of a potential application of microbial modulation and perturbation in bovine udder to prevent future instances of bovine mastitis using a group of bacteria that antagonizes pathogens but induces no strong inflammation. However, a limited number of *S. aureus* CM cases and herds may result in biological and geographical bias in this study. Therefore, further studies need to focus on the antagonistic interactions between *S. aureus* and potential probiotics as well as their pro-inflammatory effects *in vivo* and *in vitro*.

Data availability

All sequencing data is available through the NCBI Sequence Read Archive (SRA) under BioProject identifiers PRJNA752361.

Authors' contributions

JR and SD conceptualized the study. DK collected and provided milk samples and metadata. SP and DJ organized, archived, and tracked milk samples. SP performed DNA extraction, library preparation, sequencing, and data analysis and wrote the manuscript. IA contributed to microbial network analysis. All authors read and approved the final manuscript.

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Competing interests

None.

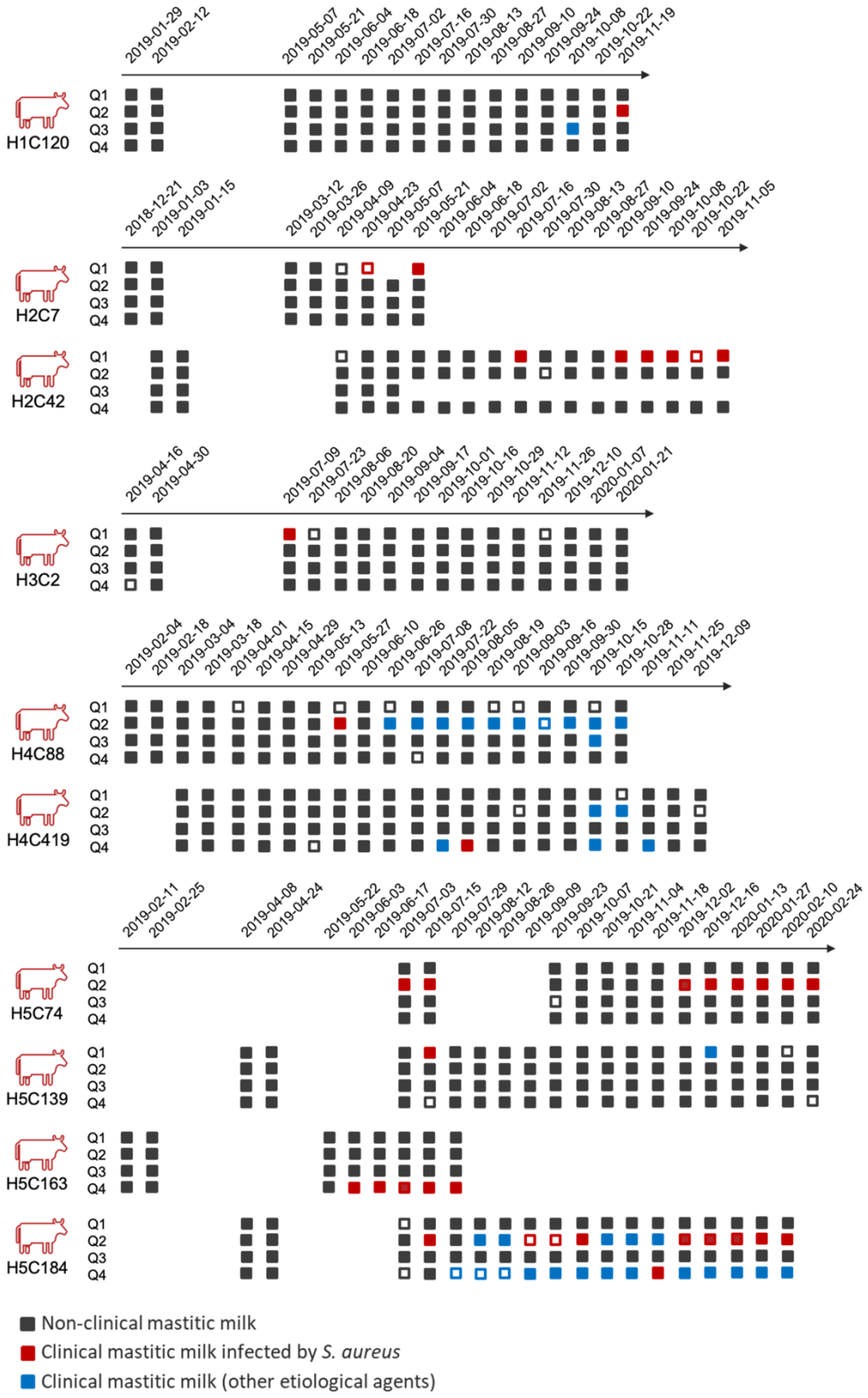

Figure 5.1 Holstein cows with *Staphylococcus aureus* clinical mastitis. This diagram illustrates 10 dairy cows diagnosed with S. aureus clinical mastitis from five dairy herds in Quebec, Canada. All mastitis occurred via natural infections. Milk samples (n = 599) were collected from all four

quarters bi-weekly. Identifiers of each cow name are 'H' for the herd and 'C' for the assigned cow number. The red and the blue square boxes represent clinical mastitis with visible symptoms infected by S. aureus and other mastitis pathogens, respectively. The black square boxes represent non-mastitic milk. The open square boxes regardless of colors indicate milk samples with no 16S rRNA data available due to missing samples (n = 16), low bacterial DNA (n = 6), and low library read size (n = 10).

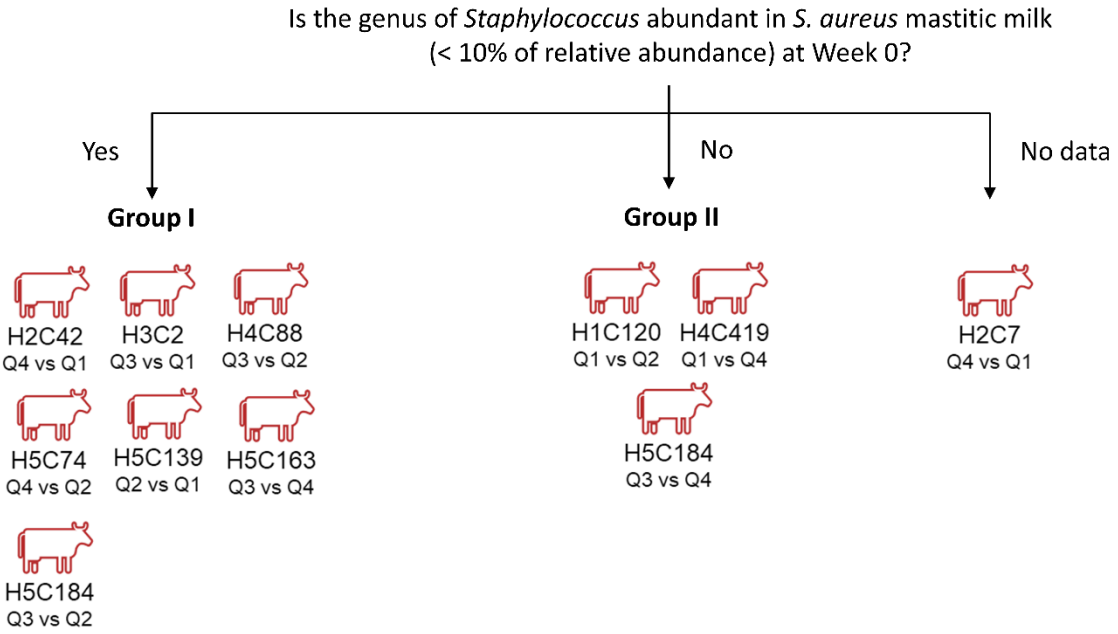

Figure 5.2 Groups of dairy cows. A total of ten dairy cows (11 quarters) affected by S. aureus clinical mastitis were grouped based on the relative abundance of Staphylococcus at Week 0. Two quarters, a healthy quarter and a CM quarter, were selected and indicated under the name of each cow. At Week 0, Staphylococcus was solely the predominant genus in mastitic milk samples in Group I while it was not detectable in Group II.

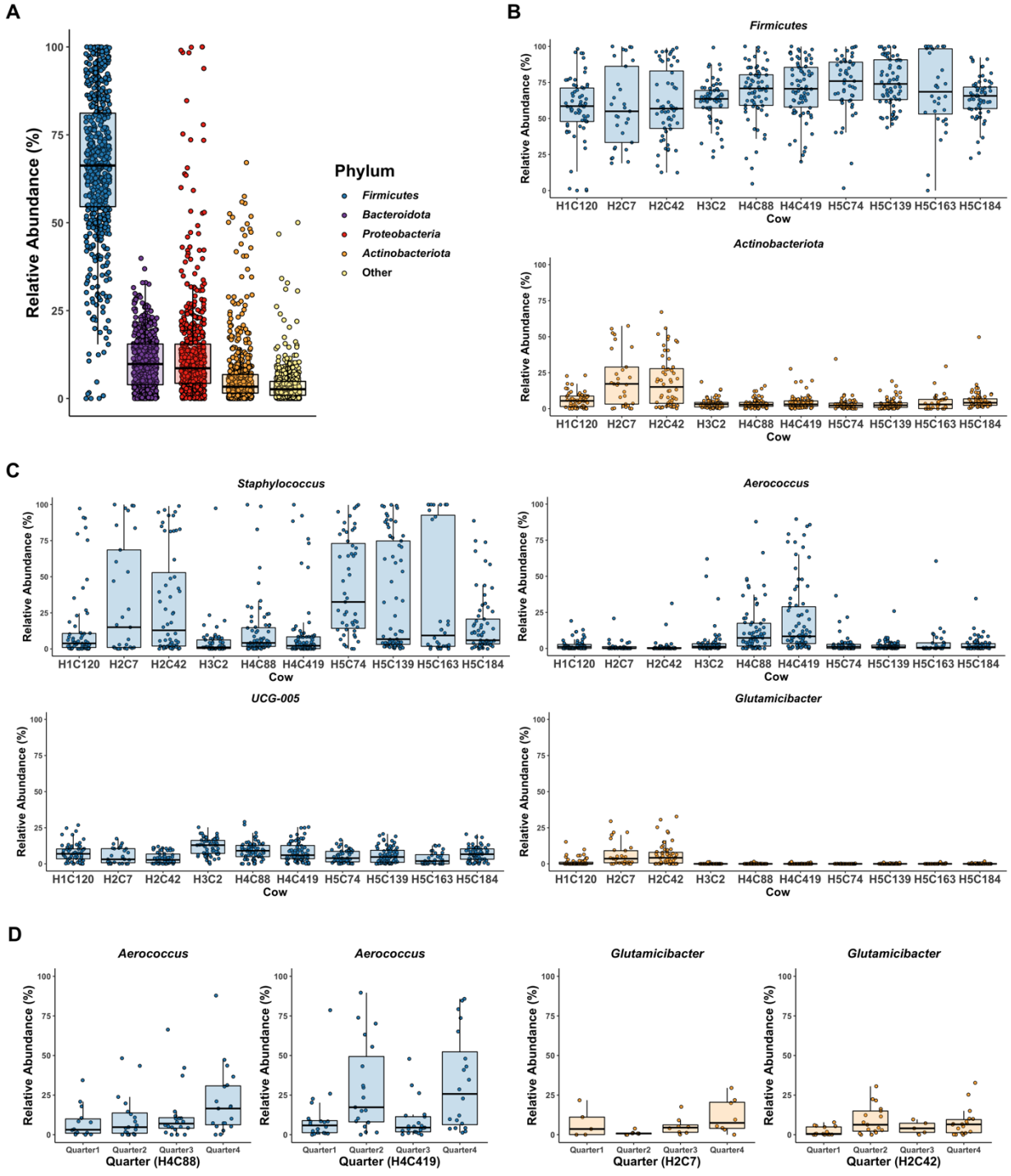

Figure 5.3 Relative abundance of the raw milk microbiota of ten cows associated with S. aureus clinical mastitis. (A) The relative abundance of each phylum in the milk samples showed four majority phyla: Firmicutes, Bacteroidota, Proteobacteria, and Actinobacteriota. (B) The relative abundance of Firmicutes and Actinobacteriota varied in ten cows. (C) At the genus level, Staphylococcus was the most abundant genus in all ten cows. The distribution of Aerococcus was high in H4C88 and H4C419 and Glutamicibacter was more abundant in H2C7 and H2C42 in other

cows. (D) The relative abundance of Aerococcus and Glutamicibacter differed at the quarter level. Identifiers of each cow name are 'H' for the herd and 'C' for cow number.

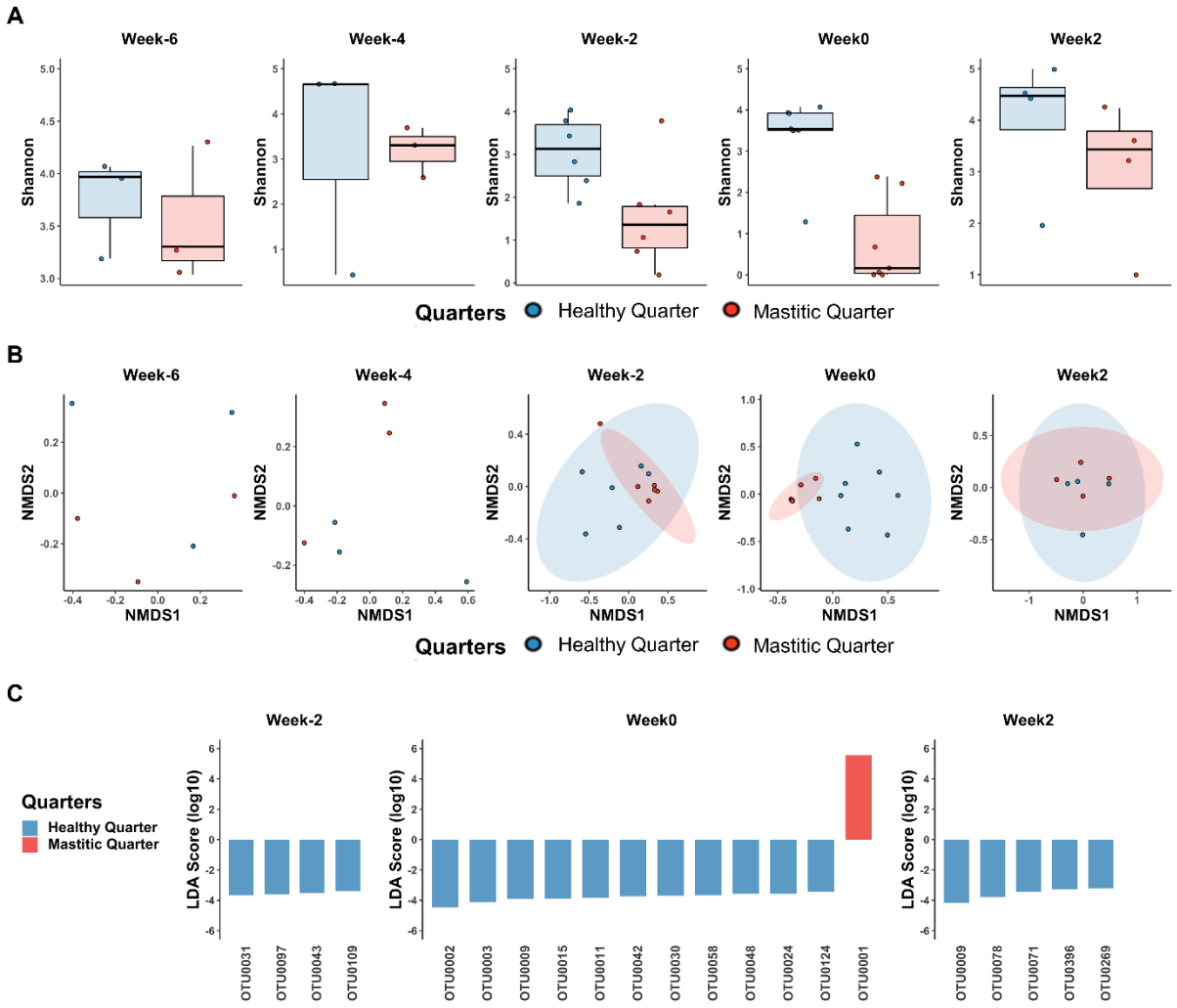

Figure 5.4 Microbial changes in alpha- and beta-diversity and biomarkers before, during, and after S. aureus clinical mastitis (CM) in Group I. Group I consisted of sick cows (n = 7) where Staphylococcus was detected in CM quarters at the time of CM diagnosis (Week 0). Both alpha-diversity (A) and beta-diversity (B) were similar between healthy and mastitic quarters at Week -6 and Week -4. Alpha-diversity in healthy quarters was significantly different from sick quarters two weeks before diagnosis (Week -2). Both alpha- and beta-diversity were significantly dissimilar between healthy and sick quarters at Week 0, which then remained as such while the mastitis continued for a few more weeks. Two weeks after the resolution of S. aureus CM (Week2), alpha- and beta-diversity had recovered, and were similar to the healthy quarters. (C) LEfSe analysis showed that at the time of diagnosis OTU0002 (Aerococcus) and OTU0001 (Staphylococcus) were highly associated with healthy and mastitic quarters, respectively. Ruminococcaceae unclassified (OTU0009) was highly associated with healthy quarters at Week 0 and Week 2.

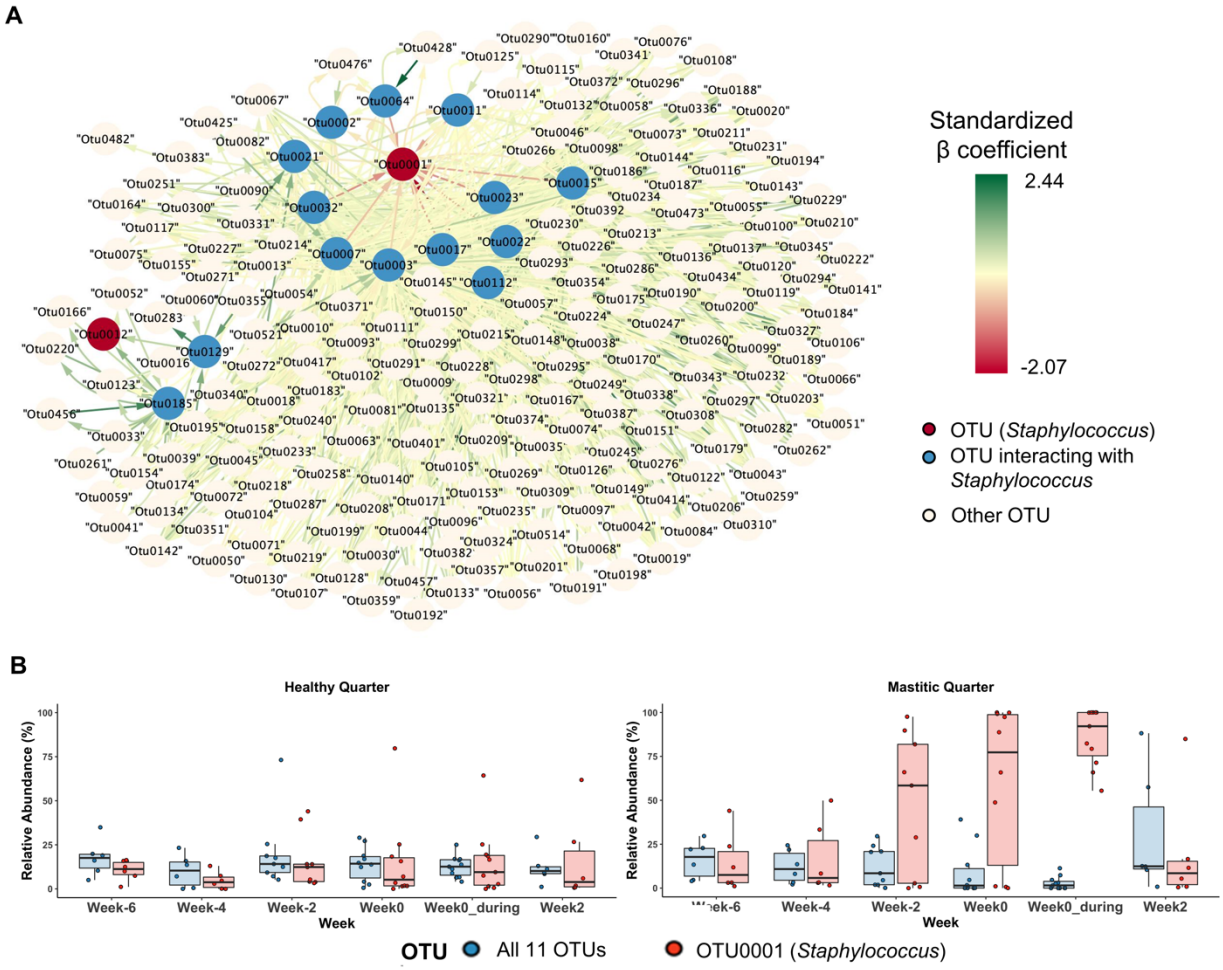

Figure 5.5 Microbial network analysis of the milk microbiota and their relative abundance. (A) The network is based on the combination of classical Spearman correlation-based network analysis corroborated with a GLM approach. Each node represents a taxonomic group at the OTU level. Arrows depict the direction of the relationship (source to target) based on the β calculation from GLM analysis. Green and red connections indicate the relative strength of the positive and negative relationships, respectively. Only 1,242 interactions where 16 OTUs including two OTUs corresponding to *Staphylococcus* were involved were shown. (B) The relative abundance of two groups (11 OTUs and OTU0001) over 8-week period including before, during, and after *S. aureus* CM was compared in healthy quarters (n = 10) and mastitic quarters (n = 11).

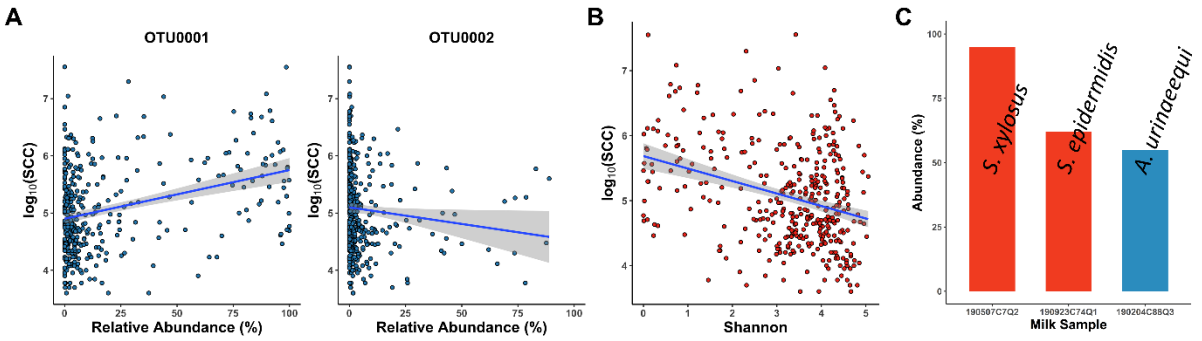

Figure 5.6 Relative abundance of milk microbiota. (A) OTU0001 and OTU0002 were predominant in milk samples with low SCC. (B) Shannon index was negatively correlated with $\log_{10}$(SCC) (Spearman's $\rho > -0.3$). (C) Shotgun metagenomic sequencing identified the bacterial species in healthy milk samples and revealed the relative abundance (%).

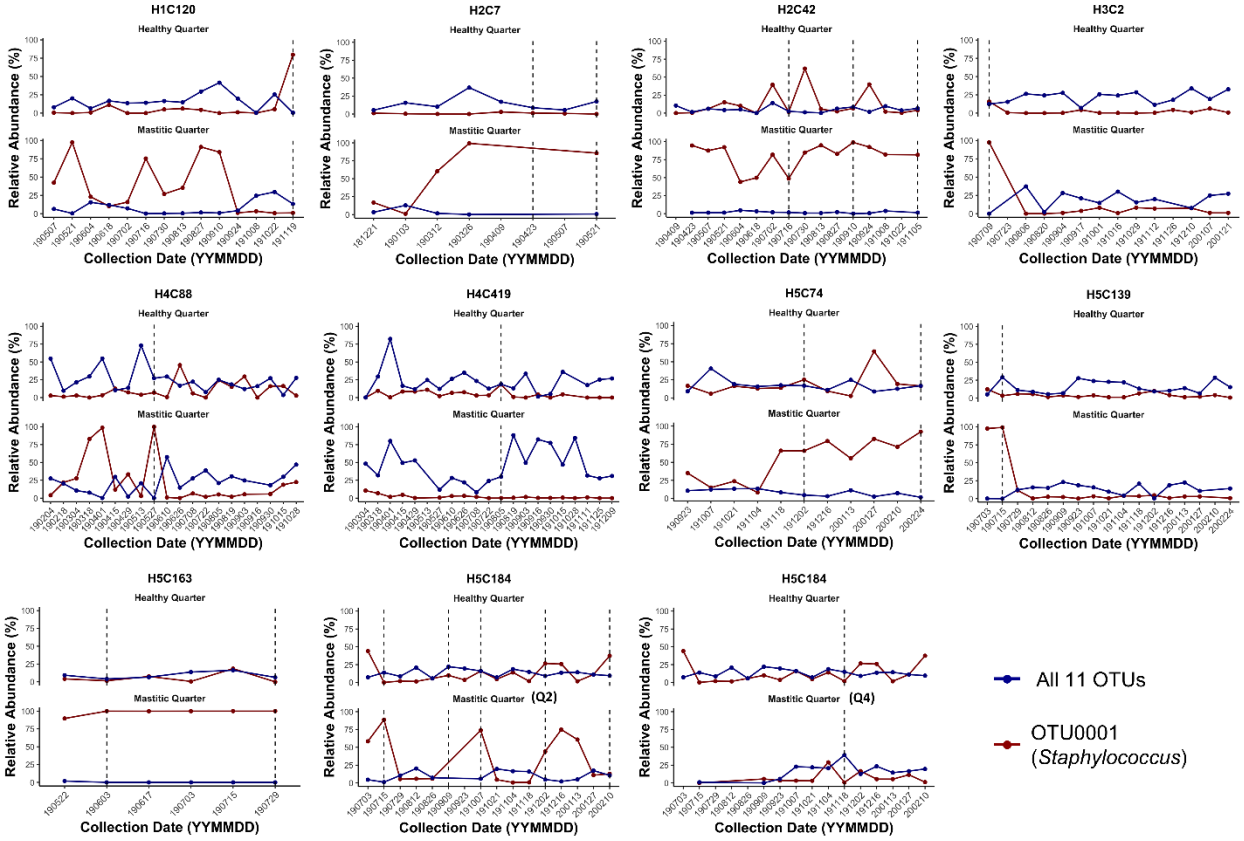

Figure 5.7 Relative abundance of 11 OTUs and OTU0001 (*Staphylococcus*) over the lactation in healthy and mastitic quarters. The line graphs depict the changes of the relative abundance of 11 OTUs and OTU0001 in two quarters (healthy vs. mastitic quarters) from each cow over the study period. The vertical dotted lines indicate either beginning or ending of *S. aureus* CM.

5.6 References

Aghamohammadi, M., Haine, D., Kelton, D. F., Barkema, H. W., Hogeveen, H., Keefe, G. P., & Dufour, S. (2018). Herd-Level Mastitis-Associated Costs on Canadian Dairy Farms. *Front Vet Sci*, *5*, 100. https://doi.org/10.3389/fvets.2018.00100

Alneberg, J., Bjarnason, B. S., de Bruijn, I., Schirmer, M., Quick, J., Ijaz, U. Z., . . . Quince, C. (2014). Binning metagenomic contigs by coverage and composition. *Nat Methods*, *11*(11), 1144-1146. https://doi.org/10.1038/nmeth.3103

Altshuler, I., Hamel, J., Turney, S., Magnuson, E., Levesque, R., Greer, C. W., & Whyte, L. G. (2019). Species interactions and distinct microbial communities in high Arctic permafrost affected cryosols are associated with the CH4 and CO2 gas fluxes. *Environ Microbiol*, *21*(10), 3711-3727. https://doi.org/10.1111/1462-2920.14715

Andrews, T., Neher, D. A., Weicht, T. R., & Barlow, J. W. (2019). Mammary microbiome of lactating organic dairy cows varies by time, tissue site, and infection status. *PLoS One*, *14*(11), e0225001. https://doi.org/10.1371/journal.pone.0225001

Angelopoulou, A., Warda, A. K., Hill, C., & Ross, R. P. (2019). Non-antibiotic microbial solutions for bovine mastitis - live biotherapeutics, bacteriophage, and phage lysins. *Crit Rev Microbiol*, *45*(5-6), 564-580. https://doi.org/10.1080/1040841X.2019.1648381

Arroyo, R., Martin, V., Maldonado, A., Jimenez, E., Fernandez, L., & Rodriguez, J. M. (2010). Treatment of infectious mastitis during lactation: antibiotics versus oral administration of Lactobacilli isolated from breast milk. *Clin Infect Dis*, *50*(12), 1551-1558. https://doi.org/10.1086/652763

Barberán, A., Bates, S. T., Casamayor, E. O., & Fierer, N. (2012). Using network analysis to explore co-occurrence patterns in soil microbial communities. *The ISME Journal*, *6*(2), 343-351. https://doi.org/10.1038/ismej.2011.119

Benjamin, A. L., Green, B. B., Hayden, L. R., Barlow, J. W., & Kerr, D. E. (2015). Cow-to-cow variation in fibroblast response to a toll-like receptor 2/6 agonist and its relation to mastitis caused by intramammary challenge with Staphylococcus aureus. *J Dairy Sci*, *98*(3), 1836-1850. https://doi.org/10.3168/jds.2014-9075

Bharti, R., & Grimm, D. G. (2019). Current challenges and best-practice protocols for microbiome analysis. *Briefings in Bioinformatics*, *22*(1), 178-193. https://doi.org/10.1093/bib/bbz155

Blin, K., Shaw, S., Kloosterman, A. M., Charlop-Powers, Z., van Wezel, G. P., Medema, M. H., & Weber, T. (2021). antiSMASH 6.0: improving cluster detection and comparison capabilities. *Nucleic Acids Res*, *49*(W1), W29-W35. https://doi.org/10.1093/nar/gkab335

Bradley, A. (2002). Bovine mastitis: an evolving disease. *Vet J*, *164*(2), 116-128. https://doi.org/10.1053/tvjl.2002.0724

Cameron, E. S., Schmidt, P. J., Tremblay, B. J.-M., Emelko, M. B., & Müller, K. M. (2021). To rarefy or not to rarefy: Enhancing diversity analysis of microbial communities through next-generation sequencing and rarefying repeatedly. *bioRxiv*, 2020.2009.2009.290049. https://doi.org/10.1101/2020.09.09.290049

Cameron, M., Barkema, H. W., De Buck, J., De Vliegher, S., Chaffer, M., Lewis, J., & Keefe, G. P. (2017). Identification of bovine-associated coagulase-negative staphylococci by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a direct transfer protocol. *J Dairy Sci*, *100*(3), 2137-2147. https://doi.org/10.3168/jds.2016-12020

Condas, L. A. Z., De Buck, J., Nobrega, D. B., Carson, D. A., Roy, J. P., Keefe, G. P., . . . Barkema, H. W. (2017). Distribution of non-aureus staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. *J Dairy Sci*, *100*(7), 5613-5627.

https://doi.org/10.3168/jds.2016-12479
De Buck, J., Ha, V., Naushad, S., Nobrega, D. B., Luby, C., Middleton, J. R., . . . Barkema, H. W. (2021). Non-aureus Staphylococci and Bovine Udder Health: Current Understanding and Knowledge Gaps. *Front Vet Sci*, *8*, 658031. https://doi.org/10.3389/fvets.2021.658031
Demontier, E., Dubé-Duquette, A., Brouillette, E., Larose, A., Ster, C., Lucier, J. F., . . . Malouin, F. (2021). Relative virulence of Staphylococcus aureus bovine mastitis strains representing the main Canadian spa types and clonal complexes as determined using in vitro and in vivo mastitis models. *J Dairy Sci*, *104*(11), 11904-11921. https://doi.org/10.3168/jds.2020-19904
Derakhshani, H., Fehr, K. B., Sepehri, S., Francoz, D., De Buck, J., Barkema, H. W., . . . Khafipour, E. (2018). Invited review: Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility. *J Dairy Sci*, *101*(12), 10605-10625. https://doi.org/10.3168/jds.2018-14860
Derakhshani, H., Plaizier, J. C., De Buck, J., Barkema, H. W., & Khafipour, E. (2020). Composition and co-occurrence patterns of the microbiota of different niches of the bovine mammary gland: potential associations with mastitis susceptibility, udder inflammation, and teat-end hyperkeratosis. *Anim Microbiome*, *2*(1), 11. https://doi.org/10.1186/s42523-020-00028-6
Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., & Xia, J. (2017). MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res*, *45*(W1), W180-w188. https://doi.org/10.1093/nar/gkx295
DiGiulio, D. B., Romero, R., Amogan, H. P., Kusanovic, J. P., Bik, E. M., Gotsch, F., . . . Relman, D. A. (2008). Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS One*, *3*(8), e3056. https://doi.org/10.1371/journal.pone.0003056
Donovan, D. M., Kerr, D. E., & Wall, R. J. (2005). Engineering disease resistant cattle. *Transgenic Res*, *14*(5), 563-567. https://doi.org/10.1007/s11248-005-0670-8
El-Sayed, A., & Kamel, M. (2021). Bovine mastitis prevention and control in the post-antibiotic era. *Trop Anim Health Prod*, *53*(2), 236. https://doi.org/10.1007/s11250-021-02680-9
Falentin, H., Rault, L., Nicolas, A., Bouchard, D. S., Lassalas, J., Lamberton, P., . . . Even, S. (2016). Bovine Teat Microbiome Analysis Revealed Reduced Alpha Diversity and Significant Changes in Taxonomic Profiles in Quarters with a History of Mastitis. *Front Microbiol*, *7*, 480. https://doi.org/10.3389/fmicb.2016.00480
Feazel, L. M., Robertson, C. E., Ramakrishnan, V. R., & Frank, D. N. (2012). Microbiome complexity and Staphylococcus aureus in chronic rhinosinusitis. *Laryngoscope*, *122*(2), 467-472. https://doi.org/10.1002/lary.22398
Fernandez, L., Cardenas, N., Arroyo, R., Manzano, S., Jimenez, E., Martin, V., & Rodriguez, J. M. (2016). Prevention of Infectious Mastitis by Oral Administration of Lactobacillus salivarius PS2 During Late Pregnancy. *Clin Infect Dis*, *62*(5), 568-573. https://doi.org/10.1093/cid/civ974
Frola, I. D., Pellegrino, M. S., Espeche, M. C., Giraudo, J. A., Nader-Macias, M. E., & Bogni, C. I. (2012). Effects of intramammary inoculation of Lactobacillus perolens CRL1724 in lactating cows' udders. *J Dairy Res*, *79*(1), 84-92. https://doi.org/10.1017/S0022029911000835
Ganda, E. K., Bisinotto, R. S., Lima, S. F., Kronauer, K., Decter, D. H., Oikonomou, G., . . . Bicalho, R. C. (2016). Longitudinal metagenomic profiling of bovine milk to assess the impact of intramammary treatment using a third-generation cephalosporin. *Sci Rep*, *6*, 37565. https://doi.org/10.1038/srep37565
Ganda, E. K., Gaeta, N., Sipka, A., Pomeroy, B., Oikonomou, G., Schukken, Y. H., & Bicalho, R. C. (2017). Normal milk microbiome is reestablished following experimental infection with

Escherichia coli independent of intramammary antibiotic treatment with a third-generation cephalosporin in bovines. *Microbiome*, *5*(1), 74. https://doi.org/10.1186/s40168-017-0291-5

Gao, J., Liu, Y. C., Wang, Y., Li, H., Wang, X. M., Wu, Y., . . . Qi, Z. L. (2020). Impact of yeast and lactic acid bacteria on mastitis and milk microbiota composition of dairy cows. *AMB Express*, *10*(1), 22. https://doi.org/10.1186/s13568-020-0953-8

Hogan, J., Gonzalez, R., Harmon, R., Nickerson, S., Oliver, S., Pankey, J., & Smith, K. (1999). Laboratory handbook on bovine mastitis. *National Mastitis Council, Madison, WI*, *78*(7), 485-488.

Hu, X., Li, S., Fu, Y., & Zhang, N. (2019). Targeting gut microbiota as a possible therapy for mastitis. *Eur J Clin Microbiol Infect Dis*, *38*(8), 1409-1423. https://doi.org/10.1007/s10096-019-03549-4

Iwase, T., Uehara, Y., Shinji, H., Tajima, A., Seo, H., Takada, K., . . . Mizunoe, Y. (2010). Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. *Nature*, *465*(7296), 346-349. https://doi.org/10.1038/nature09074

Kang, D. D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., & Wang, Z. (2019). MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ*, *7*, e7359. https://doi.org/10.7717/peerj.7359

Klostermann, K., Crispie, F., Flynn, J., Ross, R. P., Hill, C., & Meaney, W. (2008). Intramammary infusion of a live culture of Lactococcus lactis for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. *J Dairy Res*, *75*(3), 365-373. https://doi.org/10.1017/S0022029908003373

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol*, *79*(17), 5112-5120. https://doi.org/10.1128/AEM.01043-13

Krismer, B., Weidenmaier, C., Zipperer, A., & Peschel, A. (2017). The commensal lifestyle of Staphylococcus aureus and its interactions with the nasal microbiota. *Nat Rev Microbiol*, *15*(11), 675-687. https://doi.org/10.1038/nrmicro.2017.104

Leroy, S., Lebert, I., Andant, C., & Talon, R. (2020). Interaction in dual species biofilms between Staphylococcus xylosus and Staphylococcus aureus. *Int J Food Microbiol*, *326*, 108653. https://doi.org/10.1016/j.ijfoodmicro.2020.108653

Levison, L. J., Miller-Cushon, E. K., Tucker, A. L., Bergeron, R., Leslie, K. E., Barkema, H. W., & DeVries, T. J. (2016). Incidence rate of pathogen-specific clinical mastitis on conventional and organic Canadian dairy farms. *J Dairy Sci*, *99*(2), 1341-1350. https://doi.org/10.3168/jds.2015-9809

Mahmmod, Y. S., Klaas, I. C., Svennesen, L., Pedersen, K., & Ingmer, H. (2018). Communications of Staphylococcus aureus and non-aureus Staphylococcus species from bovine intramammary infections and teat apex colonization. *J Dairy Sci*, *101*(8), 7322-7333. https://doi.org/10.3168/jds.2017-14311

Maity, S., & Ambatipudi, K. (2020). Mammary microbial dysbiosis leads to the zoonosis of bovine mastitis: a One-Health perspective. *FEMS Microbiol Ecol*, *97*(1). https://doi.org/10.1093/femsec/fiaa241

Mallon, C. A., Elsas, J. D. V., & Salles, J. F. (2015). Microbial invasions: the process, patterns, and mechanisms. *Trends Microbiol*, *23*(11), 719-729. https://doi.org/10.1016/j.tim.2015.07.013

Mignacca, S. A., Dore, S., Spuria, L., Zanghi, P., Amato, B., Dupre, I., . . . Marianelli, C. (2017). Intramammary infusion of a live culture of Lactococcus lactis in ewes to treat staphylococcal mastitis. *J Med Microbiol*, *66*(12), 1798-1810. https://doi.org/10.1099/jmm.0.000641

National Mastitis Council, H. J. S. M. M. (2016). *Current concepts of bovine mastitis*.
Nurk, S., Meleshko, D., Korobeynikov, A., & Pevzner, P. A. (2017). metaSPAdes: a new versatile metagenomic assembler. *Genome Res*, *27*(5), 824-834. https://doi.org/10.1101/gr.213959.116
Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., & Kanehisa, M. (1999). KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res*, *27*(1), 29-34. https://doi.org/10.1093/nar/27.1.29
Oikonomou, G., Bicalho, M. L., Meira, E., Rossi, R. E., Foditsch, C., Machado, V. S., . . . Bicalho, R. C. (2014). Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. *PLoS One*, *9*(1), e85904. https://doi.org/10.1371/journal.pone.0085904
Oksanen, J. (2013). Vegan: ecological diversity. *R Project*, *368*, 1-11.
Olde Riekerink, R. G., Barkema, H. W., Kelton, D. F., & Scholl, D. T. (2008). Incidence rate of clinical mastitis on Canadian dairy farms. *J Dairy Sci*, *91*(4), 1366-1377. https://doi.org/10.3168/jds.2007-0757
Otto, M., Echner, H., Voelter, W., & Gotz, F. (2001). Pheromone cross-inhibition between Staphylococcus aureus and Staphylococcus epidermidis. *Infect Immun*, *69*(3), 1957-1960. https://doi.org/10.1128/IAI.69.3.1957-1960.2001
Park, S., Classen, A., Gohou, H. M., Maldonado, R., Kretschmann, E., Duvernay, C., . . . Ronholm, J. (2021). A new, reliable, and high-throughput strategy to screen bacteria for antagonistic activity against Staphylococcus aureus. *BMC Microbiol*, *21*(1), 189. https://doi.org/10.1186/s12866-021-02265-4
Park, S., & Ronholm, J. (2021). Staphylococcus aureus in Agriculture: Lessons in Evolution from a Multispecies Pathogen. *Clin Microbiol Rev*, *34*(2). https://doi.org/10.1128/CMR.00182-20
Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., & Tyson, G. W. (2015). CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res*, *25*(7), 1043-1055. https://doi.org/10.1101/gr.186072.114
Parks, D. H., Rinke, C., Chuvochina, M., Chaumeil, P.-A., Woodcroft, B. J., Evans, P. N., . . . Tyson, G. W. (2017). Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. *Nature Microbiology*, *2*(11), 1533-1542. https://doi.org/10.1038/s41564-017-0012-7
Petzl, W., Zerbe, H., Günther, J., Yang, W., Seyfert, H. M., Nürnberg, G., & Schuberth, H. J. (2008). Escherichia coli, but not Staphylococcus aureus triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. *Vet Res*, *39*(2), 18. https://doi.org/10.1051/vetres:2007057
Porcellato, D., Smistad, M., Bombelli, A., Abdelghani, A., Jorgensen, H. J., & Skeie, S. B. (2021). Longitudinal Study of the Bulk Tank Milk Microbiota Reveals Major Temporal Shifts in Composition. *Front Microbiol*, *12*, 616429. https://doi.org/10.3389/fmicb.2021.616429
Price, L. B., Liu, C. M., Melendez, J. H., Frankel, Y. M., Engelthaler, D., Aziz, M., . . . Zenilman, J. M. (2009). Community analysis of chronic wound bacteria using 16S rRNA gene-based pyrosequencing: impact of diabetes and antibiotics on chronic wound microbiota. *PLoS One*, *4*(7), e6462. https://doi.org/10.1371/journal.pone.0006462
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., . . . Glockner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*, *41*(Database issue), D590-596. https://doi.org/10.1093/nar/gks1219
Rainard, P., & Foucras, G. (2018). A Critical Appraisal of Probiotics for Mastitis Control. *Front Vet Sci*, *5*, 251. https://doi.org/10.3389/fvets.2018.00251
Rainard, P., Foucras, G., Fitzgerald, J. R., Watts, J. L., Koop, G., & Middleton, J. R. (2018). Knowledge gaps and research priorities in Staphylococcus aureus mastitis control.

Transbound Emerg Dis, *65 Suppl 1*, 149-165. https://doi.org/10.1111/tbed.12698
Sandiford, S., & Upton, M. (2012). Identification, characterization, and recombinant expression of epidermicin NI01, a novel unmodified bacteriocin produced by Staphylococcus epidermidis that displays potent activity against Staphylococci. *Antimicrob Agents Chemother*, *56*(3), 1539-1547. https://doi.org/10.1128/AAC.05397-11
Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., . . . Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol*, *75*(23), 7537-7541. https://doi.org/10.1128/AEM.01541-09
Scott, H. M., Acuff, G., Bergeron, G., Bourassa, M. W., Gill, J., Graham, D. W., . . . Wittum, T. E. (2019). Critically important antibiotics: criteria and approaches for measuring and reducing their use in food animal agriculture. *Annals of the New York Academy of Sciences*, *1441*(1), 8-16. https://doi.org/10.1111/nyas.14058
Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics*, *30*(14), 2068-2069. https://doi.org/10.1093/bioinformatics/btu153
Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60
Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., . . . Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*, *13*(11), 2498-2504. https://doi.org/10.1101/gr.1239303
Sharma, C., Rokana, N., Chandra, M., Singh, B. P., Gulhane, R. D., Gill, J. P. S., . . . Panwar, H. (2017). Antimicrobial Resistance: Its Surveillance, Impact, and Alternative Management Strategies in Dairy Animals. *Front Vet Sci*, *4*, 237. https://doi.org/10.3389/fvets.2017.00237
Skeie, S. B., Haland, M., Thorsen, I. M., Narvhus, J., & Porcellato, D. (2019). Bulk tank raw milk microbiota differs within and between farms: A moving goalpost challenging quality control. *J Dairy Sci*, *102*(3), 1959-1971. https://doi.org/10.3168/jds.2017-14083
Sung, H. S., & Jo, Y. L. (2020). Purification and Characterization of an Antibacterial Substance from Aerococcus urinaeequi Strain HS36. *J Microbiol Biotechnol*, *30*(1), 93-100. https://doi.org/10.4014/jmb.1910.10015
Team, R. C. (2013). R: A language and environment for statistical computing.
Thomas, S., Izard, J., Walsh, E., Batich, K., Chongsathidkiet, P., Clarke, G., . . . Prendergast, G. C. (2017). The Host Microbiome Regulates and Maintains Human Health: A Primer and Perspective for Non-Microbiologists. *Cancer Res*, *77*(8), 1783-1812. https://doi.org/10.1158/0008-5472.CAN-16-2929
Uritskiy, G. V., DiRuggiero, J., & Taylor, J. (2018). MetaWRAP-a flexible pipeline for genome-resolved metagenomic data analysis. *Microbiome*, *6*(1), 158. https://doi.org/10.1186/s40168-018-0541-1
van Heel, A. J., de Jong, A., Song, C., Viel, J. H., Kok, J., & Kuipers, O. P. (2018). BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. *Nucleic Acids Res*, *46*(W1), W278-W281. https://doi.org/10.1093/nar/gky383
Weiss, S., Xu, Z. Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., . . . Knight, R. (2017). Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*, *5*(1), 27. https://doi.org/10.1186/s40168-017-0237-y
WHO. (2011). *WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR): Critically Important Antimicrobials for Human Medicine. 3rd Revision 2011*.
Wood, D. E., & Salzberg, S. L. (2014). Kraken: ultrafast metagenomic sequence classification using

exact alignments. *Genome Biol*, *15*(3), R46. https://doi.org/10.1186/gb-2014-15-3-r46
Wu, Y. W., Simmons, B. A., & Singer, S. W. (2016). MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets. *Bioinformatics*, *32*(4), 605-607. https://doi.org/10.1093/bioinformatics/btv638

CHAPTER 6. GENERAL DISCUSSION

In this study, we investigated genetic characteristics of *S. aureus* originated from humans and bovine, developed a new method to screen bacteria with antagonistic activity towards *S. aureus*, and investigated raw milk microbiome associated with *S. aureus* CM. This thesis provides the evidence for clonal evolution, bacterial interactions, and disease susceptibility in the context of *S. aureus* and bovine mastitis, which ultimately aids to the knowledge in the development of early diagnostics and prophylactics/therapeutics for *S. aureus* CM.

In this thesis, we showed distinct clades of *S. aureus* from those two different hosts in phylogenomic tree (Chapter 3). Host-specific and exclusive genes were found in *S. aureus* isolates in both humans and bovine, yet there was no absolute or universal host-specific element present in all *S. aureus* isolated from the same host. We only considered the presence/absence of genes in *S. aureus* genomes to identify host-specific and host-adaptation genes. Thus, gene mutations and pseudogenes potentially associated with host adaptation and fitness were excluded. However, nucleotide mutations are also important and associated with host adaptation and pathogenicity and have been reported in *S. aureus* adapted in a new host altering its host tropism (Viana et al., 2015). Our findings on CC-specific R-M genes of *S. aureus* also provide evidence of clonal evolution and potential host jumping scenarios. Among *S. aureus* isolates originated from bovine, *spa* gene for Protein A (SpA) is often found as a pseudogene due to a premature stop codon (Herron-Olson et al., 2007). It remains unclear whether this mutation increases *S. aureus* fitness in bovine niche. However, double immunodiffusion assay showed differences in the complexes between bovine IgG-SpA and human IgG-SpA questioning the role of Protein A in bovine infection (Atkins et al., 2008). In this study, we also observed that *spa* gene in more than 50% (34/65) of IMI-associated *S. aureus*

isolates was pseudogenes while only two human-originated *S. aureus* (2/122) had premature stop condo in *spa* gene.

We showed that two isolates (2/63) carried *blaZ*, and of those only one carried *mecA*. Since β-lactams are the most common antibiotics used on Canadian dairy farms for mastitis prevention/treatment (Saini, McClure, Léger, et al., 2012), our findings showed low prevalence of *blaZ* and *mecA* in IMI-associated *S. aureus* isolates. Similarly, low prevalence of *blaZ* gene (4/119) was reported previously among *S. aureus* isolated from Canadian dairy farms, yet the phenotypical resistance was much higher (23/119) (Naushad et al., 2020). Only few STs among *S. aureus* CC97 have been reported to be positive for *blaZ* or *mecA* while human-adapted lineages such as CC5 and CC8 are highly positive for both (Kappeli et al., 2019; Klibi et al., 2018; Schmidt et al., 2017). These studies support the idea that certain *S. aureus* lineages may be more prone to obtaining specific AMR genes while other lineages may develop phenotypical resistance via random mutations. This unequal distribution of AMR genes in different lineages might be due to lineage-specific factors such as CC-specific R-M system and intra-species competitions between different CCs.

One of interesting results observed in this thesis was the intra-species competition between *S. aureus* CC151 and CC97, which are the most common CCs found in Canadian dairy farms (Chapter 3 and Chapter 4). We observed growth-inhibiting activity of CC97 against CC151 and QQ activity of CC151 towards CC97. Most of CC97 isolates (31/38) carried pCC97-1 plasmid that encodes genes for aureocin synthesis and transportation. This bacteriocin produced by CC97 is responsible for growth-inhibiting activity against CC151. On the other hand, CC151 (AIP-II producer) inhibits the QS of CC97 (AIP-I producer) interfering AIP-I binding to its cognate receptor. Our findings indicate that two major CCs in bovine niche are unlikely to colonize the same

niche at the same time point, which supports independent evolution of CC151 and CC97 as well as low chance of genetic exchanges between them.

Beside the intra-species competition, we also observed inter-species competitions in growth and QS *in vitro* (Chapter 4). *Bacillus* species mainly secreted growth-inhibiting molecules towards *S. aureus* while NAS strongly inhibited *S. aureus* QS. Members of the *Bacillus* genus produce a broad range of antimicrobial peptides that have antagonistic activity against pathogenic bacteria including *S. aureus* (Sumi et al., 2015). From draft genomes of *B. subtilis* and *B. pumilus* screened from this study (Chapter 4), we also identified gene clusters responsible for several NRPs such as surfactin and lichenysin. Similar to the intra-species antagonism, the QS of CC97 was more frequently and strongly affected by NAS than CC151, indicating *agr* type I is more vulnerable to the QQ mediated by bovine commensal bacteria. These interactions might not be observed or hardly detected via traditional methods due to low accumulation and degradation of antagonistic compounds in pure culture conditions. Indeed, with the supernatants of commensal bacteria positive to QQ from the pQS-based competition assay, the traditional competition assay failed to show antagonistic activities, suggesting the effectiveness of the new screening strategy proposed in this thesis.

In Chapter 5, one of the results observed in this thesis was three cows where the relative abundance of *Staphylococcus* was undetectable or extremely low from 16S rRNA TAS on the first week of *S. aureus* CM. Those milk samples were initially diagnosed with *S. aureus* CM via visual inspection and microbiological culture on blood agar and bacterial identification using MALDI-TOF. This discordance between microbiological culture and 16S rRNA TAS has been rarely reported previously due to the insufficient sequencing depth unable to detect rare members of the microbiota (DiGiulio et al., 2008; Feazel et al., 2012; Price et al., 2009). This discordance may result from other intrinsic factors and extrinsic factors we could not detect or notice. The latter is

likely to be the case of this study. It is worth emphasizing that there was another CM infection in the same quarter right before *S. aureus* CM in Group II. Furthermore, other bacteria including *C. bovis* and *A. viridans* were isolated with *S. aureus* from the same milk samples on the first week of *S. aureus* CM, which potentially overshadow *Staphylococcus* in those milk samples.

Another surprising result observed in Chapter 5 was that some bacterial species can be predominant in the microbial population without triggering strong inflammation. We found each of three bacterial species – *S. xylosus*, *S. epidermidis*, and *A. urinaeequi* – was highly predominant in three different milk samples from healthy quarters with low inflammation. This result is interesting because the significantly decreased microbial diversity generally implies an imbalanced microbiota, which tends to be more vulnerable to incoming or pathogenic bacteria (Maity & Ambatipudi, 2020; Mallon et al., 2015). More importantly, this specific group of bacteria can be promising candidates for the development of anti-*S. aureus* probiotics due to their antagonistic activity towards *S. aureus*. NAS such as *S. xylosus* and *S. epidermidis* are well-known antagonistic bacteria against *S. aureus* in biofilm formation, growth, and quorum-sensing (Leroy et al., 2020; Mahmmod et al., 2018). We also showed that *A. urinaeequi* isolate, originally misidentified as *A. viridian*, inhibits *S. aureus* in co-culture condition (Chapter 4).

CHAPTER 7. CONCLUSIONS, ORIGINAL CONTRIBUTIONS TO KNOWLEDGE, AND FUTURE WORK

7.1 Conclusion of Completed Research Objectives

In this thesis, *S. aureus* was investigated at the species, intra-/inter-species, and bacterial community level in the context of bovine CM. All three thesis objectives outlined in Chapter 1 were met. These objectives substantiate the hypothesis that the bovine udder microbiome hosts symbiotic bacteria and that some interactions between such symbiotic bacteria and *S. aureus* can be exploited to develop novel mastitis prophylactics/therapeutics. A summary of the completed research objectives is presented below:

Objective 1: Perform a comparative genomic analysis to understand the evolutionary relationships between bovine mammary pathogenic *S. aureus* and human pathogenic *S. aureus*

1. *S. aureus* showed distinct clonality in the constructed phylogenomic tree indicating CC-oriented evolution. Specifically, bovine-associated *S. aureus* CCs were distinct from those found in humans.
2. In *S. aureus* isolates from human and bovine origin, there were unique and exclusive genes. Human immune evasion cluster (*scn*, *chp*, *sak*) was commonly found in human-originated *S. aureus* isolates while a bovine-specific virulence factor *lukMF'* located in MGEs was common among *S. aureus* CC151 and CC97 that were highly associated with bovine CM.
3. Bovine-adapted *S. aureus* CCs carried unique R-M genes which were different from human-adapted *S. aureus* CCs. However, three potential spillover isolates indicate the possibility of *S. aureus* transmission between two hosts. Two CC97 human isolates carried human immune evasion clusters while one bovine CC8 isolate contained no human immune evasion

cluster, suggesting that acquisition and loss of this MGE might increase adaptability in these two host species.

Objective 2: Develop a new strategy using a highly stable plasmid and fluorescent gene(s) to screen bacterial isolates for antagonistic activity toward *S. aureus*

4. Two newly engineered plasmids (pQS series) encode a fluorescent gene either *gfp* or *mCherry*, the expression of which is controlled by QS promoter (*agr*P3) in *S. aureus*.
5. The expression of the fluorescent reporter proteins was detected when *S. aureus* QS is activated. The pQS series were not significantly affect *S. aureus* growth rate and highly stable without selection pressure for at least 24 hours in two major *S. aureus* CCs (CC151 and CC95) isolated from Canadian dairy farms.
6. Growth-inhibiting and QQ activity towards pQS plasmid transformants of *S. aureus* is simultaneously monitored by observing the zone of growth inhibition and fluorescent protein inhibition on agar plates in the presence of antagonistic bacteria.

Objective 3: Characterize dynamics of the mammary gland microbial community before *S. aureus* mastitis infection, during the infection, and after infection resolution in dairy cows

7. The milk microbiota of healthy quarters was distinguishable from that of sick quarters up to two weeks before CM was detected. During *S. aureus* clinical mastitis, healthy and sick quarter had significantly different alpha- and beta-diversity, while two weeks after clinical mastitis, in the milk microbiota in healthy and sick quarters were similar.
8. Most genera found in milk samples were negatively associated with the relative abundance of *Staphylococcus*, yet none of these genera showed significant correlation with SCC. Interestingly, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, and *Aerococcus*

urinaeequi were each abundant in milk from mammary glands showing low levels of inflammation.

9. Changes in bacterial populations before, during, and after *S. aureus* clinical mastitis and the correlation between the udder microbiota and inflammation suggest that the udder microbiota may be associated with the resilience and resistance to udder microbiota susceptible to mastitis pathogens. Thus, the commensal and transient microbiota of the udder may potentially affect udder health.

7.2 Claimed Original Contribution to Knowledge

S. aureus, an opportunistic pathogen in a broad range of host animals, has developed multi-drug resistance and therefore has become a global health concern. Specifically, transmission of these drug-resistant *S. aureus* between different host species is especially worrisome. Despite the importance of *S. aureus* in both human and veterinary medicine, studying *S. aureus* via genetic manipulation is remarkably difficult due to strong defense mechanisms including *S. aureus* R-M systems. In dairy cattle, defining the healthy and core udder microbiota is also challenging, which then hinders the development of microbial intervention and prevention strategies to *S. aureus* clinical mastitis. In this thesis, *S. aureus* was investigated to understand its genetic potential, antagonistic relationships, and roles in bovine udder health.

1. The phylogenomic tree of 187 genomes from *S. aureus* isolates taken from human and bovine sources and their genetic characteristics provided a snapshot of early host spillover events and intra-species horizontal gene transfer network.
2. A novel high throughput screening method was developed to identify bacteria with antagonistic activity towards *S. aureus* growth and QS simultaneously.

3. The first longitudinal cohort study of the milk microbiota associated with *S. aureus* clinical mastitis was conducted to explore microbial dynamics before, during, and after *S. aureus* clinical mastitis.

7.3 Future Work and Recommendations

This thesis characterized bovine-associated *S. aureus* and its genetic characteristics, proposed a new screening strategy of antagonistic bacteria against *S. aureus*, and examined microbial changes during lactation with or without *S. aureus* clinical mastitis. Understanding antagonistic mechanisms will lead to the development of CC-specific as well as general prevention and treatment strategies for *S. aureus* clinical mastitis. Three potential bacterial species − *S. xylosus*, *S. epidermidis*, and *A. urinaeequi* − found in dairy milk with low inflammation are suggested as candidates for alternatives to antibiotics in terms of mastitis prophylaxis. Despite the interesting data presented in this thesis, several investigations are required before implementing the aforementioned bacteria as prophylactics/therapeutics.

The next step to develop *S. aureus* clinical mastitis prophylactics/therapeutics is to isolate the highly abundant bacteria found in the milk samples with low inflammation. WGS needs to be performed to identify any potential virulence and/or antagonistic genes. Moreover, antagonism assays are required to evaluate growth and QS inhibition against mastitis pathogenic *S. aureus* strains. The level of inflammation also needs to be characterized *in vivo* to ensure that minimal inflammation will be induced in the mammary gland by the isolates. Ultimately, these bacteria and their bioactive molecules can be implemented in mammary and topical probiotics to protect the bovine udder against *S. aureus* and other mastitis pathogens.

To protect the bovine udder from mastitis pathogens including *S. aureus*, microbiota-based early diagnostics and prophylactics/therapeutics must aim to remove infecting pathogens, as well as

restore healthy microbiota to prevent future infections. CC-specific approaches would increase success rate in treatment and prevention. Diagnosing microbiota with low resilience and resistance may be a promising approach to detect clinical mastitis early. However, to be able to identify udder microbiota with low resilience and resistance, more studies need to understand and define what a healthy and core udder microbiota are composed of.

REFERENCES

Adams, D. M., Barach, J. T., & Speck, M. L. (1975). Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. *J Dairy Sci*, *58*(6), 828-834. https://doi.org/10.3168/jds.s0022-0302(75)84645-5

Addis, M. F., Tanca, A., Uzzau, S., Oikonomou, G., Bicalho, R. C., & Moroni, P. (2016). The bovine milk microbiota: insights and perspectives from -omics studies. *Mol Biosyst*, *12*(8), 2359-2372. https://doi.org/10.1039/c6mb00217j

Adegoke, A. A., & Okoh, A. I. (2014). Species diversity and antibiotic resistance properties of Staphylococcus of farm animal origin in Nkonkobe Municipality, South Africa. *Folia Microbiol (Praha)*, *59*(2), 133-140. https://doi.org/10.1007/s12223-013-0275-1

Aghamohammadi, M., Haine, D., Kelton, D. F., Barkema, H. W., Hogeveen, H., Keefe, G. P., & Dufour, S. (2018). Herd-Level Mastitis-Associated Costs on Canadian Dairy Farms. *Front Vet Sci*, *5*, 100. https://doi.org/10.3389/fvets.2018.00100

Al-Amery, K., Elhariri, M., Elsayed, A., El-Moghazy, G., Elhelw, R., El-Mahallawy, H., El Hariri, M., & Hamza, D. (2019). Vancomycin-resistant Staphylococcus aureus isolated from camel meat and slaughterhouse workers in Egypt. *Antimicrob Resist Infect Control*, *8*, 129. https://doi.org/10.1186/s13756-019-0585-4

Alawneh, J. I., James, A. S., Phillips, N., Fraser, B., Jury, K., Soust, M., & Olchowy, T. W. J. (2020). Efficacy of a Lactobacillus-Based Teat Spray on Udder Health in Lactating Dairy Cows. *Front Vet Sci*, *7*, 584436. https://doi.org/10.3389/fvets.2020.584436

Almeida, R. A., Matthews, K. R., Cifrian, E., Guidry, A. J., & Oliver, S. P. (1996). Staphylococcus aureus invasion of bovine mammary epithelial cells. *J Dairy Sci*, *79*(6), 1021-1026. https://doi.org/10.3168/jds.S0022-0302(96)76454-8

Alvarez-Sieiro, P., Montalban-Lopez, M., Mu, D., & Kuipers, O. P. (2016). Bacteriocins of lactic acid bacteria: extending the family. *Appl Microbiol Biotechnol*, *100*(7), 2939-2951. https://doi.org/10.1007/s00253-016-7343-9

Andrews, T., Neher, D. A., Weicht, T. R., & Barlow, J. W. (2019). Mammary microbiome of lactating organic dairy cows varies by time, tissue site, and infection status. *PLoS One*, *14*(11), e0225001. https://doi.org/10.1371/journal.pone.0225001

Angelopoulou, A., Warda, A. K., Hill, C., & Ross, R. P. (2019). Non-antibiotic microbial solutions for bovine mastitis - live biotherapeutics, bacteriophage, and phage lysins. *Crit Rev Microbiol*, *45*(5-6), 564-580. https://doi.org/10.1080/1040841X.2019.1648381

Anhê, F. F., Barra, N. G., & Schertzer, J. D. (2020). Glucose alters the symbiotic relationships between gut microbiota and host physiology. *American Journal of Physiology-Endocrinology and Metabolism*, *318*(2), E111-E116. https://doi.org/10.1152/ajpendo.00485.2019

Armand-Lefevre, L., Ruimy, R., & Andremont, A. (2005). Clonal comparison of Staphylococcus aureus isolates from healthy pig farmers, human controls, and pigs. *Emerg Infect Dis*, *11*(5), 711-714. https://doi.org/10.3201/eid1105.040866

Assis, B. S., Germon, P., Silva, A. M., Even, S., Nicoli, J. R., & Le Loir, Y. (2015). Lactococcus lactis V7 inhibits the cell invasion of bovine mammary epithelial cells by Escherichia coli and Staphylococcus aureus. *Benef Microbes*, *6*(6), 879-886. https://doi.org/10.3920/BM2015.0019

Atkins, K. L., Burman, J. D., Chamberlain, E. S., Cooper, J. E., Poutrel, B., Bagby, S., Jenkins, A. T., Feil, E. J., & van den Elsen, J. M. (2008). S. aureus IgG-binding proteins SpA and Sbi: host specificity and mechanisms of immune complex formation. *Mol Immunol*, *45*(6), 1600-1611.

https://doi.org/10.1016/j.molimm.2007.10.021
Baig, S., Johannesen, T. B., Overballe-Petersen, S., Larsen, J., Larsen, A. R., & Stegger, M. (2018). Novel SCCmec type XIII (9A) identified in an ST152 methicillin-resistant Staphylococcus aureus. *Infect Genet Evol*, *61*, 74-76. https://doi.org/10.1016/j.meegid.2018.03.013
Bardiau, M., Caplin, J., Detilleux, J., Graber, H., Moroni, P., Taminiau, B., & Mainil, J. G. (2016). Existence of two groups of Staphylococcus aureus strains isolated from bovine mastitis based on biofilm formation, intracellular survival, capsular profile and agr-typing. *Vet Microbiol*, *185*, 1-6. https://doi.org/10.1016/j.vetmic.2016.01.003
Bartley, J. (2002). First case of VRSA identified in Michigan. *Infect Control Hosp Epidemiol*, *23*(8), 480. https://doi.org/10.1017/s0195941700082333
Barzegari, A., Kheyrolahzadeh, K., Hosseiniyan Khatibi, S. M., Sharifi, S., Memar, M. Y., & Zununi Vahed, S. (2020). The Battle of Probiotics and Their Derivatives Against Biofilms. *Infection and drug resistance*, *13*, 659-672. https://doi.org/10.2147/IDR.S232982
Bayles, K. W., Wesson, C. A., Liou, L. E., Fox, L. K., Bohach, G. A., & Trumble, W. R. (1998). Intracellular Staphylococcus aureus escapes the endosome and induces apoptosis in epithelial cells. *Infect Immun*, *66*(1), 336-342. https://doi.org/10.1128/IAI.66.1.336-342.1998
Belkaid, Y., & Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell*, *157*(1), 121-141. https://doi.org/10.1016/j.cell.2014.03.011
Bennett, R. H., & Jasper, D. E. (1977). Bovine mycoplasma mastitis from intramammary inoculation of small numbers of Mycoplasma bovis. I. Microbiology and pathology. *Veterinary Microbiology*, *2*(4), 341-355. https://doi.org/https://doi.org/10.1016/0378-1135(77)90044-X
Berardo, N., Bohl, L., Porporatto, C., Nader-Macias, M. E. F., Bogni, C., & Pellegrino, M. (2020). Intramammary inoculation with lactic acid bacteria at dry-off triggers an immunomodulatory response in dairy cows. *Benef Microbes*, *11*(6), 561-572. https://doi.org/10.3920/bm2019.0163
Berg, G., Rybakova, D., Fischer, D., Cernava, T., Verges, M. C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G. H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J. A., Maguin, E., Mauchline, T., McClure, R., . . . Schloter, M. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome*, *8*(1), 103. https://doi.org/10.1186/s40168-020-00875-0
Bhattacharyya, D., Banerjee, J., Bandyopadhyay, S., Mondal, B., Nanda, P. K., Samanta, I., Mahanti, A., Das, A. K., Das, G., Dandapat, P., & Bandyopadhyay, S. (2016). First Report on Vancomycin-Resistant Staphylococcus aureus in Bovine and Caprine Milk. *Microb Drug Resist*, *22*(8), 675-681. https://doi.org/10.1089/mdr.2015.0330
Bloemendaal, A. L., Brouwer, E. C., & Fluit, A. C. (2010). Methicillin resistance transfer from Staphylocccus epidermidis to methicillin-susceptible Staphylococcus aureus in a patient during antibiotic therapy. *PLoS One*, *5*(7), e11841. https://doi.org/10.1371/journal.pone.0011841
Bonsaglia, E. C. R., Gomes, M. S., Canisso, I. F., Zhou, Z., Lima, S. F., Rall, V. L. M., Oikonomou, G., Bicalho, R. C., & Lima, F. S. (2017). Milk microbiome and bacterial load following dry cow therapy without antibiotics in dairy cows with healthy mammary gland. *Sci Rep*, *7*(1), 8067. https://doi.org/10.1038/s41598-017-08790-5
Boss, R., Cosandey, A., Luini, M., Artursson, K., Bardiau, M., Breitenwieser, F., Hehenberger, E., Lam, T., Mansfeld, M., Michel, A., Mosslacher, G., Naskova, J., Nelson, S., Podpecan, O., Raemy, A., Ryan, E., Salat, O., Zangerl, P., Steiner, A., & Graber, H. U. (2016). Bovine Staphylococcus aureus: Subtyping, evolution, and zoonotic transfer. *J Dairy Sci*, *99*(1), 515-528. https://doi.org/10.3168/jds.2015-9589

Bouchard, D. S., Rault, L., Berkova, N., Le Loir, Y., & Even, S. (2013). Inhibition of Staphylococcus aureus invasion into bovine mammary epithelial cells by contact with live Lactobacillus casei. *Applied and environmental microbiology*, *79*(3), 877-885. https://doi.org/10.1128/AEM.03323-12

Bugg, T. D., Wright, G. D., Dutka-Malen, S., Arthur, M., Courvalin, P., & Walsh, C. T. (1991). Molecular basis for vancomycin resistance in Enterococcus faecium BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochemistry*, *30*(43), 10408-10415. https://doi.org/10.1021/bi00107a007

Butler, J. E., Rainard, P., Lippolis, J., Salmon, H., & Kacskovics, I. (2015). Chapter 116 - The Mammary Gland in Mucosal and Regional Immunity. In J. Mestecky, W. Strober, M. W. Russell, B. L. Kelsall, H. Cheroutre, & B. N. Lambrecht (Eds.), *Mucosal Immunology (Fourth Edition)* (pp. 2269-2306). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-415847-4.00116-6

Buzzola, F. R., Alvarez, L. P., Tuchscherr, L. P., Barbagelata, M. S., Lattar, S. M., Calvinho, L., & Sordelli, D. O. (2007). Differential abilities of capsulated and noncapsulated Staphylococcus aureus isolates from diverse agr groups to invade mammary epithelial cells. *Infect Immun*, *75*(2), 886-891. https://doi.org/10.1128/IAI.01215-06

Canada, P. H. A. o. (2015). Federal action plan on antimicrobial resistance and use in Canada: building on the federal framework for action.

Canovas, J., Baldry, M., Bojer, M. S., Andersen, P. S., Grzeskowiak, P. K., Stegger, M., Damborg, P., Olsen, C. A., & Ingmer, H. (2016). Cross-Talk between Staphylococcus aureus and Other Staphylococcal Species via the agr Quorum Sensing System. *Front Microbiol*, *7*, 1733. https://doi.org/10.3389/fmicb.2016.01733

Cao, Z., Casabona, M. G., Kneuper, H., Chalmers, J. D., & Palmer, T. (2016). The type VII secretion system of Staphylococcus aureus secretes a nuclease toxin that targets competitor bacteria. *Nat Microbiol*, *2*, 16183. https://doi.org/10.1038/nmicrobiol.2016.183

Ceotto, H., Nascimento Jdos, S., Brito, M. A., & Bastos Mdo, C. (2009). Bacteriocin production by Staphylococcus aureus involved in bovine mastitis in Brazil. *Res Microbiol*, *160*(8), 592-599. https://doi.org/10.1016/j.resmic.2009.07.007

CFIA. (2013a). *Biosecurity for Canadian Dairy Farms: National Standard*. Canadian Food Inspection Agency, Ottawa, ON, Canada

CFIA. (2013b). *Biosecurity for Canadian dairy farms: Producer planning guide*. Canadian Food Inspection Agency, Ottawa, ON, Canada

Chambers, H. F., & Deleo, F. R. (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat Rev Microbiol*, *7*(9), 629-641. https://doi.org/10.1038/nrmicro2200

Chang, B. S., Moon, J. S., Kang, H. M., Kim, Y. I., Lee, H. K., Kim, J. D., Lee, B. S., Koo, H. C., & Park, Y. H. (2008). Protective effects of recombinant staphylococcal enterotoxin type C mutant vaccine against experimental bovine infection by a strain of Staphylococcus aureus isolated from subclinical mastitis in dairy cattle. *Vaccine*, *26*(17), 2081-2091. https://doi.org/10.1016/j.vaccine.2008.02.043

Cheng, W. N., & Han, S. G. (2020). Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments - A review. *Asian-Australas J Anim Sci*, *33*(11), 1699-1713. https://doi.org/10.5713/ajas.20.0156

Cherrington, C. A., Hinton, M., Mead, G. C., & Chopra, I. (1991). Organic acids: chemistry, antibacterial activity and practical applications. *Adv Microb Physiol*, *32*, 87-108. https://doi.org/10.1016/s0065-2911(08)60006-5

Coban, H. B. (2020). Organic acids as antimicrobial food agents: applications and microbial

productions. *Bioprocess and Biosystems Engineering*, *43*(4), 569-591. https://doi.org/10.1007/s00449-019-02256-w

Cong, Y., Yang, S., & Rao, X. (2020). Vancomycin resistant Staphylococcus aureus infections: A review of case updating and clinical features. *J Adv Res*, *21*, 169-176. https://doi.org/10.1016/j.jare.2019.10.005

Cosandey, A., Boss, R., Luini, M., Artursson, K., Bardiau, M., Breitenwieser, F., Hehenberger, E., Lam, T., Mansfeld, M., Michel, A., Mosslacher, G., Naskova, J., Nelson, S., Podpecan, O., Raemy, A., Ryan, E., Salat, O., Zangerl, P., Steiner, A., & Graber, H. U. (2016). Staphylococcus aureus genotype B and other genotypes isolated from cow milk in European countries. *J Dairy Sci*, *99*(1), 529-540. https://doi.org/10.3168/jds.2015-9587

Cremonesi, P., Ceccarani, C., Curone, G., Severgnini, M., Pollera, C., Bronzo, V., Riva, F., Addis, M. F., Filipe, J., Amadori, M., Trevisi, E., Vigo, D., Moroni, P., & Castiglioni, B. (2018). Milk microbiome diversity and bacterial group prevalence in a comparison between healthy Holstein Friesian and Rendena cows. *PLoS One*, *13*(10), e0205054-e0205054. https://doi.org/10.1371/journal.pone.0205054

Crispie, F., Alonso-Gomez, M., O'Loughlin, C., Klostermann, K., Flynn, J., Arkins, S., Meaney, W., Paul Ross, R., & Hill, C. (2008). Intramammary infusion of a live culture for treatment of bovine mastitis: effect of live lactococci on the mammary immune response. *J Dairy Res*, *75*(3), 374-384. https://doi.org/10.1017/S0022029908003385

Crispie, F., Twomey, D., Flynn, J., Hill, C., Ross, P., & Meaney, W. (2005). The lantibiotic lacticin 3147 produced in a milk-based medium improves the efficacy of a bismuth-based teat seal in cattle deliberately infected with Staphylococcus aureus. *J Dairy Res*, *72*(2), 159-167. https://doi.org/10.1017/s0022029905000816

Cucarella, C., Tormo, M. A., Ubeda, C., Trotonda, M. P., Monzon, M., Peris, C., Amorena, B., Lasa, I., & Penades, J. R. (2004). Role of biofilm-associated protein bap in the pathogenesis of bovine Staphylococcus aureus. *Infect Immun*, *72*(4), 2177-2185. https://doi.org/10.1128/IAI.72.4.2177-2185.2004

Cui, L., Ma, X., Sato, K., Okuma, K., Tenover, F. C., Mamizuka, E. M., Gemmell, C. G., Kim, M. N., Ploy, M. C., El-Solh, N., Ferraz, V., & Hiramatsu, K. (2003). Cell wall thickening is a common feature of vancomycin resistance in Staphylococcus aureus. *J Clin Microbiol*, *41*(1), 5-14. https://doi.org/10.1128/jcm.41.1.5-14.2003

Dahlberg, J., Williams, J. E., McGuire, M. A., Peterson, H. K., Ostensson, K., Agenas, S., Dicksved, J., & Waller, K. P. (2020). Microbiota of bovine milk, teat skin, and teat canal: Similarity and variation due to sampling technique and milk fraction. *J Dairy Sci*, *103*(8), 7322-7330. https://doi.org/10.3168/jds.2019-17783

de Freire Bastos, M. D. C., Miceli de Farias, F., Carlin Fagundes, P., & Varella Coelho, M. L. (2020). Staphylococcins: an update on antimicrobial peptides produced by staphylococci and their diverse potential applications. *Appl Microbiol Biotechnol*. https://doi.org/10.1007/s00253-020-10946-9

de Niederhausern, S., Bondi, M., Messi, P., Iseppi, R., Sabia, C., Manicardi, G., & Anacarso, I. (2011). Vancomycin-resistance transferability from VanA enterococci to Staphylococcus aureus. *Curr Microbiol*, *62*(5), 1363-1367. https://doi.org/10.1007/s00284-011-9868-6

de Oliveira, G. B., Favarin, L., Luchese, R. H., & McIntosh, D. (2015). Psychrotrophic bacteria in milk: How much do we really know? *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]*, *46*(2), 313-321. https://doi.org/10.1590/S1517-838246220130963

Denis-Robichaud, J., Kelton, D. F., Bauman, C. A., Barkema, H. W., Keefe, G. P., & Dubuc, J. (2019).

Biosecurity and herd health management practices on Canadian dairy farms. *J Dairy Sci*, *102*(10), 9536-9547. https://doi.org/10.3168/jds.2018-15921

Denis-Robichaud, J., Kelton, D. F., Bauman, C. A., Barkema, H. W., Keefe, G. P., & Dubuc, J. (2020). Gap between producers and veterinarians regarding biosecurity on Quebec dairy farms. *The Canadian veterinary journal = La revue veterinaire canadienne*, *61*(7), 757-762. https://pubmed.ncbi.nlm.nih.gov/32655160

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7296865/

Derakhshani, H., Fehr, K. B., Sepehri, S., Francoz, D., De Buck, J., Barkema, H. W., Plaizier, J. C., & Khafipour, E. (2018). Invited review: Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility. *J Dairy Sci*, *101*(12), 10605-10625. https://doi.org/10.3168/jds.2018-14860

Derakhshani, H., Plaizier, J. C., De Buck, J., Barkema, H. W., & Khafipour, E. (2018). Composition of the teat canal and intramammary microbiota of dairy cows subjected to antimicrobial dry cow therapy and internal teat sealant. *J Dairy Sci*, *101*(11), 10191-10205. https://doi.org/10.3168/jds.2018-14858

Derakhshani, H., Plaizier, J. C., De Buck, J., Barkema, H. W., & Khafipour, E. (2020). Composition and co-occurrence patterns of the microbiota of different niches of the bovine mammary gland: potential associations with mastitis susceptibility, udder inflammation, and teat-end hyperkeratosis. *Anim Microbiome*, *2*(1), 11. https://doi.org/10.1186/s42523-020-00028-6

Devriese, L. A., & Hommez, J. (1975). Epidemiology of methicillin-resistant Staphylococcus aureus in dairy herds. *Res Vet Sci*, *19*(1), 23-27. https://www.ncbi.nlm.nih.gov/pubmed/125447

DiGiulio, D. B., Romero, R., Amogan, H. P., Kusanovic, J. P., Bik, E. M., Gotsch, F., Kim, C. J., Erez, O., Edwin, S., & Relman, D. A. (2008). Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS One*, *3*(8), e3056. https://doi.org/10.1371/journal.pone.0003056

Doyle, C. J., Gleeson, D., O'Toole, P. W., & Cotter, P. D. (2017). Impacts of Seasonal Housing and Teat Preparation on Raw Milk Microbiota: a High-Throughput Sequencing Study. *Appl Environ Microbiol*, *83*(2). https://doi.org/10.1128/AEM.02694-16

El-Sayed, A., & Kamel, M. (2021). Bovine mastitis prevention and control in the post-antibiotic era. *Trop Anim Health Prod*, *53*(2), 236. https://doi.org/10.1007/s11250-021-02680-9

Ezzat Alnakip, M., Quintela-Baluja, M., Böhme, K., Fernández-No, I., Caamaño-Antelo, S., Calo-Mata, P., & Barros-Velázquez, J. (2014). The Immunology of Mammary Gland of Dairy Ruminants between Healthy and Inflammatory Conditions. *Journal of Veterinary Medicine*, *2014*, 659801. https://doi.org/10.1155/2014/659801

Falentin, H., Rault, L., Nicolas, A., Bouchard, D. S., Lassalas, J., Lamberton, P., Aubry, J. M., Marnet, P. G., Le Loir, Y., & Even, S. (2016). Bovine Teat Microbiome Analysis Revealed Reduced Alpha Diversity and Significant Changes in Taxonomic Profiles in Quarters with a History of Mastitis. *Front Microbiol*, *7*, 480. https://doi.org/10.3389/fmicb.2016.00480

Fang, K., Jin, X., & Hong, S. H. (2018). Probiotic Escherichia coli inhibits biofilm formation of pathogenic E. coli via extracellular activity of DegP. *Sci Rep*, *8*(1), 4939. https://doi.org/10.1038/s41598-018-23180-1

FAO/WHO. (2006). *Probiotics in food: Health and nutritional properties and guidelines for evaluation*.

Farrell, S., McKernan, C., Benson, T., Elliott, C., & Dean, M. (2021). Understanding farmers' and veterinarians' behavior in relation to antimicrobial use and resistance in dairy cattle: A systematic review. *Journal of Dairy Science*, *104*(4), 4584-4603. https://doi.org/https://doi.org/10.3168/jds.2020-19614

Feazel, L. M., Robertson, C. E., Ramakrishnan, V. R., & Frank, D. N. (2012). Microbiome complexity and Staphylococcus aureus in chronic rhinosinusitis. *Laryngoscope*, *122*(2), 467-472. https://doi.org/10.1002/lary.22398

Ferens, W. A., & Bohach, G. A. (2000). Persistence of Staphylococcus aureus on mucosal membranes: superantigens and internalization by host cells. *J Lab Clin Med*, *135*(3), 225-230. https://doi.org/10.1067/mlc.2000.105179

Filkins, L. M., Graber, J. A., Olson, D. G., Dolben, E. L., Lynd, L. R., Bhuju, S., & O'Toole, G. A. (2015). Coculture of Staphylococcus aureus with Pseudomonas aeruginosa Drives S. aureus towards Fermentative Metabolism and Reduced Viability in a Cystic Fibrosis Model. *J Bacteriol*, *197*(14), 2252-2264. https://doi.org/10.1128/JB.00059-15

Fitzgerald, J. R., Monday, S. R., Foster, T. J., Bohach, G. A., Hartigan, P. J., Meaney, W. J., & Smyth, C. J. (2001). Characterization of a putative pathogenicity island from bovine Staphylococcus aureus encoding multiple superantigens. *J Bacteriol*, *183*(1), 63-70. https://doi.org/10.1128/JB.183.1.63-70.2001

Flemming, H.-C., & Wuertz, S. (2019). Bacteria and archaea on Earth and their abundance in biofilms. *Nature Reviews Microbiology*, *17*(4), 247-260. https://doi.org/10.1038/s41579-019-0158-9

Fonseca, M., Heider, L. C., Leger, D., McClure, J. T., Rizzo, D., Dufour, S., Kelton, D. F., Renaud, D., Barkema, H. W., & Sanchez, J. (2021). Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR): An On-Farm Surveillance System. *Front Vet Sci*, *8*, 799622. https://doi.org/10.3389/fvets.2021.799622

Foucault, M. L., Courvalin, P., & Grillot-Courvalin, C. (2009). Fitness cost of VanA-type vancomycin resistance in methicillin-resistant Staphylococcus aureus. *Antimicrob Agents Chemother*, *53*(6), 2354-2359. https://doi.org/10.1128/AAC.01702-08

Fournier, C., Kuhnert, P., Frey, J., Miserez, R., Kirchhofer, M., Kaufmann, T., Steiner, A., & Graber, H. U. (2008). Bovine Staphylococcus aureus: association of virulence genes, genotypes and clinical outcome. *Res Vet Sci*, *85*(3), 439-448. https://doi.org/10.1016/j.rvsc.2008.01.010

Frola, I. D., Pellegrino, M. S., Espeche, M. C., Giraudo, J. A., Nader-Macias, M. E., & Bogni, C. I. (2012). Effects of intramammary inoculation of Lactobacillus perolens CRL1724 in lactating cows' udders. *J Dairy Res*, *79*(1), 84-92. https://doi.org/10.1017/S0022029911000835

Frola, I. D., Pellegrino, M. S., Magnano, G., Giraudo, J. A., Espeche, M. C., Nader-Macias, M. E., & Bogni, C. I. (2013). Histological examination of non-lactating bovine udders inoculated with Lactobacillus perolens CRL 1724. *J Dairy Res*, *80*(1), 28-35. https://doi.org/10.1017/S0022029912000581

Fromageau, A., Gilbert, F. B., Prevost, G., & Rainard, P. (2010). Binding of the Staphylococcus aureus leucotoxin LukM to its leucocyte targets. *Microb Pathog*, *49*(6), 354-362. https://doi.org/10.1016/j.micpath.2010.07.002

Ganda, E. K., Bisinotto, R. S., Lima, S. F., Kronauer, K., Decter, D. H., Oikonomou, G., Schukken, Y. H., & Bicalho, R. C. (2016). Longitudinal metagenomic profiling of bovine milk to assess the impact of intramammary treatment using a third-generation cephalosporin. *Sci Rep*, *6*, 37565. https://doi.org/10.1038/srep37565

Ganda, E. K., Gaeta, N., Sipka, A., Pomeroy, B., Oikonomou, G., Schukken, Y. H., & Bicalho, R. C. (2017). Normal milk microbiome is reestablished following experimental infection with Escherichia coli independent of intramammary antibiotic treatment with a third-generation cephalosporin in bovines. *Microbiome*, *5*(1), 74. https://doi.org/10.1186/s40168-017-0291-5

Gao, J., Liu, Y. C., Wang, Y., Li, H., Wang, X. M., Wu, Y., Zhang, D. R., Gao, S., & Qi, Z. L. (2020). Impact of yeast and lactic acid bacteria on mastitis and milk microbiota composition of dairy cows. *AMB Express*, *10*(1), 22. https://doi.org/10.1186/s13568-020-0953-8

Garzoni, C., & Kelley, W. L. (2009). Staphylococcus aureus: new evidence for intracellular persistence. *Trends Microbiol*, *17*(2), 59-65. https://doi.org/10.1016/j.tim.2008.11.005

Ghosh, C., Sarkar, P., Issa, R., & Haldar, J. (2019). Alternatives to Conventional Antibiotics in the Era of Antimicrobial Resistance. *Trends Microbiol*, *27*(4), 323-338. https://doi.org/10.1016/j.tim.2018.12.010

Goncalves, J. L., Kamphuis, C., Vernooij, H., Araujo, J. P., Jr., Grenfell, R. C., Juliano, L., Anderson, K. L., Hogeveen, H., & Dos Santos, M. V. (2020). Pathogen effects on milk yield and composition in chronic subclinical mastitis in dairy cows. *Vet J*, *262*, 105473. https://doi.org/10.1016/j.tvjl.2020.105473

Gordon, C. P., Williams, P., & Chan, W. C. (2013). Attenuating Staphylococcus aureus virulence gene regulation: a medicinal chemistry perspective. *J Med Chem*, *56*(4), 1389-1404. https://doi.org/10.1021/jm3014635

Gov, Y., Bitler, A., Dell'Acqua, G., Torres, J. V., & Balaban, N. (2001). RNAIII inhibiting peptide (RIP), a global inhibitor of Staphylococcus aureus pathogenesis: structure and function analysis. *Peptides*, *22*(10), 1609-1620. https://doi.org/10.1016/s0196-9781(01)00496-x

Gowrishankar, S., Kamaladevi, A., Ayyanar, K. S., Balamurugan, K., & Pandian, S. K. (2015). Bacillus amyloliquefaciens-secreted cyclic dipeptide - cyclo(L-leucyl-L-prolyl) inhibits biofilm and virulence production in methicillin-resistant Staphylococcus aureus. *Rsc Advances*, *5*(116), 95788-95804. https://doi.org/10.1039/c5ra11641d

Graber, H. U., Naskova, J., Studer, E., Kaufmann, T., Kirchhofer, M., Brechbuhl, M., Schaeren, W., Steiner, A., & Fournier, C. (2009). Mastitis-related subtypes of bovine Staphylococcus aureus are characterized by different clinical properties. *J Dairy Sci*, *92*(4), 1442-1451. https://doi.org/10.3168/jds.2008-1430

Grandclement, C., Tannieres, M., Morera, S., Dessaux, Y., & Faure, D. (2016). Quorum quenching: role in nature and applied developments. *FEMS Microbiol Rev*, *40*(1), 86-116. https://doi.org/10.1093/femsre/fuv038

Griesbeck-Zilch, B., Meyer, H. H., Kuhn, C. H., Schwerin, M., & Wellnitz, O. (2008). Staphylococcus aureus and Escherichia coli cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. *J Dairy Sci*, *91*(6), 2215-2224. https://doi.org/10.3168/jds.2007-0752

Grunenwald, C. M., Bennett, M. R., & Skaar, E. P. (2018). Nonconventional Therapeutics against Staphylococcus aureus. *Microbiology spectrum*, *6*(6), 10.1128/microbiolspec.GPP1123-0047-2018. https://doi.org/10.1128/microbiolspec.GPP3-0047-2018

Grunert, T., Stessl, B., Wolf, F., Sordelli, D. O., Buzzola, F. R., & Ehling-Schulz, M. (2018). Distinct phenotypic traits of Staphylococcus aureus are associated with persistent, contagious bovine intramammary infections. *Sci Rep*, *8*(1), 15968. https://doi.org/10.1038/s41598-018-34371-1

Gryaznova, M. V., Syromyatnikov, M. Y., Dvoretskaya, Y. D., Solodskikh, S. A., Klimov, N. T., Mikhalev, V. I., Zimnikov, V. I., Mikhaylov, E. V., & Popov, V. N. (2021). Microbiota of Cow's Milk with Udder Pathologies. *Microorganisms*, *9*(9). https://doi.org/10.3390/microorganisms9091974

Gunther, J., Petzl, W., Bauer, I., Ponsuksili, S., Zerbe, H., Schuberth, H. J., Brunner, R. M., & Seyfert, H. M. (2017). Differentiating Staphylococcus aureus from Escherichia coli mastitis: S. aureus triggers unbalanced immune-dampening and host cell invasion immediately after udder infection. *Sci Rep*, *7*(1), 4811. https://doi.org/10.1038/s41598-017-05107-4

Guo, X., Yu, Z., Zhao, F., Sun, Z., Kwok, L.-Y., & Li, S. (2021). Both sampling seasonality and geographic origin contribute significantly to variations in raw milk microbiota, but sampling seasonality is the more determining factor. *Journal of Dairy Science*, *104*(10), 10609-10627.

https://doi.org/https://doi.org/10.3168/jds.2021-20480
Hajfarajollah, H., Eslami, P., Mokhtarani, B., & Akbari Noghabi, K. (2018). Biosurfactants from probiotic bacteria: A review. *Biotechnol Appl Biochem*, *65*(6), 768-783. https://doi.org/10.1002/bab.1686
Hamed, M. I. (2018). Detection of methicillin-resistant and biofilm-producing Staphylococcus aureus in bovine mastitis. *Journal of Advanced Veterinary Research*, *8*(4), 95-100.
Hammer, N. D., & Skaar, E. P. (2011). Molecular mechanisms of Staphylococcus aureus iron acquisition. *Annu Rev Microbiol*, *65*, 129-147. https://doi.org/10.1146/annurev-micro-090110-102851
Haveri, M., Roslof, A., Rantala, L., & Pyorala, S. (2007). Virulence genes of bovine Staphylococcus aureus from persistent and nonpersistent intramammary infections with different clinical characteristics. *J Appl Microbiol*, *103*(4), 993-1000. https://doi.org/10.1111/j.1365-2672.2007.03356.x
Hebert, A., Sayasith, K., Senechal, S., Dubreuil, P., & Lagace, J. (2000). Demonstration of intracellular Staphylococcus aureus in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. *FEMS Microbiol Lett*, *193*(1), 57-62. https://doi.org/10.1111/j.1574-6968.2000.tb09402.x
Herron-Olson, L., Fitzgerald, J. R., Musser, J. M., & Kapur, V. (2007). Molecular correlates of host specialization in Staphylococcus aureus. *PLoS One*, *2*(10), e1120. https://doi.org/10.1371/journal.pone.0001120
Hillerton, E., & Booth, J. M. (2018). The five-point mastitis control plan—A revisory tutorial. *Proceedings of the 57th Annu. Mtg. National Mastitis Council, Tucson, AZ, USA*, *30*.
Hiramatsu, K., Ito, T., Tsubakishita, S., Sasaki, T., Takeuchi, F., Morimoto, Y., Katayama, Y., Matsuo, M., Kuwahara-Arai, K., Hishinuma, T., & Baba, T. (2013). Genomic Basis for Methicillin Resistance in Staphylococcus aureus. *Infect Chemother*, *45*(2), 117-136. https://doi.org/10.3947/ic.2013.45.2.117
Hoekstra, J., Rutten, V., Sommeling, L., van Werven, T., Spaninks, M., Duim, B., Benedictus, L., & Koop, G. (2018). High Production of LukMF' in Staphylococcus aureus Field Strains Is Associated with Clinical Bovine Mastitis. *Toxins (Basel)*, *10*(5). https://doi.org/10.3390/toxins10050200
Honda, K., & Littman, D. R. (2012). The microbiome in infectious disease and inflammation. *Annu Rev Immunol*, *30*, 759-795. https://doi.org/10.1146/annurev-immunol-020711-074937
Hoque, M. N., Istiaq, A., Clement, R. A., Sultana, M., Crandall, K. A., Siddiki, A. Z., & Hossain, M. A. (2019). Metagenomic deep sequencing reveals association of microbiome signature with functional biases in bovine mastitis. *Sci Rep*, *9*(1), 13536. https://doi.org/10.1038/s41598-019-49468-4
Hurley, W. L., & Theil, P. K. (2011). Perspectives on immunoglobulins in colostrum and milk. *Nutrients*, *3*(4), 442-474. https://doi.org/10.3390/nu3040442
Idrees, M., Sawant, S., Karodia, N., & Rahman, A. (2021). Staphylococcus aureus Biofilm: Morphology, Genetics, Pathogenesis and Treatment Strategies. *Int J Environ Res Public Health*, *18*(14). https://doi.org/10.3390/ijerph18147602
Ismail, B., & Nielsen, S. S. (2010). Invited review: Plasmin protease in milk: current knowledge and relevance to dairy industry. *J Dairy Sci*, *93*(11), 4999-5009. https://doi.org/10.3168/jds.2010-3122
Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, K., Tiensasitorn, C., & Hiramatsu, K. (2001). Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant Staphylococcus aureus. *Antimicrob*

Agents Chemother, *45*(5), 1323-1336. https://doi.org/10.1128/AAC.45.5.1323-1336.2001
Iwase, T., Uehara, Y., Shinji, H., Tajima, A., Seo, H., Takada, K., Agata, T., & Mizunoe, Y. (2010). Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. *Nature*, *465*(7296), 346-349. https://doi.org/10.1038/nature09074
IWG-SCC. (2009). Classification of staphylococcal cassette chromosome mec (SCCmec): guidelines for reporting novel SCCmec elements. *Antimicrob Agents Chemother*, *53*(12), 4961-4967. https://doi.org/10.1128/AAC.00579-09
Jensen, K., Gunther, J., Talbot, R., Petzl, W., Zerbe, H., Schuberth, H. J., Seyfert, H. M., & Glass, E. J. (2013). Escherichia coli- and Staphylococcus aureus-induced mastitis differentially modulate transcriptional responses in neighbouring uninfected bovine mammary gland quarters. *BMC Genomics*, *14*, 36. https://doi.org/10.1186/1471-2164-14-36
Kalińska, A., Wójcik, A., Slósarz, J., Kruzińska, B., Michalczuk, M., Jaworski, S., Wierzbicki, M., & Gołębiewski, M. (2018). Occurrence and aetiology of Staphylococcal mastitis-a review. *Animal Science Papers & Reports*, *36*(3).
Kalmus, P., Simojoki, H., Orro, T., Taponen, S., Mustonen, K., Holopainen, J., & Pyorala, S. (2014). Efficacy of 5-day parenteral versus intramammary benzylpenicillin for treatment of clinical mastitis caused by gram-positive bacteria susceptible to penicillin in vitro. *J Dairy Sci*, *97*(4), 2155-2164. https://doi.org/10.3168/jds.2013-7338
Kamada, N., Chen, G. Y., Inohara, N., & Nunez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol*, *14*(7), 685-690. https://doi.org/10.1038/ni.2608
Kappeli, N., Morach, M., Corti, S., Eicher, C., Stephan, R., & Johler, S. (2019). Staphylococcus aureus related to bovine mastitis in Switzerland: Clonal diversity, virulence gene profiles, and antimicrobial resistance of isolates collected throughout 2017. *J Dairy Sci*, *102*(4), 3274-3281. https://doi.org/10.3168/jds.2018-15317
Kerro Dego, O., van Dijk, J. E., & Nederbragt, H. (2002). Factors involved in the early pathogenesis of bovine Staphylococcus aureus mastitis with emphasis on bacterial adhesion and invasion. A review. *Vet Q*, *24*(4), 181-198. https://doi.org/10.1080/01652176.2002.9695135
Khanna, T., Friendship, R., Dewey, C., & Weese, J. S. (2008). Methicillin resistant Staphylococcus aureus colonization in pigs and pig farmers. *Vet Microbiol*, *128*(3-4), 298-303. https://doi.org/10.1016/j.vetmic.2007.10.006
Kirchhelle, C. (2018). Pharming animals: a global history of antibiotics in food production (1935–2017). *Palgrave Communications*, *4*(1), 96. https://doi.org/10.1057/s41599-018-0152-2
Klaas, I. C., & Zadoks, R. N. (2018). An update on environmental mastitis: Challenging perceptions. *Transbound Emerg Dis*, *65 Suppl 1*, 166-185. https://doi.org/10.1111/tbed.12704
Klibi, A., Jouini, A., Gomez, P., Slimene, K., Ceballos, S., Torres, C., & Maaroufi, A. (2018). Molecular Characterization and Clonal Diversity of Methicillin-Resistant and -Susceptible Staphylococcus aureus Isolates of Milk of Cows with Clinical Mastitis in Tunisia. *Microb Drug Resist*, *24*(8), 1210-1216. https://doi.org/10.1089/mdr.2017.0278
Klostermann, K., Crispie, F., Flynn, J., Ross, R. P., Hill, C., & Meaney, W. (2008). Intramammary infusion of a live culture of Lactococcus lactis for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. *J Dairy Res*, *75*(3), 365-373. https://doi.org/10.1017/S0022029908003373
Kobayashi, S. D., Musser, J. M., & DeLeo, F. R. (2012). Genomic analysis of the emergence of vancomycin-resistant Staphylococcus aureus. *mBio*, *3*(4). https://doi.org/10.1128/mBio.00170-12
Kock, R., Schaumburg, F., Mellmann, A., Koksal, M., Jurke, A., Becker, K., & Friedrich, A. W. (2013). Livestock-associated methicillin-resistant Staphylococcus aureus (MRSA) as causes

of human infection and colonization in Germany. *PLoS One*, *8*(2), e55040. https://doi.org/10.1371/journal.pone.0055040

Kostrzynska, M., & Bachand, A. (2006). Use of microbial antagonism to reduce pathogen levels on produce and meat products: a review. *Can J Microbiol*, *52*(11), 1017-1026. https://doi.org/10.1139/w06-058

Krol, J., Litwinczuk, Z., Brodziak, A., & Barlowska, J. (2010). Lactoferrin, lysozyme and immunoglobulin G content in milk of four breeds of cows managed under intensive production system. *Pol J Vet Sci*, *13*(2), 357-361. https://www.ncbi.nlm.nih.gov/pubmed/20731193

Kromker, V., & Leimbach, S. (2017). Mastitis treatment-Reduction in antibiotic usage in dairy cows. *Reprod Domest Anim*, *52 Suppl 3*, 21-29. https://doi.org/10.1111/rda.13032

Kuehn, J. S., Gorden, P. J., Munro, D., Rong, R., Dong, Q., Plummer, P. J., Wang, C., & Phillips, G. J. (2013). Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. *PLoS One*, *8*(4), e61959-e61959. https://doi.org/10.1371/journal.pone.0061959

Lakhundi, S., & Zhang, K. (2018). Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology. *Clin Microbiol Rev*, *31*(4). https://doi.org/10.1128/CMR.00020-18

Lammers, A., Nuijten, P. J., & Smith, H. E. (1999). The fibronectin binding proteins of Staphylococcus aureus are required for adhesion to and invasion of bovine mammary gland cells. *FEMS Microbiol Lett*, *180*(1), 103-109. https://doi.org/10.1111/j.1574-6968.1999.tb08783.x

Le, K. Y., & Otto, M. (2015). Quorum-sensing regulation in staphylococci—an overview [Mini Review]. *Frontiers in Microbiology*, *6*(1174). https://doi.org/10.3389/fmicb.2015.01174

Leroy, S., Lebert, I., Andant, C., & Talon, R. (2020). Interaction in dual species biofilms between Staphylococcus xylosus and Staphylococcus aureus. *Int J Food Microbiol*, *326*, 108653. https://doi.org/10.1016/j.ijfoodmicro.2020.108653

Leuenberger, A., Sartori, C., Boss, R., Resch, G., Oechslin, F., Steiner, A., Moreillon, P., & Graber, H. U. (2019). Genotypes of Staphylococcus aureus: On-farm epidemiology and the consequences for prevention of intramammary infections. *J Dairy Sci*, *102*(4), 3295-3309. https://doi.org/10.3168/jds.2018-15181

Levison, L. J., Miller-Cushon, E. K., Tucker, A. L., Bergeron, R., Leslie, K. E., Barkema, H. W., & DeVries, T. J. (2016). Incidence rate of pathogen-specific clinical mastitis on conventional and organic Canadian dairy farms. *J Dairy Sci*, *99*(2), 1341-1350. https://doi.org/10.3168/jds.2015-9809

Li, J., Wang, W., Xu, S. X., Magarvey, N. A., & McCormick, J. K. (2011). Lactobacillus reuteri-produced cyclic dipeptides quench agr-mediated expression of toxic shock syndrome toxin-1 in staphylococci. *Proc Natl Acad Sci U S A*, *108*(8), 3360-3365. https://doi.org/10.1073/pnas.1017431108

Li, N., Wang, Y., You, C., Ren, J., Chen, W., Zheng, H., & Liu, Z. (2018). Variation in Raw Milk Microbiota Throughout 12 Months and the Impact of Weather Conditions. *Sci Rep*, *8*(1), 2371. https://doi.org/10.1038/s41598-018-20862-8

Lima, S. F., Teixeira, A. G. V., Lima, F. S., Ganda, E. K., Higgins, C. H., Oikonomou, G., & Bicalho, R. C. (2017). The bovine colostrum microbiome and its association with clinical mastitis. *J Dairy Sci*, *100*(4), 3031-3042. https://doi.org/10.3168/jds.2016-11604

Lun, H., Yang, W., Zhao, S., Jiang, M., Xu, M., Liu, F., & Wang, Y. (2019). Altered gut microbiota and microbial biomarkers associated with chronic kidney disease. *Microbiologyopen*, *8*(4), e00678. https://doi.org/10.1002/mbo3.678

Luong, T., Sau, S., Gomez, M., Lee, J. C., & Lee, C. Y. (2002). Regulation of Staphylococcus aureus capsular polysaccharide expression by agr and sarA. *Infect Immun*, *70*(2), 444-450. https://doi.org/10.1128/IAI.70.2.444-450.2002

Lyon, G. J., Wright, J. S., Muir, T. W., & Novick, R. P. (2002). Key determinants of receptor activation in the agr autoinducing peptides of Staphylococcus aureus. *Biochemistry*, *41*(31), 10095-10104. https://doi.org/10.1021/bi026049u

Ma, R., Qiu, S., Jiang, Q., Sun, H., Xue, T., Cai, G., & Sun, B. (2017). AI-2 quorum sensing negatively regulates rbf expression and biofilm formation in Staphylococcus aureus. *Int J Med Microbiol*, *307*(4-5), 257-267. https://doi.org/10.1016/j.ijmm.2017.03.003

Mahmmod, Y. S., Klaas, I. C., Svennesen, L., Pedersen, K., & Ingmer, H. (2018). Communications of Staphylococcus aureus and non-aureus Staphylococcus species from bovine intramammary infections and teat apex colonization. *J Dairy Sci*, *101*(8), 7322-7333. https://doi.org/10.3168/jds.2017-14311

Maity, S., & Ambatipudi, K. (2020). Mammary microbial dysbiosis leads to the zoonosis of bovine mastitis: a One-Health perspective. *FEMS Microbiol Ecol*, *97*(1). https://doi.org/10.1093/femsec/fiaa241

Mallon, C. A., Elsas, J. D. V., & Salles, J. F. (2015). Microbial invasions: the process, patterns, and mechanisms. *Trends Microbiol*, *23*(11), 719-729. https://doi.org/10.1016/j.tim.2015.07.013

Mangifesta, M., Mancabelli, L., Milani, C., Gaiani, F., de'Angelis, N., de'Angelis, G. L., van Sinderen, D., Ventura, M., & Turroni, F. (2018). Mucosal microbiota of intestinal polyps reveals putative biomarkers of colorectal cancer. *Sci Rep*, *8*(1), 13974. https://doi.org/10.1038/s41598-018-32413-2

Mansson, M., Nielsen, A., Kjaerulff, L., Gotfredsen, C. H., Wietz, M., Ingmer, H., Gram, L., & Larsen, T. O. (2011). Inhibition of virulence gene expression in Staphylococcus aureus by novel depsipeptides from a marine photobacterium. *Mar Drugs*, *9*(12), 2537-2552. https://doi.org/10.3390/md9122537

Marbach, H., Mayer, K., Vogl, C., Lee, J. Y. H., Monk, I. R., Sordelli, D. O., Buzzola, F. R., Ehling-Schulz, M., & Grunert, T. (2019). Within-host evolution of bovine Staphylococcus aureus selects for a SigB-deficient pathotype characterized by reduced virulence but enhanced proteolytic activity and biofilm formation. *Sci Rep*, *9*(1), 13479. https://doi.org/10.1038/s41598-019-49981-6

Markowiak, P., & Slizewska, K. (2018). The role of probiotics, prebiotics and synbiotics in animal nutrition. *Gut Pathog*, *10*, 21. https://doi.org/10.1186/s13099-018-0250-0

Matuszewska, M., Murray, G. G. R., Harrison, E. M., Holmes, M. A., & Weinert, L. A. (2020). The Evolutionary Genomics of Host Specificity in Staphylococcus aureus. *Trends Microbiol*, *28*(6), 465-477. https://doi.org/10.1016/j.tim.2019.12.007

Mayer, K., Kucklick, M., Marbach, H., Ehling-Schulz, M., Engelmann, S., & Grunert, T. (2021). Within-Host Adaptation of Staphylococcus aureus in a Bovine Mastitis Infection Is Associated with Increased Cytotoxicity. *International Journal of Molecular Sciences*, *22*(16), 8840. https://doi.org/10.3390/ijms22168840

McDonald, J. (1970). Bovine udder infection: Time of microbial invasion of the teat sinus. Proc. VI Int. Conf. Cattle Diseases,

Melchior, M. B., Vaarkamp, H., & Fink-Gremmels, J. (2006). Biofilms: a role in recurrent mastitis infections? *Vet J*, *171*(3), 398-407. https://doi.org/10.1016/j.tvjl.2005.01.006

Melo, T. A., Dos Santos, T. F., de Almeida, M. E., Junior, L. A., Andrade, E. F., Rezende, R. P., Marques, L. M., & Romano, C. C. (2016). Inhibition of Staphylococcus aureus biofilm by Lactobacillus isolated from fine cocoa. *BMC Microbiol*, *16*(1), 250.

https://doi.org/10.1186/s12866-016-0871-8
Metzger, S. A., Hernandez, L. L., Skarlupka, J. H., Suen, G., Walker, T. M., & Ruegg, P. L. (2018). Influence of sampling technique and bedding type on the milk microbiota: Results of a pilot study. *J Dairy Sci*, *101*(7), 6346-6356. https://doi.org/10.3168/jds.2017-14212
Metzger, S. A., Hernandez, L. L., Skarlupka, J. H., Walker, T. M., Suen, G., & Ruegg, P. L. (2018). A Cohort Study of the Milk Microbiota of Healthy and Inflamed Bovine Mammary Glands From Dryoff Through 150 Days in Milk. *Frontiers in veterinary science*, *5*, 247-247. https://doi.org/10.3389/fvets.2018.00247
Mignacca, S. A., Dore, S., Spuria, L., Zanghi, P., Amato, B., Dupre, I., Armas, F., Biasibetti, E., Camperio, C., Lollai, S. A., Capucchio, M. T., Cannas, E. A., Di Marco Lo Presti, V., & Marianelli, C. (2017). Intramammary infusion of a live culture of Lactococcus lactis in ewes to treat staphylococcal mastitis. *J Med Microbiol*, *66*(12), 1798-1810. https://doi.org/10.1099/jmm.0.000641
Millette, M., Smoragiewicz, W., & Lacroix, M. (2004). Antimicrobial potential of immobilized Lactococcus lactis subsp. lactis ATCC 11454 against selected bacteria. *J Food Prot*, *67*(6), 1184-1189. https://doi.org/10.4315/0362-028x-67.6.1184
Moreno-Grua, E., Perez-Fuentes, S., Munoz-Silvestre, A., Viana, D., Fernandez-Ros, A. B., Sanz-Tejero, C., Corpa, J. M., & Selva, L. (2018). Characterization of Livestock-Associated Methicillin-Resistant Staphylococcus aureus Isolates Obtained From Commercial Rabbitries Located in the Iberian Peninsula. *Front Microbiol*, *9*, 1812. https://doi.org/10.3389/fmicb.2018.01812
Mukherjee, S., & Ramesh, A. (2015). Bacteriocin-producing strains of Lactobacillus plantarum inhibit adhesion of Staphylococcus aureus to extracellular matrix: quantitative insight and implications in antibacterial therapy. *Journal of Medical Microbiology*, *64*, 1514-1526. https://doi.org/10.1099/jmm.0.000181
Murphy, S. C., Martin, N. H., Barbano, D. M., & Wiedmann, M. (2016). Influence of raw milk quality on processed dairy products: How do raw milk quality test results relate to product quality and yield? *J Dairy Sci*, *99*(12), 10128-10149. https://doi.org/10.3168/jds.2016-11172
Nagahata, H., Mukai, T., Natsume, Y., Okuda, M., Ando, T., Hisaeda, K., Gondaira, S., & Higuchi, H. (2020). Effects of intramammary infusion of Bifidobacterium breve on mastitis pathogens and somatic cell response in quarters from dairy cows with chronic subclinical mastitis. *Anim Sci J*, *91*(1), e13406. https://doi.org/10.1111/asj.13406
Nagasawa, Y., Kiku, Y., Sugawara, K., Tanabe, F., & Hayashi, T. (2018). Exfoliation rate of mammary epithelial cells in milk on bovine mastitis caused by Staphylococcus aureus is associated with bacterial load. *Anim Sci J*, *89*(1), 259-266. https://doi.org/10.1111/asj.12886
Nataraj, B. H., Ramesh, C., & Mallappa, R. H. (2021). Characterization of biosurfactants derived from probiotic lactic acid bacteria against methicillin-resistant and sensitive Staphylococcus aureus isolates. *LWT*, *151*, 112195. https://doi.org/https://doi.org/10.1016/j.lwt.2021.112195
Naushad, S., Nobrega, D. B., Naqvi, S. A., Barkema, H. W., & De Buck, J. (2020). Genomic Analysis of Bovine Staphylococcus aureus Isolates from Milk To Elucidate Diversity and Determine the Distributions of Antimicrobial and Virulence Genes and Their Association with Mastitis. *mSystems*, *5*(4). https://doi.org/10.1128/mSystems.00063-20
Necidova, L., Bursova, S., Harustiakova, D., Bogdanovicova, K., & Lacanin, I. (2019). Effect of heat treatment on activity of staphylococcal enterotoxins of type A, B, and C in milk. *J Dairy Sci*, *102*(5), 3924-3932. https://doi.org/10.3168/jds.2018-15255
Nemeghaire, S., Roelandt, S., Argudin, M. A., Haesebrouck, F., & Butaye, P. (2013). Characterization of methicillin-resistant Staphylococcus aureus from healthy carrier chickens. *Avian Pathol*,

42(4), 342-346. https://doi.org/10.1080/03079457.2013.805183
Netz, D. J., Pohl, R., Beck-Sickinger, A. G., Selmer, T., Pierik, A. J., Bastos Mdo, C., & Sahl, H. G. (2002). Biochemical characterisation and genetic analysis of aureocin A53, a new, atypical bacteriocin from Staphylococcus aureus. *J Mol Biol*, *319*(3), 745-756. https://doi.org/10.1016/S0022-2836(02)00368-6
Netz, D. J., Sahl, H. G., Marcelino, R., dos Santos Nascimento, J., de Oliveira, S. S., Soares, M. B., & do Carmo de Freire Bastos, M. (2001). Molecular characterisation of aureocin A70, a multi-peptide bacteriocin isolated from Staphylococcus aureus. *J Mol Biol*, *311*(5), 939-949. https://doi.org/10.1006/jmbi.2001.4885
Newstead, L. L., Varjonen, K., Nuttall, T., & Paterson, G. K. (2020). Staphylococcal-Produced Bacteriocins and Antimicrobial Peptides: Their Potential as Alternative Treatments for Staphylococcus aureus Infections. *Antibiotics (Basel)*, *9*(2). https://doi.org/10.3390/antibiotics9020040
Novick, R. P., & Geisinger, E. (2008). Quorum sensing in staphylococci. *Annu Rev Genet*, *42*, 541-564. https://doi.org/10.1146/annurev.genet.42.110807.091640
O'Hara, E., Neves, A. L. A., Song, Y., & Guan, L. L. (2020). The Role of the Gut Microbiome in Cattle Production and Health: Driver or Passenger? *Annu Rev Anim Biosci*, *8*, 199-220. https://doi.org/10.1146/annurev-animal-021419-083952
Oikonomou, G., Addis, M. F., Chassard, C., Nader-Macias, M. E. F., Grant, I., Delbès, C., Bogni, C. I., Le Loir, Y., & Even, S. (2020). Milk Microbiota: What Are We Exactly Talking About? *Frontiers in Microbiology*, *11*, 60-60. https://doi.org/10.3389/fmicb.2020.00060
Oikonomou, G., Bicalho, M. L., Meira, E., Rossi, R. E., Foditsch, C., Machado, V. S., Teixeira, A. G., Santisteban, C., Schukken, Y. H., & Bicalho, R. C. (2014). Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. *PLoS One*, *9*(1), e85904. https://doi.org/10.1371/journal.pone.0085904
Oikonomou, G., Machado, V. S., Santisteban, C., Schukken, Y. H., & Bicalho, R. C. (2012). Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. *PLoS One*, *7*(10), e47671. https://doi.org/10.1371/journal.pone.0047671
Oliver, S., Gonzalez, R., Hogan, J., Jayarao, B., & Owens, W. (2004). Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. *National Mastitis Council, Verona, WI.*
Oliver, S., Headrick, S. I., Lewis, M. J., Gillespie, B., Johnson, D. L., & Almeida, R. A. (2012). Experimental Intramammary Infection with a Strain of Escherichia coli Isolated from a Cow with Persistent E. coli Mastitis. *Open Journal of Veterinary Medicine*, *2*, 186-190.
Olsen, J. E., Christensen, H., & Aarestrup, F. M. (2006). Diversity and evolution of blaZ from Staphylococcus aureus and coagulase-negative staphylococci. *J Antimicrob Chemother*, *57*(3), 450-460. https://doi.org/10.1093/jac/dki492
Otto, M., Echner, H., Voelter, W., & Gotz, F. (2001). Pheromone cross-inhibition between Staphylococcus aureus and Staphylococcus epidermidis. *Infect Immun*, *69*(3), 1957-1960. https://doi.org/10.1128/IAI.69.3.1957-1960.2001
Paduch, J. H., Lucking, J., Mansion-de Vries, E., Zinke, C., Wente, N., & Kromker, V. (2020). Prevention of Intramammary Infections by Prepartum External Application of a Teat Dip Containing Lactic Acid Bacteria with Antimicrobial Properties in Dairy Heifers. *Pathogens*, *9*(4). https://doi.org/10.3390/pathogens9040288
Paharik, A. E., Parlet, C. P., Chung, N., Todd, D. A., Rodriguez, E. I., Van Dyke, M. J., Cech, N. B., & Horswill, A. R. (2017). Coagulase-Negative Staphylococcal Strain Prevents Staphylococcus aureus Colonization and Skin Infection by Blocking Quorum Sensing. *Cell*

Host Microbe, *22*(6), 746-756 e745. https://doi.org/10.1016/j.chom.2017.11.001
Painter, K. L., Strange, E., Parkhill, J., Bamford, K. B., Armstrong-James, D., & Edwards, A. M. (2015). Staphylococcus aureus adapts to oxidative stress by producing H2O2-resistant small-colony variants via the SOS response. *Infect Immun*, *83*(5), 1830-1844. https://doi.org/10.1128/IAI.03016-14
Palma, E., Tilocca, B., & Roncada, P. (2020). Antimicrobial Resistance in Veterinary Medicine: An Overview. *International Journal of Molecular Sciences*, *21*(6), 1914. https://www.mdpi.com/1422-0067/21/6/1914
Parente, E., Ricciardi, A., & Zotta, T. (2020). The microbiota of dairy milk: A review. *International Dairy Journal*, *107*, 104714. https://doi.org/https://doi.org/10.1016/j.idairyj.2020.104714
Park, S., Classen, A., Gohou, H. M., Maldonado, R., Kretschmann, E., Duvernay, C., Kim, G. J., & Ronholm, J. (2021). A new, reliable, and high-throughput strategy to screen bacteria for antagonistic activity against Staphylococcus aureus. *BMC Microbiol*, *21*(1), 189. https://doi.org/10.1186/s12866-021-02265-4
Park, S., & Ronholm, J. (2021). Staphylococcus aureus in Agriculture: Lessons in Evolution from a Multispecies Pathogen. *Clin Microbiol Rev*, *34*(2). https://doi.org/10.1128/CMR.00182-20
Paulrud, C. O. (2005). Basic concepts of the bovine teat canal. *Vet Res Commun*, *29*(3), 215-245. https://doi.org/10.1023/b:verc.0000047496.47571.41
Pena, J. L., Goncalves Schwarz, D. G., Willian de Lima Brasil, A., Licursi de Oliveira, L., Albuquerque Caldeira, J. L., & Scatamburlo Moreira, M. A. (2020). Differences in the coinfective process of Staphylococcus aureus and Streptococcus agalactiae in bovine mammary epithelial cells infected by Mycobacterium avium subsp. paratuberculosis. *Microb Pathog*, *149*, 104476. https://doi.org/10.1016/j.micpath.2020.104476
Peng, H. Y., Tiantong, A., Chen, S. E., Piamya, P., Liu, W. B., Peh, H. C., Lee, J. W., Chen, M. T., Nagahata, H., & Chang, C. J. (2013). Ultrasonicated Enterococcus faecium SF68 enhances neutrophil free radical production and udder innate immunity of drying-off dairy cows. *J Dairy Res*, *80*(3), 349-359. https://doi.org/10.1017/S0022029913000319
Peng, P., Baldry, M., Gless, B. H., Bojer, M. S., Espinosa-Gongora, C., Baig, S. J., Andersen, P. S., Olsen, C. A., & Ingmer, H. (2019). Effect of Co-inhabiting Coagulase Negative Staphylococci on S. aureus agr Quorum Sensing, Host Factor Binding, and Biofilm Formation. *Front Microbiol*, *10*, 2212. https://doi.org/10.3389/fmicb.2019.02212
Peton, V., Bouchard, D. S., Almeida, S., Rault, L., Falentin, H., Jardin, J., Jan, G., Hernandez, D., Francois, P., Schrenzel, J., Azevedo, V., Miyoshi, A., Berkova, N., Even, S., & Le Loir, Y. (2014). Fine-tuned characterization of Staphylococcus aureus Newbould 305, a strain associated with mild and chronic mastitis in bovines. *Vet Res*, *45*, 106. https://doi.org/10.1186/s13567-014-0106-7
Piepers, S., De Vliegher, S., de Kruif, A., Opsomer, G., & Barkema, H. W. (2009). Impact of intramammary infections in dairy heifers on future udder health, milk production, and culling. *Vet Microbiol*, *134*(1-2), 113-120. https://doi.org/10.1016/j.vetmic.2008.09.017
Piewngam, P., & Otto, M. (2020). Probiotics to prevent Staphylococcus aureus disease? *Gut Microbes*, *11*(1), 94-101. https://doi.org/10.1080/19490976.2019.1591137
Piewngam, P., Zheng, Y., Nguyen, T. H., Dickey, S. W., Joo, H.-S., Villaruz, A. E., Glose, K. A., Fisher, E. L., Hunt, R. L., Li, B., Chiou, J., Pharkjaksu, S., Khongthong, S., Cheung, G. Y. C., Kiratisin, P., & Otto, M. (2018). Pathogen elimination by probiotic Bacillus via signalling interference. *Nature*, *562*(7728), 532-537. https://doi.org/10.1038/s41586-018-0616-y
Pinzon-Sanchez, C., & Ruegg, P. L. (2011). Risk factors associated with short-term post-treatment outcomes of clinical mastitis. *J Dairy Sci*, *94*(7), 3397-3410. https://doi.org/10.3168/jds.2010-

3925
Pohlmann-Dietze, P., Ulrich, M., Kiser, K. B., Doring, G., Lee, J. C., Fournier, J. M., Botzenhart, K., & Wolz, C. (2000). Adherence of Staphylococcus aureus to endothelial cells: influence of capsular polysaccharide, global regulator agr, and bacterial growth phase. *Infect Immun*, *68*(9), 4865-4871. https://doi.org/10.1128/IAI.68.9.4865-4871.2000
Porcellato, D., Meisal, R., Bombelli, A., & Narvhus, J. A. (2020). A core microbiota dominates a rich microbial diversity in the bovine udder and may indicate presence of dysbiosis. *Sci Rep*, *10*(1), 21608. https://doi.org/10.1038/s41598-020-77054-6
Price, L. B., Liu, C. M., Melendez, J. H., Frankel, Y. M., Engelthaler, D., Aziz, M., Bowers, J., Rattray, R., Ravel, J., Kingsley, C., Keim, P. S., Lazarus, G. S., & Zenilman, J. M. (2009). Community analysis of chronic wound bacteria using 16S rRNA gene-based pyrosequencing: impact of diabetes and antibiotics on chronic wound microbiota. *PLoS One*, *4*(7), e6462. https://doi.org/10.1371/journal.pone.0006462
Price, L. B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, P. S., Pearson, T., Waters, A. E., Foster, J. T., Schupp, J., Gillece, J., Driebe, E., Liu, C. M., Springer, B., Zdovc, I., Battisti, A., Franco, A., Zmudzki, J., Schwarz, S., . . . Aarestrup, F. M. (2012). Staphylococcus aureus CC398: host adaptation and emergence of methicillin resistance in livestock. *mBio*, *3*(1). https://doi.org/10.1128/mBio.00305-11
Pushkaran, A. C., Nataraj, N., Nair, N., Götz, F., Biswas, R., & Mohan, C. G. (2015). Understanding the Structure-Function Relationship of Lysozyme Resistance in Staphylococcus aureus by Peptidoglycan O-Acetylation Using Molecular Docking, Dynamics, and Lysis Assay. *J Chem Inf Model*, *55*(4), 760-770. https://doi.org/10.1021/ci500734k
Qazi, S., Middleton, B., Muharram, S. H., Cockayne, A., Hill, P., O'Shea, P., Chhabra, S. R., Camara, M., & Williams, P. (2006). N-acylhomoserine lactones antagonize virulence gene expression and quorum sensing in Staphylococcus aureus. *Infect Immun*, *74*(2), 910-919. https://doi.org/10.1128/IAI.74.2.910-919.2006
Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiol Rev*, *37*(5), 664-698. https://doi.org/10.1111/1574-6976.12030
Rainard, P. (2017). Mammary microbiota of dairy ruminants: fact or fiction? *Vet Res*, *48*(1), 25. https://doi.org/10.1186/s13567-017-0429-2
Rainard, P., & Foucras, G. (2018). A Critical Appraisal of Probiotics for Mastitis Control. *Front Vet Sci*, *5*, 251. https://doi.org/10.3389/fvets.2018.00251
Regev-Yochay, G., Trzcinski, K., Thompson, C. M., Malley, R., & Lipsitch, M. (2006). Interference between Streptococcus pneumoniae and Staphylococcus aureus: In vitro hydrogen peroxide-mediated killing by Streptococcus pneumoniae. *Journal of bacteriology*, *188*(13), 4996-5001. https://doi.org/10.1128/JB.00317-06
Ren, D. Y., Li, C., Qin, Y. Q., Yin, R. L., Li, X., Tian, M. Y., Du, S. W., Guo, H. H., Liu, C. X., Zhu, N., Sun, D. D., Li, Y., & Jin, N. Y. (2012). Inhibition of Staphylococcus aureus adherence to Caco-2 cells by lactobacilli and cell surface properties that influence attachment. *Anaerobe*, *18*(5), 508-515. https://doi.org/10.1016/j.anaerobe.2012.08.001
Renault, V., Humblet, M.-F., Pham, P. N., & Saegerman, C. (2021). Biosecurity at Cattle Farms: Strengths, Weaknesses, Opportunities and Threats. *Pathogens*, *10*(10), 1315. https://www.mdpi.com/2076-0817/10/10/1315
Reuben, R. C., Roy, P. C., Sarkar, S. L., Rubayet Ul Alam, A. S. M., & Jahid, I. K. (2020). Characterization and evaluation of lactic acid bacteria from indigenous raw milk for potential probiotic properties. *J Dairy Sci*, *103*(2), 1223-1237. https://doi.org/10.3168/jds.2019-17092

Rode, T. M., Moretro, T., Langsrud, S., Langsrud, O., Vogt, G., & Holck, A. (2010). Responses of Staphylococcus aureus exposed to HCl and organic acid stress. *Can J Microbiol*, *56*(9), 777-792. https://doi.org/10.1139/w10-057

Rogers, G. B., Hoffman, L. R., Johnson, M. W., Mayer-Hamblett, N., Schwarze, J., Carroll, M. P., & Bruce, K. D. (2011). Using bacterial biomarkers to identify early indicators of cystic fibrosis pulmonary exacerbation onset. *Expert review of molecular diagnostics*, *11*(2), 197-206. https://doi.org/10.1586/erm.10.117

Rollin, G., Tan, X., Tros, F., Dupuis, M., Nassif, X., Charbit, A., & Coureuil, M. (2017). Intracellular Survival of Staphylococcus aureus in Endothelial Cells: A Matter of Growth or Persistence. *Front Microbiol*, *8*, 1354. https://doi.org/10.3389/fmicb.2017.01354

Rooks, M. G., & Garrett, W. S. (2016). Gut microbiota, metabolites and host immunity. *Nat Rev Immunol*, *16*(6), 341-352. https://doi.org/10.1038/nri.2016.42

Ruegg, P. L. (2017). A 100-Year Review: Mastitis detection, management, and prevention. *J Dairy Sci*, *100*(12), 10381-10397. https://doi.org/10.3168/jds.2017-13023

Saini, V., McClure, J. T., Léger, D., Dufour, S., Sheldon, A. G., Scholl, D. T., & Barkema, H. W. (2012). Antimicrobial use on Canadian dairy farms. *J Dairy Sci*, *95*(3), 1209-1221. https://doi.org/10.3168/jds.2011-4527

Saini, V., McClure, J. T., Leger, D., Keefe, G. P., Scholl, D. T., Morck, D. W., & Barkema, H. W. (2012). Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. *J Dairy Sci*, *95*(8), 4319-4332. https://doi.org/10.3168/jds.2012-5373

Sakwinska, O., Giddey, M., Moreillon, M., Morisset, D., Waldvogel, A., & Moreillon, P. (2011). Staphylococcus aureus host range and human-bovine host shift. *Appl Environ Microbiol*, *77*(17), 5908-5915. https://doi.org/10.1128/AEM.00238-11

Sandgren, C. H., Waller, K. P., & Emanuelson, U. (2008). Therapeutic effects of systemic or intramammary antimicrobial treatment of bovine subclinical mastitis during lactation. *Vet J*, *175*(1), 108-117. https://doi.org/10.1016/j.tvjl.2006.12.005

Sartori, C., Boss, R., Bodmer, M., Leuenberger, A., Ivanovic, I., & Graber, H. U. (2018). Sanitation of Staphylococcus aureus genotype B-positive dairy herds: A field study. *J Dairy Sci*, *101*(8), 6897-6914. https://doi.org/10.3168/jds.2017-13937

Schijffelen, M. J., Boel, C. H., van Strijp, J. A., & Fluit, A. C. (2010). Whole genome analysis of a livestock-associated methicillin-resistant Staphylococcus aureus ST398 isolate from a case of human endocarditis. *BMC Genomics*, *11*, 376. https://doi.org/10.1186/1471-2164-11-376

Schlusselhuber, M., Godard, J., Sebban, M., Bernay, B., Garon, D., Seguin, V., Oulyadi, H., & Desmasures, N. (2018). Characterization of Milkisin, a Novel Lipopeptide With Antimicrobial Properties Produced By Pseudomonas sp. UCMA 17988 Isolated From Bovine Raw Milk. *Front Microbiol*, *9*, 1030. https://doi.org/10.3389/fmicb.2018.01030

Schmidt, T., Kock, M. M., & Ehlers, M. M. (2017). Molecular Characterization of Staphylococcus aureus Isolated from Bovine Mastitis and Close Human Contacts in South African Dairy Herds: Genetic Diversity and Inter-Species Host Transmission. *Front Microbiol*, *8*, 511. https://doi.org/10.3389/fmicb.2017.00511

Scott, H. M., Acuff, G., Bergeron, G., Bourassa, M. W., Gill, J., Graham, D. W., Kahn, L. H., Morley, P. S., Salois, M. J., Simjee, S., Singer, R. S., Smith, T. C., Storrs, C., & Wittum, T. E. (2019). Critically important antibiotics: criteria and approaches for measuring and reducing their use in food animal agriculture. *Annals of the New York Academy of Sciences*, *1441*(1), 8-16. https://doi.org/10.1111/nyas.14058

Selva, L., Viana, D., Regev-Yochay, G., Trzcinski, K., Corpa, J. M., Lasa, I., Novick, R. P., & Penades, J. R. (2009). Killing niche competitors by remote-control bacteriophage induction.

Proceedings of the National Academy of Sciences of the United States of America, *106*(4), 1234-1238. https://doi.org/10.1073/pnas.0809600106

Sharma, D., & Saharan, B. S. (2016). Functional characterization of biomedical potential of biosurfactant produced by Lactobacillus helveticus. *Biotechnol Rep (Amst)*, *11*, 27-35. https://doi.org/10.1016/j.btre.2016.05.001

Sharma, J., Sundar, D., & Srivastava, P. (2021). Biosurfactants: Potential Agents for Controlling Cellular Communication, Motility, and Antagonism. *Front Mol Biosci*, *8*, 727070. https://doi.org/10.3389/fmolb.2021.727070

Sharun, K., Dhama, K., Tiwari, R., Gugjoo, M. B., Iqbal Yatoo, M., Patel, S. K., Pathak, M., Karthik, K., Khurana, S. K., Singh, R., Puvvala, B., Amarpal, Singh, R., Singh, K. P., & Chaicumpa, W. (2021). Advances in therapeutic and managemental approaches of bovine mastitis: a comprehensive review. *Vet Q*, *41*(1), 107-136. https://doi.org/10.1080/01652176.2021.1882713

Silva, C. C. G., Silva, S. P. M., & Ribeiro, S. C. (2018). Application of Bacteriocins and Protective Cultures in Dairy Food Preservation. *Front Microbiol*, *9*, 594. https://doi.org/10.3389/fmicb.2018.00594

Smith, E. M., Needs, P. F., Manley, G., & Green, L. E. (2014). Global distribution and diversity of ovine-associated Staphylococcus aureus. *Infect Genet Evol*, *22*, 208-215. https://doi.org/10.1016/j.meegid.2013.09.008

Souza, R. F. S., Jardin, J., Cauty, C., Rault, L., Bouchard, D. S., Bermúdez-Humarán, L. G., Langella, P., Monedero, V., Seyffert, N., Azevedo, V., Le Loir, Y., & Even, S. (2017). Contribution of sortase SrtA2 to Lactobacillus casei BL23 inhibition of Staphylococcus aureus internalization into bovine mammary epithelial cells. *PLoS One*, *12*(3), e0174060-e0174060. https://doi.org/10.1371/journal.pone.0174060

Spaniol, J. S., Oltramari, C. E., Locatelli, M., Volpato, A., Campigotto, G., Stefani, L. M., & Da Silva, A. S. (2015). Influence of probiotic on somatic cell count in milk and immune system of dairy cows. *Comparative Clinical Pathology*, *24*(3), 677-681.

Spoor, L. E., McAdam, P. R., Weinert, L. A., Rambaut, A., Hasman, H., Aarestrup, F. M., Kearns, A. M., Larsen, A. R., Skov, R. L., & Fitzgerald, J. R. (2013). Livestock origin for a human pandemic clone of community-associated methicillin-resistant Staphylococcus aureus. *mBio*, *4*(4). https://doi.org/10.1128/mBio.00356-13

Sugrue, I., Tobin, C., Ross, R. P., Stanton, C., & Hill, C. (2019). Chapter 12 - Foodborne Pathogens and Zoonotic Diseases. In L. A. Nero & A. F. De Carvalho (Eds.), *Raw Milk* (pp. 259-272). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-810530-6.00012-2

Sumi, C. D., Yang, B. W., Yeo, I. C., & Hahm, Y. T. (2015). Antimicrobial peptides of the genus Bacillus: a new era for antibiotics. *Can J Microbiol*, *61*(2), 93-103. https://doi.org/10.1139/cjm-2014-0613

Sung, J. M., & Lindsay, J. A. (2007). Staphylococcus aureus strains that are hypersusceptible to resistance gene transfer from enterococci. *Antimicrob Agents Chemother*, *51*(6), 2189-2191. https://doi.org/10.1128/aac.01442-06

Taponen, S., Liski, E., Heikkila, A. M., & Pyorala, S. (2017). Factors associated with intramammary infection in dairy cows caused by coagulase-negative staphylococci, Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Corynebacterium bovis, or Escherichia coli. *J Dairy Sci*, *100*(1), 493-503. https://doi.org/10.3168/jds.2016-11465

Taponen, S., McGuinness, D., Hiitiö, H., Simojoki, H., Zadoks, R., & Pyörälä, S. (2019). Bovine milk microbiome: a more complex issue than expected. *Veterinary Research*, *50*(1), 44. https://doi.org/10.1186/s13567-019-0662-y

Tiantong, A., Peng, H. Y., Chen, S. E., Piamya, P., Liu, W. B., Chen, M. T., Yu, C., Nagahata, H., & Chang, C. J. (2015). Intramammary infusion of an Enterococcus faecium SF68 preparation promoted the involution of drying off Holstein cows partly related to neutrophil-associated matrix metalloproteinase 9. *Anim Sci J*, *86*(1), 111-119. https://doi.org/10.1111/asj.12243

Toledo-Silva, B., de Souza, F. N., Mertens, K., Piepers, S., Haesebrouck, F., & De Vliegher, S. (2021). Bovine-associated non-aureus staphylococci suppress Staphylococcus aureus biofilm dispersal in vitro yet not through agr regulation. *Vet Res*, *52*(1), 114. https://doi.org/10.1186/s13567-021-00985-z

Tong, J., Zhang, H., Yang, D., Zhang, Y., Xiong, B., & Jiang, L. (2018). Illumina sequencing analysis of the ruminal microbiota in high-yield and low-yield lactating dairy cows. *PLoS One*, *13*(11), e0198225. https://doi.org/10.1371/journal.pone.0198225

Tsubakishita, S., Kuwahara-Arai, K., Sasaki, T., & Hiramatsu, K. (2010). Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. *Antimicrob Agents Chemother*, *54*(10), 4352-4359. https://doi.org/10.1128/AAC.00356-10

Tuchscherr, L. P., Buzzola, F. R., Alvarez, L. P., Caccuri, R. L., Lee, J. C., & Sordelli, D. O. (2005). Capsule-negative Staphylococcus aureus induces chronic experimental mastitis in mice. *Infect Immun*, *73*(12), 7932-7937. https://doi.org/10.1128/IAI.73.12.7932-7937.2005

Tuffs, S. W., Haeryfar, S. M. M., & McCormick, J. K. (2018). Manipulation of Innate and Adaptive Immunity by Staphylococcal Superantigens. *Pathogens*, *7*(2). https://doi.org/10.3390/pathogens7020053

Turner, C. E., & Sriskandan, S. (2015). Panton-Valentine leucocidin expression by Staphylococcus aureus exposed to common antibiotics. *J Infect*, *71*(3), 338-346. https://doi.org/10.1016/j.jinf.2015.05.008

Ulhuq, F. R., Gomes, M. C., Duggan, G. M., Guo, M., Mendonca, C., Buchanan, G., Chalmers, J. D., Cao, Z., Kneuper, H., Murdoch, S., Thomson, S., Strahl, H., Trost, M., Mostowy, S., & Palmer, T. (2020). A membrane-depolarizing toxin substrate of the Staphylococcus aureus type VII secretion system mediates intraspecies competition. *Proc Natl Acad Sci U S A*, *117*(34), 20836-20847. https://doi.org/10.1073/pnas.2006110117

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A., & Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proc Natl Acad Sci U S A*, *112*(18), 5649-5654. https://doi.org/10.1073/pnas.1503141112

Viana, D., Comos, M., McAdam, P. R., Ward, M. J., Selva, L., Guinane, C. M., Gonzalez-Munoz, B. M., Tristan, A., Foster, S. J., Fitzgerald, J. R., & Penades, J. R. (2015). A single natural nucleotide mutation alters bacterial pathogen host tropism. *Nat Genet*, *47*(4), 361-366. https://doi.org/10.1038/ng.3219

Voggu, L., Schlag, S., Biswas, R., Rosenstein, R., Rausch, C., & Gotz, F. (2006). Microevolution of cytochrome bd oxidase in Staphylococci and its implication in resistance to respiratory toxins released by Pseudomonas. *J Bacteriol*, *188*(23), 8079-8086. https://doi.org/10.1128/JB.00858-06

Voss, A., Loeffen, F., Bakker, J., Klaassen, C., & Wulf, M. (2005). Methicillin-resistant Staphylococcus aureus in pig farming. *Emerg Infect Dis*, *11*(12), 1965-1966. https://doi.org/10.3201/eid1112.050428

Vrieling, M., Koymans, K. J., Heesterbeek, D. A., Aerts, P. C., Rutten, V. P., de Haas, C. J., van Kessel, K. P., Koets, A. P., Nijland, R., & van Strijp, J. A. (2015). Bovine Staphylococcus aureus Secretes the Leukocidin LukMF' To Kill Migrating Neutrophils through CCR1. *mBio*, *6*(3), e00335. https://doi.org/10.1128/mBio.00335-15

Wang, B., & Muir, T. W. (2016). Regulation of Virulence in Staphylococcus aureus: Molecular

Mechanisms and Remaining Puzzles. *Cell Chem Biol*, *23*(2), 214-224. https://doi.org/10.1016/j.chembiol.2016.01.004

Wang, Y., Nan, X., Zhao, Y., Jiang, L., Wang, M., Wang, H., Zhang, F., Xue, F., Hua, D., Liu, J., Yao, J., & Xiong, B. (2021). Rumen microbiome structure and metabolites activity in dairy cows with clinical and subclinical mastitis. *J Anim Sci Biotechnol*, *12*(1), 36. https://doi.org/10.1186/s40104-020-00543-1

Wang, Y., Nan, X., Zhao, Y., Wang, H., Wang, M., Jiang, L., Zhang, F., Xue, F., Hua, D., Li, K., Liu, J., Yao, J., & Xiong, B. (2020). Coupling 16S rDNA Sequencing and Untargeted Mass Spectrometry for Milk Microbial Composition and Metabolites from Dairy Cows with Clinical and Subclinical Mastitis. *J Agric Food Chem*, *68*(31), 8496-8508. https://doi.org/10.1021/acs.jafc.0c03738

Watkins, K. E., & Unnikrishnan, M. (2020). Evasion of host defenses by intracellular Staphylococcus aureus. *Adv Appl Microbiol*, *112*, 105-141. https://doi.org/10.1016/bs.aambs.2020.05.001

Watts, J. L. (1988). Etiological agents of bovine mastitis. *Vet Microbiol*, *16*(1), 41-66. https://doi.org/10.1016/0378-1135(88)90126-5

Weinert, L. A., Welch, J. J., Suchard, M. A., Lemey, P., Rambaut, A., & Fitzgerald, J. R. (2012). Molecular dating of human-to-bovid host jumps by Staphylococcus aureus reveals an association with the spread of domestication. *Biol Lett*, *8*(5), 829-832. https://doi.org/10.1098/rsbl.2012.0290

Wellenberg, G. J., van der Poel, W. H., & Van Oirschot, J. T. (2002). Viral infections and bovine mastitis: a review. *Vet Microbiol*, *88*(1), 27-45. https://doi.org/10.1016/s0378-1135(02)00098-6

WHO. (2010). Biosecurity: An integrated approach to manage risk to human animal and plant life and health. *International Food Safety Authorities Network (INFOSAN)*.

Wilson, G. J., Tuffs, S. W., Wee, B. A., Seo, K. S., Park, N., Connelley, T., Guinane, C. M., Morrison, W. I., & Fitzgerald, J. R. (2018). Bovine Staphylococcus aureus Superantigens Stimulate the Entire T Cell Repertoire of Cattle. *Infect Immun*, *86*(11). https://doi.org/10.1128/IAI.00505-18

Wright, J. S., 3rd, Jin, R., & Novick, R. P. (2005). Transient interference with staphylococcal quorum sensing blocks abscess formation. *Proc Natl Acad Sci U S A*, *102*(5), 1691-1696. https://doi.org/10.1073/pnas.0407661102

Wu, S. W., de Lencastre, H., & Tomasz, A. (2001). Recruitment of the mecA gene homologue of Staphylococcus sciuri into a resistance determinant and expression of the resistant phenotype in Staphylococcus aureus. *J Bacteriol*, *183*(8), 2417-2424. https://doi.org/10.1128/JB.183.8.2417-2424.2001

Wu, Z., Li, F., Liu, D., Xue, H., & Zhao, X. (2015). Novel Type XII Staphylococcal Cassette Chromosome mec Harboring a New Cassette Chromosome Recombinase, CcrC2. *Antimicrob Agents Chemother*, *59*(12), 7597-7601. https://doi.org/10.1128/AAC.01692-15

Xue, M. Y., Sun, H. Z., Wu, X. H., Liu, J. X., & Guan, L. L. (2020). Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome*, *8*(1), 64. https://doi.org/10.1186/s40168-020-00819-8

Yamada, T., Tochimaru, N., Nakasuji, S., Hata, E., Kobayashi, H., Eguchi, M., Kaneko, J., Kamio, Y., Kaidoh, T., & Takeuchi, S. (2005). Leukotoxin family genes in Staphylococcus aureus isolated from domestic animals and prevalence of lukM-lukF-PV genes by bacteriophages in bovine isolates. *Vet Microbiol*, *110*(1-2), 97-103. https://doi.org/10.1016/j.vetmic.2005.07.006

Yamashita, D., Sugawara, T., Takeshita, M., Kaneko, J., Kamio, Y., Tanaka, I., Tanaka, Y., & Yao, M. (2014). Molecular basis of transmembrane beta-barrel formation of staphylococcal pore-forming toxins. *Nat Commun*, *5*, 4897. https://doi.org/10.1038/ncomms5897

Yan, X., Gu, S., Cui, X., Shi, Y., Wen, S., Chen, H., & Ge, J. (2019). Antimicrobial, anti-adhesive and anti-biofilm potential of biosurfactants isolated from Pediococcus acidilactici and Lactobacillus plantarum against Staphylococcus aureus CMCC26003. *Microb Pathog*, *127*, 12-20. https://doi.org/10.1016/j.micpath.2018.11.039

Yoon, Y., Lee, S., & Choi, K.-H. (2016). Microbial benefits and risks of raw milk cheese. *Food Control*, *63*, 201-215. https://doi.org/https://doi.org/10.1016/j.foodcont.2015.11.013

Younes, J. A., van der Mei, H. C., van den Heuvel, E., Busscher, H. J., & Reid, G. (2012). Adhesion Forces and Coaggregation between Vaginal Staphylococci and Lactobacilli. *PLoS One*, *7*(5). https://doi.org/ARTN e36917

10.1371/journal.pone.0036917

Young, W., Hine, B. C., Wallace, O. A., Callaghan, M., & Bibiloni, R. (2015). Transfer of intestinal bacterial components to mammary secretions in the cow. *PeerJ*, *3*, e888. https://doi.org/10.7717/peerj.888

Younis, A., Krifucks, O., Fleminger, G., Heller, E. D., Gollop, N., Saran, A., & Leitner, G. (2005). Staphylococcus aureus leucocidin, a virulence factor in bovine mastitis. *J Dairy Res*, *72*(2), 188-194. https://doi.org/10.1017/s002202990500083x

Yu, J., Ren, Y., Xi, X., Huang, W., & Zhang, H. (2017). A Novel Lactobacilli-Based Teat Disinfectant for Improving Bacterial Communities in the Milks of Cow Teats with Subclinical Mastitis. *Front Microbiol*, *8*, 1782. https://doi.org/10.3389/fmicb.2017.01782

Yung, D. B. Y., Sircombe, K. J., & Pletzer, D. (2021). Friends or enemies? The complicated relationship between Pseudomonas aeruginosa and Staphylococcus aureus. *Molecular Microbiology*, *116*(1), 1-15. https://doi.org/https://doi.org/10.1111/mmi.14699

Zapotoczna, M., O'Neill, E., & O'Gara, J. P. (2016). Untangling the Diverse and Redundant Mechanisms of Staphylococcus aureus Biofilm Formation. *PLoS Pathog*, *12*(7), e1005671. https://doi.org/10.1371/journal.ppat.1005671

Zeder, M. (2009). The Neolithic Macro-(R)evolution: Macroevolutionary Theory and the Study of Culture Change. *Journal of Archaeological Research*, *17*, 1-63.

Zeder, M. A. (2008). Domestication and early agriculture in the Mediterranean Basin: Origins, diffusion, and impact. *Proc Natl Acad Sci U S A*, *105*(33), 11597-11604. https://doi.org/10.1073/pnas.0801317105

Zhang, R., Huo, W., Zhu, W., & Mao, S. (2015). Characterization of bacterial community of raw milk from dairy cows during subacute ruminal acidosis challenge by high-throughput sequencing. *J Sci Food Agric*, *95*(5), 1072-1079. https://doi.org/10.1002/jsfa.6800

Zhao, X., & Lacasse, P. (2008). Mammary tissue damage during bovine mastitis: causes and control. *J Anim Sci*, *86*(13 Suppl), 57-65. https://doi.org/10.2527/jas.2007-0302

Zhong, Y., Xue, M., & Liu, J. (2018). Composition of Rumen Bacterial Community in Dairy Cows With Different Levels of Somatic Cell Counts. *Front Microbiol*, *9*, 3217. https://doi.org/10.3389/fmicb.2018.03217

Zou, D., Kaneko, J., Narita, S., & Kamio, Y. (2000). Prophage, phiPV83-pro, carrying panton-valentine leukocidin genes, on the Staphylococcus aureus P83 chromosome: comparative analysis of the genome structures of phiPV83-pro, phiPVL, phi11, and other phages. *Biosci Biotechnol Biochem*, *64*(12), 2631-2643. https://doi.org/10.1271/bbb.64.2631

Appendix 1

No.	Strain name	Accession no. NCBI	Genome structure[a]	Country of origin	Host[b]	Disease/clinical information	ST	CC	year	Seq.[d]
1	Sa1	JAANBU000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
2	Sa3	JAANBR000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
3	Sa4	JAANCG000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
4	Sa5	JAANCB000000000	Draft	Canada	B	Intramammary infection	Unknown	Unknown	2007	I
5	Sa6	JAANCH000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
6	Sa7	JAANBN000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
7	Sa8	JAANCD000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2008	I
8	Sa9	JAANBZ000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
9	Sa10	JAANCE000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
10	Sa11	JAANCA000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
11	Sa12	JAANCC000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2008	I
12	Sa14	JAANCF000000000	Draft	Canada	B	Intramammary infection	ST3028	CC97	2007	I
13	Sa16	JAANBM000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
14	Sa17	JAANBL000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
15	Sa18	JAANBK000000000	Draft	Canada	B	Intramammary infection	ST2185	CC151	2008	I
16	Sa19	JAANBW000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2008	I
17	Sa21	JAANBX000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
18	Sa22	JAANBY000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
19	Sa23	JAANBP000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
20	Sa24	JAANBS000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
21	Sa25	JAANBO000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
22	Sa27	JAANBI000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2019	I
23	Sa28	JAANBH000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2019	I
24	Sa29	JAANBG000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2019	I
25	Sa30	JAANBF000000000	Draft	Canada	B	Intramammary infection	ST352	CC97	2019	I
26	Sa31	JAANBE000000000	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I

27	Sa1158c	SRR11471981	Draft	Canada	B	Intramammary infection	ST8	CC8	2008	I
28	Sa2112	SRR11471976	Draft	Canada	B	Intramammary infection	ST2187	CC97	2007	I
29	Sa2117	SRR11471989	Draft	Canada	B	Intramammary infection	ST2187	CC97	2007	I
30	Sa2539	SRR11471965	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
31	Sa2605	SRR11471994	Draft	Canada	B	Intramammary infection	ST126	CC126	2008	I
32	Sa2911	SRR11471986	Draft	Canada	B	Intramammary infection	ST2187	CC97	2008	I
33	Sa2925	SRR11471988	Draft	Canada	B	Intramammary infection	ST2187	CC97	2007	I
34	Sa2946	SRR11471975	Draft	Canada	B	Intramammary infection	ST2187	CC97	2007	I
35	Sa2954	SRR11471977	Draft	Canada	B	Intramammary infection	ST2187	CC97	2007	I
36	Sa2970	SRR11471973	Draft	Canada	B	Intramammary infection	ST2270	CC126	2007	I
37	Sa2978	SRR11471964	Draft	Canada	B	Intramammary infection	ST151	CC151	2008	I
38	Sa3003	SRR11471960	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
39	Sa3014	SRR11471958	Draft	Canada	B	Intramammary infection	ST352	CC97	2008	I
40	Sa3022	SRR11471957	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
41	Sa3071	SRR11471985	Draft	Canada	B	Intramammary infection	ST2187	CC97	2008	I
42	Sa3129	SRR11471990	Draft	Canada	B	Intramammary infection	ST2270	CC126	2008	I
43	Sa3131	SRR11471991	Draft	Canada	B	Intramammary infection	ST2270	CC126	2008	I
44	Sa3137	SRR11471992	Draft	Canada	B	Intramammary infection	ST2270	CC126	2008	I
45	Sa3151	SRR11471971	Draft	Canada	B	Intramammary infection	ST2187	CC97	2008	I
46	Sa3154	SRR11471968	Draft	Canada	B	Intramammary infection	ST351	CC151	2007	I
47	Sa3199	SRR11471956	Draft	Canada	B	Intramammary infection	ST352	CC97	2008	I
48	Sa3213	SRR11471970	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
49	Sa3247	SRR11471974	Draft	Canada	B	Intramammary infection	ST2187	CC97	2008	I
50	Sa3302	SRR11471972	Draft	Canada	B	Intramammary infection	ST2187	CC97	2008	I
51	Sa3336	SRR11471993	Draft	Canada	B	Intramammary infection	ST2187	CC97	2008	I
52	Sa3353	SRR11471959	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
53	Sa3370	SRR11471963	Draft	Canada	B	Intramammary infection	ST151	CC151	2008	I
54	Sa3454	SRR11471984	Draft	Canada	B	Intramammary infection	ST352	CC97	2008	I
55	Sa3456	SRR11471983	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
56	Sa3468	SRR11471982	Draft	Canada	B	Intramammary infection	ST352	CC97	2008	I
57	Sa3489	SRR11471978	Draft	Canada	B	Intramammary infection	ST352	CC97	2008	I
58	Sa3493	SRR11471962	Draft	Canada	B	Intramammary infection	ST352	CC97	2008	I

59	Sa3498	SRR11471966	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
60	Sa3511	SRR11471969	Draft	Canada	B	Intramammary infection	ST151	CC151	2007	I
61	Sa3532	SRR11471980	Draft	Canada	B	Intramammary infection	ST352	CC97	2007	I
62	Sa3603	SRR11471979	Draft	Canada	B	Intramammary infection	ST352	CC97	2008	I
63	Sa3763	SRR11471987	Draft	Canada	B	Intramammary infection	ST2187	CC97	2007	I
64	RF122	NC_007622	Draft	Ireland	B	Intramammary infection	ST151	CC151	1993	
65	Newbould 305	NZ_AKYW01000001 - NZ_AKYW01000028	High	Canada	B	Clinical mastitis	ST115	CC97	1958	I
66	04-002	NZ_CP038021	High	USA	H	Abdominal wound	ST30	CC30	2004	PB
67	08-02119	NZ_CP015645	High	Germany	H	Wound infection	ST582	CC15	2008	PB, I
68	08-02300	NZ_CP015646	High	Germany	H	Wound infection	ST7		2008	PB, I
69	08BA02176	NC_018608	High	Canada	H	LA-MRSA origin, soft tissue infection of skin	ST398		2008	R
70	11819-97	NC_017351, NC_017350	High	Denmark	H	CA-MRSA, skin abscess	ST80		1997	I, R
71	135	NZ_CP022720, NZ_CP022721	High	Germany	H	Bacteremia	ST15	CC15	1994	I, PB
72	16445	NZ_CP043302, NZ_CP043303	High	USA	H		ST8	CC8	2019	O
73	293G	NZ_CP019591	High	Canada	H		ST398			I, PB
74	502A	NZ_CP007454, NZ_CP007455	High	USA	H	Colonization	ST5	CC5	1963	PB
75	5118.N	NZ_CP016855, NZ_CP016854, NZ_CP025482	High	USA	H	Nose from military trainee	ST8	CC8	2010	I
76	6850	NC_022222	High		H	Bacteremia	ST50			R, SG
77	ATCC_25923	NZ_CP009361, NZ_CP009362	High	USA	H		ST243	CC30	1945	PB
78	ATCC_6538	NZ_CP020020, NZ_CP020021	High	Germany	H	Pleural fluid	ST464	CC97	1884	PB, I
79	ATCC_BAA-39	NZ_CP033505	High	Kazakhstan	H		ST464	CC97	2017	PB
80	BSN9R	NZ_CP042348, NZ_CP042349	High	USA	H	Infective endocarditis	ST8	CC8	2018	O, I
81	BSN9S	NZ_CP042346, NZ_CP042347	High	USA	H	Infective endocarditis	ST8	CC8	2018	O, I
82	Be62	NZ_CP012013, NZ_CP012014	High	Brazil	H	Blood stream infection	ST239	CC8	1996	R
83	Bmb9393	NC_021670.1, NC_021657.1	High	Brazil	H	Blood stream infection	ST239	CC8	1993	R
84	C2406	CP019590	High	Canada	H	USA300, Necrotizing pneumonia	ST8	CC8		PB
85	C3948	CP020957	High	Canada	H	MSSA	ST8	CC8		PB

86	C8879	NZ_CP020956	High	Canada	H	MRSA	ST8	CC8		PB
87	CA-347	NC_021554, NC_021552	High	USA	H	USA600	ST45	CC45	2005	I, PB
88	CA12	NZ_CP007672, NZ_CP007673	High	Colombia	H	Bacteremia	ST8	CC8	2007	PB
89	CA15	NZ_CP007674, NZ_CP007675	High	Colombia	H	Bacteremia	ST8	CC8	2007	PB
90	CC5	NZ_CP021105	High	Brazil	H	Respiratory tract infection	ST5	CC5	2014	I
91	CC8	NZ_AP017377	High	Russia	H		ST8	CC8	2007	PB
92	CHU15-056	NZ_CP021171	High	Brazil	H	Bloodstream infection	ST5	CC5	2015	I
93	CMRSA3	CP029685, MH470063	High	Canada	H	MRSA	ST241	CC8		I, PB
94	CMRSA6	CP027788	High	Canada	H	MRSA	ST239	CC8		I, PB
95	CN1	NC_022226, NC_022227, NC_022228	High	South Korea	H	PVL-negative CA-MRSA	ST72	CC8		R
96	COL	NC_002951, NC_006629	High	USA	H	MRSA	ST250	CC8	1976	SG
97	Col52-A5	NZ_CP040560, NZ_CP040561	High	Colombia	H	Colonization	ST923	CC8	2017	PB
98	DAR4145	NZ_CP010526	High	India	H	MRSA	ST772	CC1	2009	PB
99	DSM_20231	NZ_CP011526, NZ_CP011527	High		H		ST8	CC8	1884	PB
100	E16SA093	NZ_CP031131	High	South Korea	H	Blood stream infection	ST72	CC8	2016	PB
101	ECT-R2	NC_017343, NC_017346, NC_017344	High	Sweden	H	MR-MSSA	ST5	CC5	2005	R
102	EDCC5464	NZ_CP022291	High	Germany	H	Implant associated bone infection	ST22	CC22	2015	PB
103	F17SA003	NZ_CP031130	High	South Korea	H	Blood stream infection	ST72	CC8	2017	PB
104	FCFHV36	NZ_CP011147	High	Brazil	H	Osteomyelitis	ST105	CC5	2010	I
105	FORC_012	NZ_CP010998	High	South Korea	H	Sputum	ST72	CC8	2009	I, PB
106	FORC_045	NZ_CP017115	High	South Korea	H		ST1	CC1	2014	PB
107	FORC_061	NZ_CP022607, NZ_CP022608	High	South Korea	H	Food poisoning	ST72	CC8	2017	PB
108	FORC_062	NZ_CP022582	High	South Korea	H	Food poisoning	ST5	CC5	2017	PB
109	GN1	NZ_AP018349	High	Japan	H	MSSA	ST50		2005	PB
110	GN3	NZ_AP017891	High	Japan	H	MSSA	ST50		2005	PB
111	GR2	NZ_CP010402, NZ_CP010403, NZ_CP010404	High	Greece	H		ST80		2006	R, I
112	Gv88	NZ_CP012018, NZ_CP012017	High	Brazil	H	Wound infection	ST239	CC8	1997	R

113	H-EMRSA-15	NZ_CP007659	High	Belgium	H	Abscess	ST22	CC22	2011	I
114	H489	NZ_CP020959	High	Canada	H	MSSA	ST8	CC8		PB
115	HC1340	NZ_CP012011	High	Brazil	H	Nasa colonization	ST239	CC8	2001	R
116	HOU1444-VR	NZ_CP012593, NZ_CP012594, NZ_CP012595, NZ_CP012596	High	Brazil	H	Bacteremia	ST5	CC5	2012	PB
117	HO_5096_0412	NC_017763	High	UK	H	Fatal neonatal infection	ST22	CC22	2005	I
118	HUV05	NZ_CP007676, NZ_CP007677, NZ_CP007678, NZ_CP007679	High	Colombia	H	Bacteremia	ST8	CC8	2006	PB
119	HZW450	NZ_CP020741	High	China	H	Impetigo	ST59		2016	PB
120	IT1-S	NZ_CP028468, NZ_CP028469	High	Italy	H	Endocarditis	ST22	CC22	2013	I
121	IT4-R	NZ_CP028470, NZ_CP028471	High	Italy	H	Endocarditis	ST22	CC22	2013	I
122	J01	NZ_CP040619, NZ_CP040620, NZ_CP040621	High	USA	H	Infective endocarditis	ST8	CC8	2011	I, PB
123	JE2	NZ_CP020619	High	USA	H	Soft tissue infections	ST8	CC8	2013	I, PB
124	JH4899	NZ_AP014921, NZ_AP014922	High	Japan	H	Invasive growth	ST8	CC8	2013	I
125	JK3137	CP020960	High	Canada	H	MRSA	ST8	CC8		PB
126	JKD6004-DR	NZ_CP040625	High	USA	H		ST239	CC8	2019	I
127	JKD6004	NZ_CP040622	High	Australia	H	Pacemaker abscess	ST239	CC8	2004	I, PB
128	JKD6008	CP002120	High	New Zealand	H	MRSA, VSSA	ST239	CC8	2003	R, SG, SL
129	JMUB1273	NZ_AP018922	High	Japan	H	Subcutaneous abscess	ST188	CC1	2016	I, O
130	JMUB3031	NZ_AP018923, NZ_AP018924	High	Japan	H	Subcutaneous abscess	ST1	CC1	2017	PB, I
131	JP02758	NZ_AP017922, NZ_AP017923	High	Japan	H	Super biofilm-elaborating	Unknown	Unknown	2005	PB
132	KG-03	NZ_AP019542	High	Japan	H	Persistent bacteremia	ST5	CC5	2015	PB, I
133	KG-18	NZ_AP019543, NZ_AP019544	High	Japan	H	VISA	ST5	CC5	2015	PB, I
134	KG-22	NZ_AP019545, NZ_AP019546	High	Japan	H	VISA	ST5	CC5	2015	PB, I
135	KUH140046	NZ_AP020313, NZ_AP020314	High	Japan	H	MRSA	ST8	CC8	2014	I, O
136	KUH140331	NZ_AP020316, NZ_AP020317	High	Japan	H	MRSA	ST188	CC1	2014	I, O
137	KUH180062	NZ_AP020320, NZ_AP020321	High	Japan	H		ST764	CC5	2018	I, O

138	KUN1163	NZ_AP020324, NZ_AP020325	High	Japan	H		ST764	CC5	2006	I, O
139	M013	NC_016928, NZ_CP039996	High	Taiwan	H	CA-MRSA	ST59		2002	R
140	M121	NZ_CP007670, NZ_CP007671	High	Colombia	H	Healthy volunteer	ST8	CC8	2004	I, PB
141	M92	CP015447	High	Canada	H	MRSA, Colonization	ST5354	CC8		PB
142	MI	NZ_AP017320, NZ_AP017321	High		H	VISA, Peritonitis	ST5	CC5	1997	I, PB
143	MRSA252	NC_002952	High	UK	H	MRSA, Fatal bacteremia	ST36	CC30	1997	
144	MS4	NZ_CP009828	High	China	H	Wound exudate of bone fracture	ST338		2012	I, PB
145	MSSA476	BX571857, BX571858	High		H	Osteomyelitis and bacteremia	ST1	CC1	1998	
146	MW2	BA000033, AP004832	High	USA	H	CA-MRSA, septicaemia and septic arthritis	ST1	CC1	1998	SG
147	Mu50	BA000017, AP003367	High	Japan	H	Surgical wound infection, VRSA	ST5	CC5	1997	SG
148	N315	BA000018, AP003139	High	Japan	H	Pharyngeal smear, *mecA* but methicillin susceptible	ST5	CC5	1982	
149	NCCP16830	NZ_CP043843, NZ_CP043844	High	South Korea	H	Diabetes, urine	ST513		2008	PB, IT
150	NCTC8325	NC_007795	High		H	Toxic-shock syndrome, staphylococcal scarlet fever	ST8	CC8		
151	NML151290	MEGZ00000001-MEGZ00000008	High	Canada	H	Endocarditis and septic arthritis	ST25			I
152	NP66	NZ_CP041037	High	South Africa	H	Pus aspirate	ST12		2018	PB
153	Newman	NC_009641	High	UK	H		ST254	CC8	1952	SG
154	Newman_D2C	NZ_CP023391	High		H	Osteomyelitis	ST254	CC8	1970	I
155	PCFH-226	NZ_CP035005, NZ_CP035006	High	South Korea	H	Healthy, hand	ST541		2017	PB
156	R46	NZ_CP039164, NZ_CP039165, NZ_CP039166	High	Pakistan	H		ST113	CC8	2017	I
157	RIVM1607	NZ_CP013619, NZ_CP013620	High	Netherlands	H		ST398		2008	R, I
158	RIVM3897	NZ_CP013621	High	Netherlands	H	LA-MRSA, nosocomial transmission	ST398		2008	R, I
159	RK14	NZ_CP011528, NZ_CP011529	High	Germany	H	Staphylococcal food poisoning	ST27	CC8	2008	I, PB
160	S57	NZ_CP030136	High	Brazil	H	Osteomyelitis	ST9	CC1	2010	PB
161	SA268	NZ_CP006630	High	China	H		ST59		2012	I
162	SAW1	NZ_CP045468, NZ_CP045469, NZ_CP045470, NZ_CP045471	High	China	H	Endocarditis	ST59		2018	O

163	SR153	NZ_CP048643, NZ_CP048644, NZ_CP048645, NZ_CP048646	High	China	H	Acute pancreatitis	ST5	CC5	2013	PB
164	SR434	NZ_CP019563, NZ_CP019564, NZ_CP019565, NZ_CP019566, NZ_CP019567	High	China	H	Skin abscess	ST88		2015	PB
165	SVH7513	NZ_CP029166, NZ_CP029167, NZ_CP029165	High	Australia	H	Cellulitis	ST612	CC8	2009	PB, I
166	Seattle_1945	NZ_CP021907, NZ_CP021908	High	Germany	H	PVL+	ST243	CC30	2013	I
167	T0131	NC_017347	High	China	H	MRSA	ST239	CC8	2006	R
168	TCH60	NC_017342, NC_017345,	High		H	MRSA	ST4618	CC30		R
169	TUM9458	NZ_AP019305	High		H		ST2389	CC5	2008	I, O
170	TUM9463	NZ_AP019306	High		H		ST2389	CC5	2009	I, O
171	TW20	NC_017331, NC_017352, NC_017332	High	UK	H	MRSA	ST239	CC8	2003	
172	Tager_104	NZ_CP012409	High	USA	H	Cutaneous abscess	ST49		1947	I, PB
173	UCI28	NZ_CP018768, NZ_CP018769	High	USA	H	No contact with swine	ST5	CC5	2009	PB, I
174	UCI62	NZ_CP018766, NZ_CP018767	High	USA	H	No contact with swine	ST5	CC5	2010	PB, I
175	USA300_FPR3757	CP000255, CP000256, CP000257, CP000258	High	USA	H	MRSA	ST8	CC8		SG
176	USA300_TCH1516	NC_010079, NC_010063, NC_012417	High	USA	H	CA-MRSA	ST8	CC8		R
177	USA500	NZ_CP007499, NZ_CP007500	High	USA	H		ST8	CC8		I, PB
178	USA_100	NZ_CP029474, NZ_CP029475	High	USA	H		ST5	CC5	2015	I, O
179	UTSW_MRSA_55	NZ_CP013231, NZ_CP013227, NZ_CP013228, NZ_CP013229, NZ_CP013230	High	USA	H	Osteomyelitis, MRSA	ST8	CC8	2013	PB
180	V2200	NZ_CP007657,	High	Venezuela	H	Osteomyelitis	ST923	CC8	2007	PB
181	VGC1	NZ_CP039448, NZ_CP039449, NZ_CP039450	High	Taiwan	H	Pneumonia and bacteremia	ST59		2013	I, O
182	WCH-SK2	NZ_CP031537	High	Australia	H	Pneumoniae, SSTI, Cystic fibrosis	ST239	CC8	2009	PB

183	WCUH29	NZ_CP039156	High	Poland	H	MRSA, Osteomyelitis	ST5	CC5	2004	PB
184	XQ	NZ_CP013137	High	China	H	Acute skin infections	ST121		2009	IT
185	Z172	NC_022604, NC_022610, NC_022605	High	Taiwan	H	VISA, Bacteremia	ST239	CC8	2010	I, PB
186	ZJ5499	NZ_CP011685	High	China	H	Pulmonary infection	ST5	CC5	2010	I
187	ZY05	NZ_CP045472, NZ_CP045473	High	China	H	Toxic shock syndrome	ST338		2016	PB

[a] Draft: draft genome, High: highly assembled genome

[b] B: bovine, H: human

[c] SG: shotgun sequencing, PB: PacBio, I: Illumina, R: Roche (454), SL: SOLiD, O: Oxford nanopore, IT: Ion Torrent

Appendix 2

Sample	Mastitis Association	Sample Type[a]	Mastitis Score	SCC (x 1,000 cells/mL)
Sa6	No	L1	0	214
Sa4	No	L1	0	886
Sa14	No	L1	0	77
Sa10	Yes	M1	3	missing value
Sa8	Yes	M1	2	missing value
Sa12	Yes	M1	3	missing value
Sa5	No	V1	missing value	4133
Sa11	No	T1	0	445
Sa9	No	L2	0	11
Sa22	Yes	M1	1	missing value
Sa21	No	T1	0	231
Sa19	Yes	M2	missing value	missing value
Sa1	No	L1	0	1008
Sa24	Yes	M1	2	missing value
Sa3	No	L2	1	3534
Sa23	Yes	M1	2	missing value
Sa25	Yes	M2	missing value	4571
Sa7	No	T1	0	126
Sa16	Yes	M1	2	missing value
Sa17	No	T1	å	8483
Sa18	Yes	M1	1	missing value
Sa27	Yes	M1	2	missing value
Sa28	Yes	M1	1	missing value
Sa29	Yes	M1	2	missing value
Sa30	Yes	M1	1	missing value
Sa31	Yes	M1	3	missing value
Sa2112	No	L1	0	5162
Sa2605	No	T2	0	-2
Sa2946	No	T1	0	499
Sa2954	No	V1	0	2611
Sa2970	No	T1	0	3134
Sa2978	No	L1	0	1452
Sa3151	No	T1	0	1616
Sa3247	Yes	M2	0	2706
Sa3302	No	T2	0	6038
Sa3336	No	L3	0	928
Sa3489	No	L1	0	11
Sa3532	No	L3	missing value	149
Sa3603	No	L3	0	1713
Sa3763	Yes	M1	1	missing value
Sa3511	Yes	M1	1	missing value

Sa2539	No	L1	0	398
Sa3370	No	L1	0	3525
Sa3003	No	L1	0	2620
Sa3014	Yes	M2	missing value	47
Sa3199	Yes	M1	2	missing value
Sa3131	No	T1	0	2188
Sa2117	No	L1	0	404
Sa2911	No	L1	0	489
Sa3454	Yes	M1	1	missing value
Sa3468	Yes	M1	1	missing value
Sa3493	No	L1	0	11
Sa3353	No	L1	0	1273
Sa3022	Yes	M1	2	missing value
Sa3137	No	T1	0	1577
Sa3129	No	T1	0	198
Sa2925	No	L1	0	972
Sa3071	No	L1	0	9
Sa3456	Yes	M1	missing value	7159
Sa1158c	No	L2	0	440
Sa3213	Yes	M1	2	missing value
Sa3154	Yes	M1	2	missing value
Sa3498	No	L1	0	missing value

[a] M1: day of diagnostic, M2: +14 days after diagnostic, L1-L3: during lactation, T1 and T2: before drying-off, V1: after calving

Appendix 3

Primers	Nucleotides Sequence (5'-3')	Target gene
blaZ-F	CAAAGATGATATAGTTGCTTATTCTCC	*blaZ*
blaZ_R	TGCTTGACCACTTTTATCAGC	*blaZ*
hsdM_F	ATGTCTATTACTGAAAAACAACG	*hsdM*
hsdM_R	TTACTCATCTTTCAACACCC	*hsdM*
CC97_hsdSb_F	ATAAGAGTGATAAATTTAACCCTC	*hsdS*
CC97_hsdSb_R	GCTGCAATTCAATTAGTTTTTCATTTC	*hsdS*
USA500_hsdSa_F	AGATAGAGTAATTAGGAAAAATAAAAAC	*hsdS*
USA500_hsdSa_R	TTTTTTAATTGTTTATATTTTAAGTTCC	*hsdS*

Appendix 4

VFclass	Virulence factors	Related genes
Adherence	Autolysin	*atl*
	Cell wall associated fibronectin binding protein	*ebh*

Enzyme	Clumping factor A	*clfA*
	Clumping factor B	*clfB*
	Elastin binding protein	*ebp*
	Fibrinogen binding protein	*efb*
	Fibronectin binding proteins	*fnbA*
		fnbB
	Intercellular adhesin	*icaA, icaB, icaC, icaD, icaR*
	Ser-Asp rich fibrinogen-binding proteins	*sdrC, sdrD, sdrE*
	Staphylococcal protein A	*spa*
	Cysteine protease	*sspB, sspC*
	Hyaluronate lyase	*hysA*
	Lipase	*geh, lip*
	Serine V8 protease	*sspA*
	Serine protease	*splA, splB, splC, splD, splE, splF*
	Staphylocoagulase	*coa*
	von Willebrand factor-binding protein	*vWbp-bov*
	Thermonuclease	*nuc*
Immune evasion	Capsule	*capA, cap8B, cap8C, cap8D, cap8E, cap8F, cap8G, cap8H, cap8I, cap8J, ca8K, cap8L, cap8M, capN, cap8O, cap8P, cap5H, cap5I, cap5J, cap5K*
	AdsA	*adsA*
	SCIN	*scn*
	Sbi	*sbi*
Secretion system	Type VII secretion system	*esxA, esaA, essA, esaB, essB, essC, esxC, esxB, essE, esxD, essD, esaG*
Toxin	Alpha hemolysin	*hly/hla*
	Beta hemolysin	*hlb*
	Delta hemolysin	*hld*
	Enterotoxin Z	*selz*
	Enterotoxin C bovine variant	*sec-bov*
	Enterotoxin G	*seg*
	Enterotoxin-like K	*selk*
	Enterotoxin-like L	*sell*
	Enterotoxin-like M	*selm*
	Enterotoxin-like N	*seln*
	Enterotoxin-like O	*selo*
	Enterotoxin-like U	*selu*
	Exfoliative toxin type A	*eta*
	Exotoxin	*set11, set16, set17, set18, set19, set20, set21, set22, set23, set24, set25, set26, set30, set34, set36*
	Gamma hemolysin	*hlgA, hlgB, hlgC*
	Leukotoxin M	*lukM*
	Leukotoxin F'	*lukF'*
	Leukotoxin D	*lukD*
	Leukotoxin E	*lukE*

	Toxic shock syndrome toxin	*tst*

Function	AMR factors	*Related genes*
Aminoglycosides resistance	AAC3	*aac3*
	AAC6-PRIME	*aac6-prime*
	APH3-PRIME	*aph3-prime*
MDR regulator	ARLR	*arlR*
	ARLS	*arlS*
Penicillin resistance	BLAI	*blaI*
	BLAR	*blaR*
	BLAZ	*blaZ*
Phenicol resistance	DHAP	*dhaP*
Fosfomycin resistance	FOSB	*fosB*
Biocide resistance	LMRS	*lmrS*
Lincosamide resistance	LNUA	*linA*
beta-lactam resistance	MECA	*mecA*
	MECR	*mecR*
Multi-drug resistance	MEPA	*mepA*
	MEPB	*mepB*
	MEPR	*mepR*
MDR regulator	MGRA	*mgrA*
Biocide resistance	NORA	*norA*
	NORB	*norB*
Biocide resistance	QACC	*qacC*
MLS resistance	RLMH	*rlmH*
Tetracycline resistance	TET38	*tet38*
	TETK	*tetK*
	TETM	*tetM*

Appendix 5 Bacteria used in screening antagonistic bacteria using the pQS-based methods and their growth-inhibiting (GI) and quorum-quenching (QQ) activity toward *S. aureus*

Test No.	Bacteria	Sa25 (CC97)	Sa27 (CC151)
1	*Lactococcus lactis* subsp. *lactis* ATCC 11454	GI	GI
3	*Staphylococcus epidermidis* ATCC 14990	QQ (variable)	
5	*Staphylococcus aureus* (Sa25)		GI
6	*Staphylococcus aureus* (Sa27)	QQ	
7	*Bacillus pumilus*	GI	GI
8	*Bacillus altitudinis*	GI	GI
9	*Bacillus paralicheniformis*		
10	*Bacillus licheniformis*		
11	*Bacillus subtilis*	GI	GI
12	*Staphylococcus pasteuri*	QQ	
13	*Staphylococcus hominis*	QQ	
14	*Staphylococcus saprophyticus*	QQ	

15	*Staphylococcus chromogenes*	QQ	QQ
16	*Staphylococcus vitulinus*		
17	*Staphylococcus haemolyticus*		
18	*Staphylococcus gallinarum*	QQ	QQ
19	*Staphylococcus cohnii*		
20	*Staphylococcus xylosus*		
21	*Staphylococcus hyicus*	QQ	
22	*Staphylococcus simulans*	QQ	QQ
23	*Staphylococcus epidermidis*		
24	*Staphylococcus sciuri*		
25	*Staphylococcus capitis*		
26	*Staphylococcus arlettae*	QQ	QQ
27	*Staphylococcus warneri*	QQ	
28	*Staphylococcus equorum*	QQ	QQ
29	*Staphylococcus succinus*	QQ	
30	*Staphylococcus hominis*		
31	*Staphylococcus devriesei*	QQ	QQ
32	*Aerococcus viridans*	GI	GI
33	*Rothia aerolata*		
34	*Corynebacterium frankenforstense*		
38	*Pantoea species*		
43	*Staphylococcus chromogenes*	QQ	QQ
44	*Staphylococcus caprae*	QQ	
45	*Staphylococcus saprophyticus*	QQ	QQ
46	*Staphylococcus haemolyticus*		
50	*Bacillus* species		
51	*Bacillus licheniformis*		
53	*Staphylococcus chromogenes*	QQ	QQ
54	*Staphylococcus chromogenes*	QQ	QQ
55	*Bacillus licheniformis*		
56	*Bacillus licheniformis*		
57	*Staphylococcus haemolyticus*		
58	*Staphylococcus chromogenes*	QQ	QQ
59	*Bacillus licheniformis*		
60	*Staphylococcus chromogenes*	QQ	QQ
61	*Staphylococcus saprophyticus*	QQ	
62	*Bacillus licheniformis*		
63	*Bacillus subtilis*	GI	GI
64	*Staphylococcus saprophyticus*	QQ	
65	*Bacillus* species	GI	GI
66	*Staphylococcus saprophyticus*	QQ	
67	*Staphylococcus saprophyticus*	QQ	
68	*Bacillus licheniformis*	QQ	
69	*Bacillus pumilus*	GI	GI
70	*Bacillus pumilus*	GI	GI
71	*Staphylococcus pasteuri*	QQ	

72	*Bacillus pumilus*	GI	GI
73	*Staphylococcus hominis*		
74	*Pantoea species*		
75	*Bacillus pumilus*	GI	GI
76	*Staphylococcus saprophyticus*	QQ	
77	*Bacillus pumilus*	GI	GI
78	*Bacillus species*		
79	*Staphylococcus chromogenes*	QQ	QQ
80	*Rothia aerolata*	QQ	
81	*Rothia aerolata*		
82	*Rothia aerolata*		
83	Unknown1	GI	GI
84	*Bacillus pumilus*	GI	GI
85	*Bacillus licheniformis*		
86	*Bacillus licheniformis*		
87	*Staphylococcus pasteuri*	QQ	
88	*Staphylococcus saprophyticus*	QQ	
89	*Staphylococcus saprophyticus*	QQ	
90	*Bacillus licheniformis*		
91	*Bacillus licheniformis*		
92	*Bacillus licheniformis*		
93	Unknown2	QQ	
94	*Staphylococcus vitulinus*		
95	*Bacillus licheniformis*		
96	*Bacillus licheniformis*		
97	Unknown3	QQ	
98	Unknown4		

Appendix 6 Metadata of all milk samples (n = 583) collected from the cows diagnosed with *S. aureus* clinical mastitis

Sample ID	Herd	Cow	Quarter	Collection Date (YYYY-MM-DD)	SCC (x 1,000)	DIM
191216C139Q2	5	139	2	2019-12-16	No data	171
191216C139Q3	5	139	3	2019-12-16	No data	171
191216C139Q4	5	139	4	2019-12-16	No data	171
200113C139Q1	5	139	1	2020-01-13	200	199
200113C139Q2	5	139	2	2020-01-13	57	199
200113C139Q3	5	139	3	2020-01-13	381	199
200113C139Q4	5	139	4	2020-01-13	556	199
191202C139Q1	5	139	1	2019-12-02	201	157
191202C139Q2	5	139	2	2019-12-02	44	157
191202C139Q3	5	139	3	2019-12-02	690	157
191202C139Q4	5	139	4	2019-12-02	758	157
191216C139Q1	5	139	1	2019-12-16	No data	171

190703C139Q1	5	139	1	2019-07-03	363	5
190703C139Q2	5	139	2	2019-07-03	2036	5
190703C139Q3	5	139	3	2019-07-03	106	5
190703C139Q4	5	139	4	2019-07-03	35548	5
190715C139Q1	5	139	1	2019-07-15	No data	17
190715C139Q2	5	139	2	2019-07-15	75	17
190715C139Q3	5	139	3	2019-07-15	210	17
190729C139Q1	5	139	1	2019-07-29	No data	31
190729C139Q2	5	139	2	2019-07-29	No data	31
190729C139Q3	5	139	3	2019-07-29	No data	31
190729C139Q4	5	139	4	2019-07-29	No data	31
190812C139Q1	5	139	1	2019-08-12	517	45
190812C139Q2	5	139	2	2019-08-12	38	45
200127C139Q1	5	139	1	2020-01-27	12	213
200127C139Q2	5	139	2	2020-01-27	5	213
200127C139Q3	5	139	3	2020-01-27	22	213
200127C139Q4	5	139	4	2020-01-27	51	213
190408C139Q1	5	139	1	2019-04-08	75	300
190408C139Q2	5	139	2	2019-04-08	32	300
190408C139Q3	5	139	3	2019-04-08	83	300
190424C139Q1	5	139	1	2019-04-24	178	316
190424C139Q2	5	139	2	2019-04-24	87	316
190424C139Q3	5	139	3	2019-04-24	210	316
190424C139Q4	5	139	4	2019-04-24	77	316
190812C139Q3	5	139	3	2019-08-12	376	45
190812C139Q4	5	139	4	2019-08-12	703	45
190826C139Q1	5	139	1	2019-08-26	696	59
190826C139Q3	5	139	3	2019-08-26	895	59
190826C139Q4	5	139	4	2019-08-26	571	59
190909C139Q1	5	139	1	2019-09-09	No data	73
190909C139Q3	5	139	3	2019-09-09	100	73
190909C139Q4	5	139	4	2019-09-09	191	73
190923C139Q1	5	139	1	2019-09-23	501	87
190923C139Q2	5	139	2	2019-09-23	84	87
190923C139Q3	5	139	3	2019-09-23	638	87
190923C139Q4	5	139	4	2019-09-23	611	87
191007C139Q1	5	139	1	2019-10-07	No data	101
191007C139Q2	5	139	2	2019-10-07	No data	101
191007C139Q3	5	139	3	2019-10-07	No data	101
191007C139Q4	5	139	4	2019-10-07	No data	101
191021C139Q1	5	139	1	2019-10-21	494	115
191021C139Q2	5	139	2	2019-10-21	158	115
191021C139Q3	5	139	3	2019-10-21	659	115
191021C139Q4	5	139	4	2019-10-21	718	115

191104C139Q1	5	139	1	2019-11-04	No data	129
191104C139Q3	5	139	3	2019-11-04	No data	129
191104C139Q4	5	139	4	2019-11-04	No data	129
191118C139Q1	5	139	1	2019-11-18	325	143
191118C139Q2	5	139	2	2019-11-18	50	143
191118C139Q3	5	139	3	2019-11-18	444	143
191118C139Q4	5	139	4	2019-11-18	462	143
200210C139Q2	5	139	2	2020-02-10	No data	227
200210C139Q3	5	139	3	2020-02-10	No data	227
200210C139Q4	5	139	4	2020-02-10	No data	227
200224C139Q1	5	139	1	2020-02-24	139	241
200224C139Q2	5	139	2	2020-02-24	16	241
200224C139Q3	5	139	3	2020-02-24	1111	241
190408C139Q4	5	139	4	2019-04-08	35	300
190826C139Q2	5	139	2	2019-08-26	107	59
190909C139Q2	5	139	2	2019-09-09	17	73
191104C139Q2	5	139	2	2019-11-04	No data	129
190211C163Q1	5	163	1	2019-02-11	No data	273
190211C163Q2	5	163	2	2019-02-11	No data	273
190211C163Q4	5	163	4	2019-02-11	No data	273
190225C163Q1	5	163	1	2019-02-25	No data	287
190225C163Q2	5	163	2	2019-02-25	No data	287
190225C163Q3	5	163	3	2019-02-25	No data	287
190225C163Q4	5	163	4	2019-02-25	No data	287
190522C163Q2	5	163	2	2019-05-22	718	14
190522C163Q4	5	163	4	2019-05-22	12107	14
190603C163Q2	5	163	2	2019-06-03	No data	26
190603C163Q4	5	163	4	2019-06-03	No data	26
190617C163Q1	5	163	1	2019-06-17	8	40
190617C163Q2	5	163	2	2019-06-17	41	40
190617C163Q3	5	163	3	2019-06-17	9	40
190617C163Q4	5	163	4	2019-06-17	No data	40
190703C163Q2	5	163	2	2019-07-03	6163	56
190703C163Q3	5	163	3	2019-07-03	52	56
190703C163Q4	5	163	4	2019-07-03	No data	56
190715C163Q1	5	163	1	2019-07-15	50	68
190715C163Q2	5	163	2	2019-07-15	1066	68
190715C163Q4	5	163	4	2019-07-15	No data	68
190729C163Q2	5	163	2	2019-07-29	No data	82
190729C163Q3	5	163	3	2019-07-29	No data	82
190729C163Q4	5	163	4	2019-07-29	No data	82
190211C163Q3	5	163	3	2019-02-11	No data	273
190522C163Q1	5	163	1	2019-05-22	31	14
190522C163Q3	5	163	3	2019-05-22	35	14

190603C163Q1	5	163	1	2019-06-03	No data	26
190603C163Q3	5	163	3	2019-06-03	No data	26
190703C163Q1	5	163	1	2019-07-03	49	56
190715C163Q3	5	163	3	2019-07-15	20	68
190729C163Q1	5	163	1	2019-07-29	No data	82
190408C184Q1	5	184	1	2019-04-08	93	301
190408C184Q2	5	184	2	2019-04-08	No data	301
190408C184Q3	5	184	3	2019-04-08	166	301
190408C184Q4	5	184	4	2019-04-08	No data	301
190424C184Q1	5	184	1	2019-04-24	198	317
190424C184Q2	5	184	2	2019-04-24	No data	317
190424C184Q3	5	184	3	2019-04-24	434	317
190424C184Q4	5	184	4	2019-04-24	No data	317
190703C184Q2	5	184	2	2019-07-03	No data	3
190703C184Q3	5	184	3	2019-07-03	330	3
190715C184Q3	5	184	3	2019-07-15	22	15
190715C184Q4	5	184	4	2019-07-15	35891	15
190729C184Q1	5	184	1	2019-07-29	No data	29
190729C184Q2	5	184	2	2019-07-29	No data	29
190729C184Q3	5	184	3	2019-07-29	No data	29
190812C184Q1	5	184	1	2019-08-12	17	43
190812C184Q2	5	184	2	2019-08-12	No data	43
190812C184Q3	5	184	3	2019-08-12	46	43
190826C184Q1	5	184	1	2019-08-26	57	57
190826C184Q2	5	184	2	2019-08-26	No data	57
190826C184Q3	5	184	3	2019-08-26	21	57
190715C184Q2	5	184	2	2019-07-15	No data	15
190909C184Q1	5	184	1	2019-09-09	47	71
190909C184Q3	5	184	3	2019-09-09	48	71
190909C184Q4	5	184	4	2019-09-09	No data	71
190923C184Q1	5	184	1	2019-09-23	25	85
190923C184Q3	5	184	3	2019-09-23	9	85
190923C184Q4	5	184	4	2019-09-23	No data	85
191007C184Q1	5	184	1	2019-10-07	No data	99
191007C184Q2	5	184	2	2019-10-07	No data	99
191007C184Q3	5	184	3	2019-10-07	No data	99
191007C184Q4	5	184	4	2019-10-07	No data	99
191021C184Q1	5	184	1	2019-10-21	69	113
191021C184Q2	5	184	2	2019-10-21	No data	113
191021C184Q3	5	184	3	2019-10-21	18	113
191021C184Q4	5	184	4	2019-10-21	No data	113
191104C184Q1	5	184	1	2019-11-04	No data	127
191104C184Q2	5	184	2	2019-11-04	No data	127
191104C184Q3	5	184	3	2019-11-04	No data	127

191104C184Q4	5	184	4	2019-11-04	No data	127
191118C184Q1	5	184	1	2019-11-18	95	141
191118C184Q2	5	184	2	2019-11-18	No data	141
191118C184Q3	5	184	3	2019-11-18	49	141
191118C184Q4	5	184	4	2019-11-18	No data	141
191202C184Q1	5	184	1	2019-12-02	17	155
191202C184Q3	5	184	3	2019-12-02	20	155
191202C184Q4	5	184	4	2019-12-02	No data	155
191216C184Q1	5	184	1	2019-12-16	No data	169
191216C184Q3	5	184	3	2019-12-16	No data	169
191216C184Q4	5	184	4	2019-12-16	No data	169
200113C184Q1	5	184	1	2020-01-13	35	197
200113C184Q3	5	184	3	2020-01-13	28	197
200113C184Q4	5	184	4	2020-01-13	No data	197
200127C184Q1	5	184	1	2020-01-27	11	211
200127C184Q2	5	184	2	2020-01-27	No data	211
200127C184Q3	5	184	3	2020-01-27	7	211
200127C184Q4	5	184	4	2020-01-27	No data	211
200210C184Q1	5	184	1	2020-02-10	6	225
200210C184Q2	5	184	2	2020-02-10	No data	225
200210C184Q3	5	184	3	2020-02-10	4	225
200210C184Q4	5	184	4	2020-02-10	No data	225
191202C184Q2	5	184	2	2019-12-02	No data	155
191216C184Q2	5	184	2	2019-12-16	No data	169
200113C184Q2	5	184	2	2020-01-13	No data	197
190703C74Q1	5	74	1	2019-07-03	99	280
190703C74Q2	5	74	2	2019-07-03	No data	280
190703C74Q3	5	74	3	2019-07-03	2086	280
190703C74Q4	5	74	4	2019-07-03	913	280
190715C74Q1	5	74	1	2019-07-15	197	292
190715C74Q2	5	74	2	2019-07-15	No data	292
190715C74Q3	5	74	3	2019-07-15	1298	292
190715C74Q4	5	74	4	2019-07-15	129	292
190923C74Q1	5	74	1	2019-09-23	143	13
190923C74Q2	5	74	2	2019-09-23	2170	13
190923C74Q4	5	74	4	2019-09-23	32	13
191007C74Q1	5	74	1	2019-10-07	No data	27
191007C74Q2	5	74	2	2019-10-07	No data	27
191007C74Q3	5	74	3	2019-10-07	No data	27
191007C74Q4	5	74	4	2019-10-07	No data	27
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191105C42Q4	2	42	4	2019-11-05	No data	224
190129C120Q1	1	120	1	2019-01-29	158	259
190129C120Q2	1	120	2	2019-01-29	108	259
190129C120Q3	1	120	3	2019-01-29	179	259
190129C120Q4	1	120	4	2019-01-29	191	259
190212C120Q1	1	120	1	2019-02-12	No data	273
190212C120Q2	1	120	2	2019-02-12	No data	273
190212C120Q3	1	120	3	2019-02-12	No data	273
190212C120Q4	1	120	4	2019-02-12	No data	273
190507C120Q1	1	120	1	2019-05-07	29	7
190507C120Q2	1	120	2	2019-05-07	734	7
190507C120Q3	1	120	3	2019-05-07	427	7
190507C120Q4	1	120	4	2019-05-07	115	7
190521C120Q2	1	120	2	2019-05-21	1273	21
190521C120Q3	1	120	3	2019-05-21	134	21
190521C120Q4	1	120	4	2019-05-21	13	21
190604C120Q1	1	120	1	2019-06-04	14	35
190604C120Q2	1	120	2	2019-06-04	4989	35
190604C120Q3	1	120	3	2019-06-04	185	35
190604C120Q4	1	120	4	2019-06-04	13	35
190618C120Q1	1	120	1	2019-06-18	No data	49
190618C120Q2	1	120	2	2019-06-18	No data	49
190618C120Q3	1	120	3	2019-06-18	No data	49
190618C120Q4	1	120	4	2019-06-18	No data	49
190702C120Q1	1	120	1	2019-07-02	16	63
190702C120Q2	1	120	2	2019-07-02	4493	63
190702C120Q3	1	120	3	2019-07-02	48	63
190702C120Q4	1	120	4	2019-07-02	27	63
190716C120Q1	1	120	1	2019-07-16	107	77
190716C120Q2	1	120	2	2019-07-16	5960	77
190716C120Q3	1	120	3	2019-07-16	136	77
190716C120Q4	1	120	4	2019-07-16	627	77
190730C120Q1	1	120	1	2019-07-30	No data	91
190730C120Q2	1	120	2	2019-07-30	No data	91

190730C120Q3	1	120	3	2019-07-30	No data	91
190730C120Q4	1	120	4	2019-07-30	1054	91
190813C120Q1	1	120	1	2019-08-13	16	105
190813C120Q2	1	120	2	2019-08-13	4872	105
190813C120Q3	1	120	3	2019-08-13	64	105
190813C120Q4	1	120	4	2019-08-13	290	105
190827C120Q1	1	120	1	2019-08-27	5	119
190827C120Q2	1	120	2	2019-08-27	289	119
190827C120Q3	1	120	3	2019-08-27	230	119
190827C120Q4	1	120	4	2019-08-27	433	119
190910C120Q1	1	120	1	2019-09-10	17	133
190910C120Q2	1	120	2	2019-09-10	2495	133
190910C120Q3	1	120	3	2019-09-10	45	133
190910C120Q4	1	120	4	2019-09-10	66	133
190924C120Q1	1	120	1	2019-09-24	43	147
190924C120Q2	1	120	2	2019-09-24	93	147
190924C120Q3	1	120	3	2019-09-24	114	147
190924C120Q4	1	120	4	2019-09-24	157	147
191008C120Q1	1	120	1	2019-10-08	No data	161
191008C120Q2	1	120	2	2019-10-08	No data	161
191008C120Q3	1	120	3	2019-10-08	No data	161
191008C120Q4	1	120	4	2019-10-08	No data	161
191022C120Q1	1	120	1	2019-10-22	77	175
191022C120Q2	1	120	2	2019-10-22	249	175
191022C120Q3	1	120	3	2019-10-22	2041	175
191022C120Q4	1	120	4	2019-10-22	629	175
191119C120Q1	1	120	1	2019-11-19	282	203
191119C120Q2	1	120	2	2019-11-19	No data	203
191119C120Q3	1	120	3	2019-11-19	No data	203
191119C120Q4	1	120	4	2019-11-19	427	203
190507C42Q2	2	42	2	2019-05-07	16	42
190618C42Q4	2	42	4	2019-06-18	No data	84
190715C184Q1	5	184	1	2019-07-15	69	15
190415C419Q1	4	419	1	2019-04-15	28	50
190805C419Q1	4	419	1	2019-08-05	15	162
190903C419Q1	4	419	1	2019-09-03	26	191
190409C7Q4	2	7	4	2019-04-09	57	35
190521C7Q4	2	7	4	2019-05-21	56	77
190423C42Q2	2	42	2	2019-04-23	75	28
190521C42Q2	2	42	2	2019-05-21	37	56
190521C42Q4	2	42	4	2019-05-21	18	56
190702C42Q4	2	42	4	2019-07-02	12	98
190521C120Q1	1	120	1	2019-05-21	11	21

Appendix 7 Microbial changes before, during, and after S. aureus clinical mastitis in Group I and Group II

	Group	Index	Week	Statistic	p-value
Mann-Whitney test	Group I	chao1	Week-6	3	0.7
			Week-4	6	0.7
			Week-2	23	0.4848485
			Week0	40	0.0530303
			Week2	12	0.3428571
		shannon	Week-6	5	1
			Week-4	6	0.7
			Week-2	32	0.025974026
			Week0	47	0.002331002
			Week2	13	0.2
		simpson	Week-6	7	0.4
			Week-4	6	0.7
			Week-2	28	0.132034632
			Week0	47	0.002331002
			Week2	12	0.342857143
	Group II	chao1	Week-6	5	1
			Week-4	4	1
			Week-2	5	1
			Week0	1	0.2
			Week2	1	0.6666667
		shannon	Week-6	4	1
			Week-4	3	0.7
			Week-2	9	0.1
			Week0	2	0.4
			Week2	2	1
		simpson	Week-6	3	0.7
			Week-4	3	0.7
			Week-2	9	0.1
			Week0	2	0.4
			Week2	2	1

	Group	Index	Week	F	p-value
PERMANOVA	Group I	Bray-Curtis	Week-6	0.8040262	0.7
			Week-4	1.1446422	0.3
			Week-2	2.3223155	0.036
			Week0	7.076521	0.001

			Week2	0.63088558	0.776	
			Week-6	0.8533165	0.9	
			Week-4	0.9156798	0.8	
	Group II	Bray-Curtis	Week-2	0.6890413	0.9	
			Week0	0.9960621	0.5	
			Week2	0.5952046	1	

	Group	Week	OTU	LDA	p-value	Genus
LEfSe	Group I	Week-2	Otu0031	-3.68395	0.0201042	Paracoccus
			Otu0043	-3.52303	0.025986	Rikenellaceae_RC9_gut_group
			Otu0097	-3.59557	0.0222352	Lachnospiraceae_unclassified
			Otu0109	-3.40605	0.0322932	Pseudomonas
		Week0	Otu0001	5.57875	0.0017257	Staphylococcus
			Otu0002	-4.47119	0.0344454	Aerococcus
			Otu0003	-4.14313	0.0247064	UCG-005
			Otu0009	-3.92332	0.00774698	Ruminococcaceae_unclassified
			Otu0011	-3.82692	0.0185344	Bifidobacterium
			Otu0015	-3.87708	0.00782032	Bacteroides
			Otu0024	-3.57011	0.019402	Lachnospiraceae_NK3A20_group
			Otu0030	-3.69768	0.00779795	Christensenellaceae_R-7_group
			Otu0042	-3.74164	0.0402087	Rikenellaceae_RC9_gut_group
			Otu0048	-3.57596	0.0250689	Aerococcus
			Otu0058	-3.66517	0.00916163	Rikenellaceae_RC9_gut_group
			Otu0124	-3.41739	0.0250689	Treponema
		Week2	Otu0009	-4.16958	0.0433167	Ruminococcaceae_unclassified
			Otu0071	-3.44034	0.0472209	uncultured
			Otu0078	-3.79497	0.0472209	Fervidobacterium
			Otu0269	-3.22266	0.0472209	Firmicutes_unclassified
			Otu0396	-3.27798	0.0472209	UCG-010_ge
	Group II	Week-6	Otu0013	-4.07553	0.0463056	Psychrobacter
			Otu0061	4.09669	0.0495388	Turicibacter
		Week-4	Otu0008	-4.37133	0.0369074	Escherichia-Shigella
			Otu0016	4.00976	0.0495388	Intrasporangiaceae_unclassified
			Otu0039	3.77495	0.0369074	Aerococcaceae_unclassified
		Week-2	Otu0049	-3.90676	0.0463056	Ruminococcus
			Otu0140	3.86917	0.0369074	Parasutterella
		Week0	Otu0001	-5.27807	0.0495388	Staphylococcus
			Otu0023	3.67466	0.0463056	Lachnospiraceae_unclassified

Otu0033	3.98651	0.0463056	Ornithinimicrobium
Otu0040	3.14597	0.0369074	Acinetobacter
Otu0055	3.6125	0.0463056	Alistipes
Otu0066	3.3347	0.0369074	Monoglobus
Otu0127	3.22297	0.0369074	Prevotellaceae_UCG-001

Appendix 8 Etiological agents isolated from clinical mastitic milk samples

Sample	Reason	Severity	Phenotype	Species 1	CFU (Species 1)	Species 2	CFU (Species 2)
191119C120Q2	Clinical mastitis	1	Contaminated	*Staphylococcus aureus*	1		
191008C120Q3	Clinical mastitis	1	Pure	*Escherichia coli*	10		
190423C7Q1	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190521C7Q1	Clinical mastitis	1	Mixed	*Staphylococcus aureus*	10	*Staphylococcus aureus*	10
191105C42Q1	Clinical mastitis	2	Pure	*Staphylococcus aureus*	10		
190716C42Q1	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190910C42Q1	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190924C42Q1	Clinical mastitis	2	Pure	*Staphylococcus aureus*	10		
191008C42Q1	Clinical mastitis	2	Pure	*Staphylococcus aureus*	10		
191022C42Q1	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190709C2Q1	Clinical mastitis	2	Pure	*Staphylococcus aureus*	10		
190722C88Q2	Clinical mastitis	1	Pure	No identification possible	5		
190903C88Q2	Clinical mastitis	1	Pure	*Staphylococcus spp*	1		
190805C88Q2	Clinical mastitis	1	No growth				
191015C88Q2	Clinical mastitis	1	Pure	*Staphylococcus spp*	10		
190930C88Q2	Clinical mastitis	2	Pure	*Staphylococcus epidermidis*	7		
190916C88Q2	Clinical mastitis	1	Pure	*Escherichia coli*	8		
190708C88Q2	Clinical mastitis	2	Pure	*Corynebacterium camporealensis*	1		
190626C88Q2	Clinical mastitis	1	Contaminated		1		
190527C88Q2	Clinical mastitis	2	Pure	*Staphylococcus aureus*	10		
190819C88Q2	Clinical mastitis	2	Pure	*Corynebacterium spp*	2		
191028C88Q2	Clinical mastitis	2	Mixed	*Aerococcus spp*	4	*Staphylococcus epidermidis*	5
191015C88Q3	Clinical mastitis	1	Pure	*Klebsiella pneumoniae*	1		
190805C419Q4	Clinical mastitis	1	Mixed	*Staphylococcus aureus*	1	*Corynebacterium bovis*	4
190722C419Q4	Clinical mastitis	1	Pure	*Corynebacterium bovis*	10		
191015C419Q4	Clinical mastitis	1	Contaminated		1		
191111C419Q4	Clinical mastitis	1	Pure	*Aerococcus viridans*	1		
191015C419Q2	Clinical mastitis	1	No growth				
191028C419Q2	Clinical mastitis	1	Mixed	*Corynebacterium spp*	2	*Aerococcus viridans*	4
191202C74Q2	Clinical mastitis	1	Mixed	*Staphylococcus aureus*	10	No identification possible	1
191216C74Q2	Clinical mastitis	1	Mixed	*Staphylococcus aureus*	10	*Staphylococcus aureus*	10
200127C74Q2	Clinical mastitis	2	Mixed	*Staphylococcus aureus*	10	*Staphylococcus aureus*	10

200210C74Q2	Clinical mastitis	2	Contaminated	*Staphylococcus aureus*	1	*Staphylococcus spp*	
200224C74Q2	Clinical mastitis	1	Mixed	*Staphylococcus aureus*	10	*Staphylococcus aureus*	1
200113C74Q2	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190715C74Q2	Clinical mastitis	2	Mixed	*Staphylococcus aureus*	10	*Streptococcus dysgalactiae*	10
190703C74Q2	Clinical mastitis	1	Mixed	*Streptococcus dysgalactiae*	10	*Staphylococcus aureus*	4
191216C139Q1	Clinical mastitis	1	No growth				
190715C139Q1	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190703C163Q4	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190715C163Q4	Clinical mastitis	2	Pure	*Staphylococcus aureus*	10		
190603C163Q4	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190617C163Q4	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190729C163Q4	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
191202C184Q2	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
191104C184Q2	Clinical mastitis	2	Mixed	*Aerococcus viridans*	4	No identification possible	6
191118C184Q2	Clinical mastitis	2	Mixed	*Staphylococcus sciuri*	3	*Aerococcus viridans*	10
200210C184Q2	Clinical mastitis	2	Mixed	*Staphylococcus aureus*	10	*Staphylococcus aureus*	8
200127C184Q2	Clinical mastitis	2	Mixed	*Staphylococcus aureus*	10	*Staphylococcus aureus*	10
200113C184Q2	Clinical mastitis	2	Pure	*Staphylococcus aureus*	10		
191216C184Q2	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190715C184Q2	Clinical mastitis	2	Pure	*Staphylococcus aureus*	10		
190909C184Q2	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
190923C184Q2	Clinical mastitis	1	Pure	*Staphylococcus aureus*	10		
191021C184Q2	Clinical mastitis	1	No growth	No identification possible	0	No identification possible	
191007C184Q2	Clinical mastitis	2	Pure	*Staphylococcus aureus*	10		
190812C184Q2	Clinical mastitis	2	No growth				
190826C184Q2	Clinical mastitis	1	No growth				
191202C184Q4	Clinical mastitis	1	Pure	*Staphylococcus epidermidis*	1		
191118C184Q4	Clinical mastitis	2	Mixed	*Aerococcus viridans*	10	*Staphylococcus aureus*	10
191104C184Q4	Clinical mastitis	2	Mixed	*Staphylococcus epidermidis*	3	*Corynebacterium amycolatum*	1
200210C184Q4	Clinical mastitis	2	Mixed	No identification possible	1	*Staphylococcus haemolyticus*	1
191216C184Q4	Clinical mastitis	1	No growth				
200127C184Q4	Clinical mastitis	2	Contaminated	*Staphylococcus equorum*	1		
200113C184Q4	Clinical mastitis	2	Mixed	*Aerococcus viridans*	1	*Corynebacterium spp*	10
190909C184Q4	Clinical mastitis	1	Mixed	*Streptococcus dysgalactiae*	10	*Streptococcus dysgalactiae*	10
190923C184Q4	Clinical mastitis	1	No growth				
191007C184Q4	Clinical mastitis	2	No growth				
191021C184Q4	Clinical mastitis	1	Contaminated				
190729C184Q4	Clinical mastitis	1	Pure	*Streptococcus dysgalactiae*	10		
190812C184Q4	Clinical mastitis	2	Pure	*Streptococcus dysgalactiae*	10		
190826C184Q4	Clinical mastitis	1	Pure	*Streptococcus dysgalactiae*	10		

Appendix 9 Microbial network analysis in milk samples (n = 583)

Target	Source	Spearman rho	Spearman p	Pseudo R2 env only	Pseudo R2 w bio	bio beta	bio SE	anova dev	anova p
Staphylococcus	Aerococcus	-0.3351	4.96E-17	0.640649	0.658545	-0.00013	3.52E-05	103999.3	1.16E-06
Staphylococcus	UCG-005	-0.4044	9.75E-25	0.640649	0.74661	-0.00044	3.49E-05	615778.2	7.16E-48
Staphylococcus	Acinetobacter	-0.1192	0.003643	0.640649	0.669021	-0.00049	0.000102	164878.6	2.34E-08
Staphylococcus	Ruminococcaceae_unclassified	-0.1718	2.60E-05	0.640649	0.683501	-0.00097	0.000142	249030	1.43E-14
Staphylococcus	Bacteroides	-0.3073	1.97E-14	0.640649	0.708172	-0.00105	0.000126	392397.9	2.21E-21
Staphylococcus	Rikenellaceae_RC9_gut_group	-0.298	1.27E-13	0.640649	0.727435	-0.00081	7.31E-05	504342.5	1.10E-36
Staphylococcus	Lachnospiraceae_unclassified	-0.4526	2.76E-31	0.640649	0.736372	-0.00079	8.68E-05	556279.3	9.36E-25
Staphylococcus	Lachnospiraceae_NK3A20_group	-0.2329	9.49E-09	0.640649	0.675087	-0.00134	0.000223	200133.7	5.23E-12
Staphylococcus	Christensenellaceae_R-7_group	-0.3101	1.11E-14	0.640649	0.722646	-0.00109	0.000109	476513	2.21E-31
Staphylococcus	Oscillospirales_ge	-0.281	3.19E-12	0.640649	0.71258	-0.00167	0.000184	418016.6	1.10E-26
Staphylococcus	UCG-010_ge	-0.2571	2.09E-10	0.640649	0.725831	-0.00091	8.78E-05	495025.1	2.97E-33
Aerococcus	Staphylococcus	-0.3351	4.96E-17	0.633152	0.657143	-7.17E-05	1.64E-05	59487.94	2.31E-08
UCG-005	Staphylococcus	-0.4044	9.75E-25	0.36234	0.502168	-5.96E-05	6.01E-06	106002.4	3.20E-36
UCG-005	Ruminococcaceae_unclassified	0.4655	3.16E-33	0.36234	0.392389	0.00029	5.44E-05	22779.68	1.88E-07
UCG-005	Bacteroides	0.6384	3.31E-69	0.36234	0.442524	0.000394	4.34E-05	60787.16	1.10E-18
UCG-005	Rikenellaceae_RC9_gut_group	0.6418	3.86E-70	0.36234	0.465846	0.000312	2.99E-05	78467.4	3.07E-24
UCG-005	Lachnospiraceae_unclassified	0.6431	1.61E-70	0.36234	0.433218	0.000224	2.65E-05	53732.26	1.54E-16
UCG-005	Christensenellaceae_R-7_group	0.5876	2.41E-56	0.36234	0.413619	0.00032	4.68E-05	38873.94	5.27E-12
UCG-005	Oscillospirales_ge	0.5997	3.51E-59	0.36234	0.44149	0.000517	5.58E-05	60002.66	1.71E-18
UCG-005	UCG-010_ge	0.618	9.76E-64	0.36234	0.45312	0.000301	3.01E-05	68819.46	2.45E-21
Acinetobacter	Staphylococcus	-0.1192	0.003643	0.433808	0.488558	-5.57E-05	1.07E-05	27118.76	2.28E-10
Ruminococcaceae_unclassified	Staphylococcus	-0.1718	2.60E-05	0.345429	0.405227	-4.81E-05	8.41E-06	16448.08	1.36E-11
Ruminococcaceae_unclassified	UCG-005	0.4655	3.16E-33	0.345429	0.408463	0.000187	2.86E-05	17337.99	5.83E-11
Ruminococcaceae_unclassified	Bacteroides	0.39	5.61E-23	0.345429	0.357223	0.000205	7.06E-05	3244.087	0.004548
Ruminococcaceae_unclassified	Rikenellaceae_RC9_gut_group	0.4435	5.78E-30	0.345429	0.361395	0.00018	5.33E-05	4391.6	0.000938
Ruminococcaceae_unclassified	Lachnospiraceae_unclassified	0.4391	2.40E-29	0.345429	0.373547	0.000183	4.07E-05	7734.289	1.04E-05
Ruminococcaceae_unclassified	Lachnospiraceae_NK3A20_group	0.4491	9.05E-31	0.345429	0.384941	0.000472	8.23E-05	10868.08	6.73E-08

Ruminococcaceae_unclassified	Christensenellaceae_R-7_group	0.5267	1.23E-43	0.345429	0.416205	0.000465	6.62E-05	19467.74	1.04E-12
Ruminococcaceae_unclassified	Oscillospirales_ge	0.4125	9.23E-26	0.345429	0.362625	0.000366	0.000103	4729.878	0.000656
Bacteroides	Staphylococcus	-0.3073	1.97E-14	0.330425	0.413859	-5.43E-05	7.82E-06	24551.19	9.99E-18
Bacteroides	UCG-005	0.6384	3.31E-69	0.330425	0.450136	0.000226	2.24E-05	35226.11	2.83E-24
Bacteroides	Rikenellaceae_RC9_gut_group	0.5696	2.65E-52	0.330425	0.426236	0.000344	3.67E-05	28193.28	7.68E-20
Bacteroides	Lachnospiraceae_unclassified	0.5389	5.59E-46	0.330425	0.420626	0.000296	3.26E-05	26542.52	4.09E-19
Bacteroides	Christensenellaceae_R-7_group	0.4705	5.43E-34	0.330425	0.348104	0.000219	5.87E-05	5202.205	0.000194
Bacteroides	Oscillospirales_ge	0.5274	9.30E-44	0.330425	0.381404	0.000466	6.76E-05	15000.99	9.81E-11
Bacteroides	UCG-010_ge	0.5239	4.18E-43	0.330425	0.388397	0.000277	3.76E-05	17058.82	4.12E-12
Rikenellaceae_RC9_gut_group	Staphylococcus	-0.298	1.27E-13	0.301775	0.422289	-5.96E-05	6.97E-06	47510.08	3.33E-26
Rikenellaceae_RC9_gut_group	UCG-005	0.6418	3.86E-70	0.301775	0.444751	0.000235	2.10E-05	56365.33	1.10E-29
Rikenellaceae_RC9_gut_group	Bacteroides	0.5696	2.65E-52	0.301775	0.394127	0.000468	5.14E-05	36407.98	6.09E-19
Rikenellaceae_RC9_gut_group	Lachnospiraceae_unclassified	0.5534	7.02E-49	0.301775	0.370934	0.00025	3.21E-05	27264.39	2.50E-14
Rikenellaceae_RC9_gut_group	Christensenellaceae_R-7_group	0.5406	2.61E-46	0.301775	0.342005	0.000314	5.58E-05	15859.87	1.49E-08
Rikenellaceae_RC9_gut_group	Oscillospirales_ge	0.5042	1.46E-39	0.301775	0.391163	0.000587	6.37E-05	35239.49	1.34E-18
Rikenellaceae_RC9_gut_group	UCG-010_ge	0.6323	1.59E-67	0.301775	0.443448	0.000403	3.37E-05	55851.64	1.03E-31
Lachnospiraceae_unclassified	Staphylococcus	-0.4526	2.76E-31	0.365226	0.476669	-5.42E-05	6.34E-06	51478.67	6.73E-26
Lachnospiraceae_unclassified	UCG-005	0.6431	1.61E-70	0.365226	0.441924	0.000156	1.93E-05	35428.98	7.78E-16
Lachnospiraceae_unclassified	Ruminococcaceae_unclassified	0.4391	2.40E-29	0.365226	0.381999	0.000226	5.95E-05	7748.07	0.000194
Lachnospiraceae_unclassified	Bacteroides	0.5389	5.59E-46	0.365226	0.437045	0.000392	4.82E-05	33175.26	3.03E-15
Lachnospiraceae_unclassified	Rikenellaceae_RC9_gut_group	0.5534	7.02E-49	0.365226	0.425023	0.000255	3.48E-05	27621.93	1.23E-12
Lachnospiraceae_unclassified	Christensenellaceae_R-7_group	0.5241	3.81E-43	0.365226	0.388896	0.000226	5.11E-05	10933.9	9.88E-06
Lachnospiraceae_unclassified	Oscillospirales_ge	0.489	5.56E-37	0.365226	0.40919	0.000393	6.03E-05	20308	9.58E-10
Lachnospiraceae_unclassified	UCG-010_ge	0.4747	1.19E-34	0.365226	0.402103	0.000204	3.46E-05	17034.54	2.62E-08
Lachnospiraceae_NK3A20_group	Staphylococcus	-0.2329	9.49E-09	0.357945	0.407172	-5.68E-05	1.10E-05	10953.37	7.86E-10
Lachnospiraceae_NK3A20_group	Ruminococcaceae_unclassified	0.4491	9.05E-31	0.357945	0.396719	0.000423	7.81E-05	8627.554	8.85E-08
Lachnospiraceae_NK3A20_group	Rikenellaceae_RC9_gut_group	0.353	7.67E-19	0.357945	0.371744	0.000193	6.07E-05	3070.438	0.001896
Lachnospiraceae_NK3A20_group	Christensenellaceae_R-7_group	0.4336	1.40E-28	0.357945	0.409338	0.000456	7.45E-05	11435.35	5.83E-10
Christensenellaceae_R-7_group	Staphylococcus	-0.3101	1.11E-14	0.364986	0.478821	-6.42E-05	7.71E-06	34439.33	1.05E-25
Christensenellaceae_R-7_group	UCG-005	0.5876	2.41E-56	0.364986	0.454216	0.000187	2.16E-05	26995.5	3.19E-18
Christensenellaceae_R-7_group	Ruminococcaceae_unclassified	0.5267	1.23E-43	0.364986	0.425381	0.000417	5.68E-05	18271.83	8.36E-13

Christensenellaceae_R-7_group	Bacteroides	0.4705	5.43E-34	0.364986	0.388584	0.000245	5.50E-05	7139.395	1.36E-05
Christensenellaceae_R-7_group	Rikenellaceae_RC9_gut_group	0.5406	2.61E-46	0.364986	0.419125	0.000259	3.78E-05	16379.01	2.45E-11
Christensenellaceae_R-7_group	Lachnospiraceae_unclassified	0.5241	3.81E-43	0.364986	0.401431	0.000178	3.21E-05	11025.82	5.14E-08
Christensenellaceae_R-7_group	Lachnospiraceae_NK3A20_group	0.4336	1.40E-28	0.364986	0.403637	0.000399	6.67E-05	11693.4	1.42E-08
Christensenellaceae_R-7_group	Oscillospirales_ge	0.4949	5.83E-38	0.364986	0.414773	0.000448	6.52E-05	15062.36	1.05E-10
Christensenellaceae_R-7_group	UCG-010_ge	0.4705	5.40E-34	0.364986	0.393177	0.000203	4.07E-05	8528.804	1.99E-06
Oscillospirales_ge	Staphylococcus	-0.281	3.19E-12	0.305514	0.411905	-6.42E-05	1.07E-05	21259.74	1.97E-14
Oscillospirales_ge	UCG-005	0.5997	3.51E-59	0.305514	0.424482	0.000227	3.36E-05	23772.75	1.12E-11
Oscillospirales_ge	Bacteroides	0.5274	9.30E-44	0.305514	0.366075	0.000414	6.79E-05	12101.62	3.00E-09
Oscillospirales_ge	Rikenellaceae_RC9_gut_group	0.5042	1.46E-39	0.305514	0.405077	0.000354	4.32E-05	19895.26	1.34E-15
Oscillospirales_ge	Lachnospiraceae_unclassified	0.489	5.56E-37	0.305514	0.365753	0.000243	4.07E-05	12037.33	5.35E-09
Oscillospirales_ge	Christensenellaceae_R-7_group	0.4949	5.83E-38	0.305514	0.358759	0.000395	6.57E-05	10639.64	1.75E-09
Oscillospirales_ge	UCG-010_ge	0.4731	2.08E-34	0.305514	0.41721	0.000349	3.81E-05	22319.71	3.52E-18
UCG-010_ge	Staphylococcus	-0.2571	2.09E-10	0.231007	0.370001	-6.68E-05	8.10E-06	48670.63	6.24E-26
UCG-010_ge	UCG-005	0.618	9.76E-64	0.231007	0.364431	0.000234	2.39E-05	46720.39	4.75E-23
UCG-010_ge	Bacteroides	0.5239	4.18E-43	0.231007	0.289835	0.000396	6.09E-05	20599.29	2.33E-10
UCG-010_ge	Rikenellaceae_RC9_gut_group	0.6323	1.59E-67	0.231007	0.381741	0.000418	3.77E-05	52781.75	1.14E-27
UCG-010_ge	Lachnospiraceae_unclassified	0.4747	1.19E-34	0.231007	0.27586	0.000211	3.73E-05	15705.85	3.24E-08
UCG-010_ge	Christensenellaceae_R-7_group	0.4705	5.40E-34	0.231007	0.24809	0.000219	6.58E-05	5981.72	0.000889
UCG-010_ge	Oscillospirales_ge	0.4731	2.08E-34	0.231007	0.338897	0.000609	6.50E-05	37779.2	3.79E-19