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Developing a Non-aureus Staphylococcus Intramammary Probiotic as a Preventative Measure for Bovine Mastitis

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Developing a Non-aureus Staphylococcus Intramammary Probiotic as a Preventative Measure for Bovine Mastitis

by

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Abstract

Bovine mastitis is the most common and economically important disease affecting the dairy industry. Intramammary infection (IMI) with *Staphylococcus aureus* is the leading cause of contagious mastitis. Interestingly, non-*aureus* staphylococci (NAS) are frequently found in cows with subclinical mastitis, but with a severity less than with *S. aureus.* Antibiotics are the main method for preventing and treating mastitis. Misuse and overuse of antibiotics have resulted in the emergence of antimicrobial-resistant bacteria, and thus, alternative treatments are required. Bacteriocins, antimicrobial peptides produced by bacteria, are a promising alternative. We hypothesized that by creating a NAS probiotic through genetically engineering a bacteriocin gene cluster into its genome, it will be able to inhibit *S. aureus* and prevent mastitis. To achieve this, we needed to find a persistent and non-inflammatory NAS strain that can colonize cow mammary glands by using an experimental mammary infusion model. After finding a persistent and non-inflammatory NAS, we will perform a bacteriocin gene cluster knock-in using allelic replacement. Finally, the probiotic will then be characterized through gene expression and killing assays. This thesis aimed to create an alternative treatment to prevent the growth of mastitis pathogens during the dry period. Ultimately, this will lower the usage of antibiotics and give us another preventative tool against mastitis.

We identified *S. warneri* 2993 as the most persistent and non-inflammatory NAS but unfortunately, we were not able to perform our bacteriocin gene knock-in. Instead, we recommend future studies to re-attempt this gene knock-in but with a different bacteriocin gene cluster for an increased likelihood of success. The probiotic will then need testing in *in vivo* IMI experiments in mice and then in cows following the protocols we have designed.

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List of Abbreviations

ABR Antibiotic Resistance

AdoMet S-adenosylmethionine

AMR Antimicrobial Resistance

ATc Anhydrotetracycline

BCP 1-bromo-3-chloropropane

BDCT Blanket Dry Cow Therapy

BHI Brain and Heart Infusion

BTSCC Bulk Tank Somatic Cell Count

crRNA CRISPR RNA

FDF Final Dilution Factor

GMO Genetically Modified Organism

IMI Intramammary Infection

LAB Lactic Acid Bacteria

MRSA Methicillin-resistant *Staphylococcus aureus*

NAS Non-*aureus* staphylococci

NRT No Reverse Transcriptase

PCPA Para-chlorophenylalanine

PDF Plating Dilution Factor

PMN Polymorphonucleocytes

RM Restriction Modification

RFP Red Fluorescent Protein

RT-qPCR Reverse Transcription Quantitative Polymerase Chain Reaction

SCC Somatic Cell Count

SDCT Selective Dry Cow Treatment

TCDM Total Cost of Mastitis around the Dry Cow Period

TSB Tryptic Soy Broth

TSDF Total Series Dilution Factor

tracrRNA Trans-activating RNA

VF Virulence Factor

VSACC Veterinary Services Animal Care Committee

Introduction

Udder health is a constant concern for dairy farmers due to numerous deleterious impacts of intramammary infections (IMI) on milk quality and quantity. Mastitis is the most common and economically important disease affecting the Canadian dairy industry with an estimated loss of $600 million per year[2,3]. This is due to a substantial loss in milk production, treatment costs, culling, changes in product quality, and risk of other disease[2,3]. Nickerson and Ryman (2019) state there are currently three methods for dealing with cows that have mastitis: spontaneous recovery, culling, and antibiotic treatment[4]. These methods are typically used in combination, and they are not an independent approach to be used by itself. Spontaneous cure occurs when the cow's immune system engages and eliminates the infection without the use of antibiotics. This occurs in only 20% of confirmed infections for mild or recently acquired infections with coliform bacteria[5], but very rarely in well-established or chronic infections with *Staphylococcus aureus*. Relying solely on spontaneous recovery to treat mastitis is inefficient and can be an animal welfare issue. The second method is culling which is only useful in eliminating the infection if the cow does not respond to repeated attempts of therapy or the treatment costs outweigh the pros. When treatment costs are projected to be greater than the value of the milk produced by the cow, veterinary fees and drug costs, and cost of labor, culling would be the more economical option rather than treatment. The third and most effective method is treating subclinical or clinical mastitis with antibiotics. Antibiotics such as ceftiofur, penicillins, cephalosporins, and novobiocin are used to treat mastitis, and it is estimated that 80% of all antibiotics used in dairy cattle are for prevention or treatment of mastitis[6]. Fortunately, for well-managed mastitis treatment, there is little evidence that supports increased antimicrobial resistance (AMR) due to the treatment[7]. In contrast, careless overuse of antibiotics is what is responsible for the increased reports of AMR[7]. It is important to

note that the route of antibiotic administration matters in whether AMR arise[7]. There is a positive association between systemic use of antibiotic and AMR while there is no association between intramammary or intrauterine usage and AMR[7]. Overall, one of the most effective method of controlling mastitis is through preventative measures.

Blanket Dry Cow Therapy

Blanket dry cow therapy (BDCT) has been a prominent preventative measure for mastitis over the years[8]. This management practice works by applying a broad-spectrum antibiotic intramammarily to all cows in a herd at the end of their lactation. As it is a blanket treatment, all cows will receive the treatment regardless of they have had a previous history of mastitis or are infected or not. This practice helps to increase the elimination of existing IMIs in the dry period and prevent the occurrence of new IMIs. The use of BDCT has been effective in reducing the prevalence of contagious mastitis pathogens and also reducing bulk tank somatic cell count (BTSCC)[9,10]. By reducing the risk of the contagious pathogens, this ultimately lowers the overall incidence of mastitis in the herd and can increase milk production by reducing the negative impact of subclinical and clinical mastitis on milk yield. Thus, as BDCT is such an effective measure, a majority of farmers have adapted this into their mastitis control program. Unfortunately, using blanket antibiotic treatment comes with several risks. The first risk is the overuse and misuse of antibiotics may lead to antimicrobial resistant bacteria[11,12]. This proves detrimental as they may cause infections that are difficult or not possible to treat with commonly used antibiotics, leading to more intensive and costly treatments affecting not only the infected cow, but other cows, farmers, and the entire ecosystem if an outbreak occurs. The second risk comes from the effect the broad-spectrum antibiotic has on the cow's udder microbiome. A cow's microbiome is composed many different beneficial genera such as *Staphylococcus* and *Lactobacillus,* and a broad-spectrum antibiotic may affect the ideal proportions of each bacterium. For this reason, some Nordic

countries, Canada, and other countries have switched to selective dry cow therapy (SDCT) as an alternative to BDCT[13-15].

Selective Dry Cow Therapy

SDCT as opposed to BDCT refers to the selective treatment of cows with broad-spectrum antibiotic during the dry period. The goal is SDCT is to selectively treat cows that are at a higher risk of developing mastitis, rather than a blanket antibiotic treatment to all cows. This would include cows that have a previous history of clinical mastitis, a high somatic cell count, or are suspected to become infected. This will alleviate the risks associated with BDCT such as the emergence of antibiotic resistant bacteria or the broad-spectrum effects on the udder microbiota. While some producers may not particularly embrace the idea of SDCT as it could lower the milk production and increase SCC, research shows that this is not the case. Studies have demonstrated that SDCT does not negatively affect milk production and milk quality in comparison to BDCT[13,16]. Furthermore, SCDT proves as efficient as BDCT for curing existing IMIs at dry off and preventing the occurrence of new IMIs in low SCC herds (BTSCC under 250 000 cells/ml)[17]. Another additional benefit while negligible is the lower economical cost of SCDT in comparison to BDCT. In a study analyzing the data from the Dairy Herd Improvement Association and individual dairy herds in California, they demonstrated that SDCT was cheaper than BDCT for cows with a medium SCC[18]. The total cost of mastitis around the dry period (TCDM) for BDCT was $54.7 per primiparous dry cow and $58.5 per multiparous dry cow annually. Contrastingly, for SDCT, the TCDM was $52.4 per primiparous dry cow and $58.2 per multiparous dry cow[18]. Overall, SDCT is a promising alternative to BDCT and should be seriously considered to alleviate the risks with BDCT.

Prevention of Mastitis without Antibiotics

Some of the most effective measures to prevent IMI are improved sanitation and clean hygiene

during the dry period and initiation of milking, which have also been associated with a lower incidence of clinical mastitis[6]. Proper sanitation includes appropriate bedding and clean surfaces for the cow, such as sand compared to straw, sawdust, or compost, as it supports less bacterial growth[6]. Good pre-milking udder hygiene can drastically reduce the amount of teat skin bacteria compared to no preparation or wiping with a dry towel[19]. By sanitizing the udders with a pre-milking disinfectant, wash sanitizer, and manual drying with paper towels, there is an 85% reduction in teat skin bacteria compared to no preparation[19]. Similarly, combining pre-milking hygiene with a post-milking teat disinfectant further reduces the risk of bacterial infections[20].

Internal teat sealants are another alternative that mimics the natural keratin plug that forms in the teat canal at the beginning of the dry period[6]. By closing the entrance to the teat canal, bacteria are prevented from ascending into the udder and causing IMI[21]. When comparing internal teat sealants to untreated cows, it reduces the risk of IMI in the dry period by 73%[21].

The use of antagonistic metabolites produced by bacteria can be used to target mastitis-causing pathogens. Researchers have suggested using *Lactobacillus perolens* as a probiotic to prevent mastitis[22,23]. Frola *et al*. (2011) suggested using *L. perolens* CRL1724 as an intramammary probiotic to prevent mastitis[23]. *L. perolens* CRL1724 produces two antagonistic metabolites: lactic acid and hydrogen peroxide which was able to inhibit 12/14 mastitis-causing pathogens *in-vitro*. To test which concentration of *L. perolens* CRL1724 can be added into the mammary gland without any significant inflammation, 1 mL of 10^3, 10^6, and 10^9 cfu/mL was infused into an individual quarter. 10^3 and 10^6 cfu/mL resulted in no physical inflammation of the teat or visible changes to the milk compared to the non-treated quarter. Conversely, the milk in the 10^9 cfu/mL infusion had clots and lumps which disappeared 48 h after the initial infusion[23]. Another antagonistic metabolite that has the potential to target mastitis-causing pathogens are bacteriocins.

Bacteriocin Classification

The classification of bacteriocins has been altered many times over the past decades from the Klaenhammer classification scheme to the Cotter scheme[24]. Currently, the most popular classification is the universal scheme of bacteriocin classification which is a combination of the two previous schemes[24]. Bacteriocins are divided into classes I, II, III, and IV which reflect the structural and chemical similarities and differences of the molecules[24]. Class I, also known as lantibiotics, are small peptides that are smaller than 5 kDa[25]. This group has unusual amino acid residues such as lanthionine and methyl lanthionine that result from post-translational modifications[25]. Lantibiotics can be further subdivided into subclass A and subclass B which are amphipathic peptides that are elongated and flexible or smaller and globular, respectively[26,27]. Class II bacteriocins are thermostable and generally amphiphilic peptides that are smaller than 10 kDa without any post-translational modifications[25]. Most of the class II bacteriocins are active through membrane permeabilization and leaking cytoplasmic molecules through the target's membrane[25]. This antimicrobial activity is presumed to be mediated by specific receptors on the membrane, allowing for more potent activity than non-specific antimicrobial peptides[25]. Class III bacteriocins are large heat-labile peptides and are larger than 10 kDa[25,28]. Their mode of action is different from other bacteriocins as they act through lysis of sensitive cells by catalyzing cell-wall hydrolysis[28]. Lastly, class IV bacteriocins are cyclic peptides[24]. Their circular structure is hypothesized to provide strong stability towards high pH and temperatures[29]. Class IV bacteriocins are presumed to act in a non-specific manner where their antimicrobial activity comes from high bacteriocin concentrations[29]. However, at low bacteriocin concentrations, they can likely act specifically on membrane receptors as well[29]. Once attached to the membrane, pores are formed leading to leakage of cytoplasmic molecules and ions[29].

Bacteriocin Mechanisms of Action

Bacteriocins are ribosomally synthesized and post-translationally modified peptides produced by either Gram-positive or Gram-negative bacteria that are bactericidal or bacteriostatic against closely related bacteria [30,31]. Bacteriocins are antimicrobial peptides that typically do not harm the producing bacterium themselves as they have specific immunity genes [28,30]. These peptides have varying modes of action categorized into different classes of bacteriocin [30]. Class I bacteriocins such as nisin work by having a high affinity to lipid II, a cell wall precursor, and uses it to dock. After dockage, large pores are formed in the cell membrane which prevents proper peptidoglycan synthesis[32-34]. Lipid II is needed to transport peptidoglycan subunits from the cytoplasm to the cell wall. However, once docked by nisin, it prevents correct cell wall synthesis and ultimately results in cell death. Nisin has another function of inserting itself into the membrane causing depolarization of the membrane and subsequently, creates pores that also lead to cell death[32-34]. It is important to note that the precise mechanisms for all the different class I bacteriocins have not been identified and may vary from nisin. For example, lacticin 3147 has this dual activity but it is distributed across two peptides[35,36], or mersacidin which only has the lipid II binding activity[37]. Class II bacteriocins mechanism of action works by inducing membrane permeabilization resulting in a more leaky membrane[38]. This subsequently causes leakage of molecules from the membrane affecting the proton motive force across the membrane[30]. Ultimately, this kills the bacterium due to the pore formation. Lastly, bacteriolysins (class III bacteriocins) has a mode of action that is distinct from class I and class II bacteriocins. Their mode of action is through lysis of cells by catalysing cell-wall hydrolysis instead of interacting with the cell membrane[39-41].

Innate Bacteriocin Immunity

An important feature, as mentioned earlier, is that these bacteriocin-producing pathogens

generally all contain the cognate immunity genes. These genes protect the bacterium from the antimicrobial properties of the bacteriocin and there are several mechanisms in which they work. Class I and II bacteriocins can be protected against through two different systems separately or together. The first system involves having an immunity protein whose mechanism is hypothesized to antagonistically compete for a receptor used by the bacteriocin, thus preventing pore formation[42]. For example, the immunity protein that confers protection for *Lactococcus lactis* against its own bacteriocin is through the protein's interaction with a putative receptor for lactococcin A[42]. This ultimately prevents lactococcin from creating a pore allowing for immunity. The immunity protein depending on the species can also confer immunity through target shielding by accumulating near the membrane-cell wall interface, thus, preventing the bacteriocin from creating pores[43]. The second system involves pumping the bacteriocin out of the producer's membrane through a specialized ABC-transporter system, thus, allowing for resistance[44,45]. Overall, the innate bacteriocin resistance likely works through either sequestering the bacteriocins into the environment or by outcompeting the bacteriocin for a receptor. It is important to mention that these immunity mechanisms are highly specific and may not protect against other bacteriocins.

Bacteriocin Resistance

Sometimes, bacteria such as can gain resistance to class II bacteriocins through alterations to the cell wall in terms of membrane charge and fluidity, cell wall thickness, cell wall charge, or a combination of these factors[46-48]. This type of increased resistance can be seen in *Listeria monocytogenes* as it has increased membrane fluidity by containing shorter, unsaturated acyl chains[49]. Furthermore, highly resistant strains of *L. monocytogenes* had a higher alanine content in teichoic acid which is used for their cell wall surface components, resulting in a more positive cell wall charge contributing to more bacteriocin resistance[44]. Another contributing factor to *L. monocytogenes'*

resistance comes from having reduced expression of the mannose permase of the phosphotransferase system[50]. The membrane-located complex that forms as a result of this phosphotransferase system is used as a bacteriocin receptor[51]. Interestingly, some bacteria such as *Streptococcus pneumoniae*, can develop a higher MIC from 0.4 to 6.4 mg/L following serial exposures to class II bacteriocins[52]. However, the mechanism of which *S. pneumoniae* developed this bacteriocin resistance is not known.

Genetic editing inside Staphylococcus

Genetic editing in *Staphylococcus* species involves modifying its DNA to alter its behavior and characteristics. This can be especially useful when developing new treatments or vaccines as the bacterium is made less virulent through gene knock-outs. Furthermore, it could be applied to creating, genetically modified organisms (GMOs) for agriculture, medical applications such as producing antibiotics, increase our basic research on understanding bacterial mechanisms, or into a probiotic through gene knock-ins. There are several methods for genetic editing *Staphylococcus* such as relying on transformation and allelic replacement, CRISPR-Cas9 and homology-directed repair, or transposon mutagenesis to introduce random mutations.

Transformation is the process of introducing exogenous DNA into the bacterium using a plasmid. Then, the DNA of interest is integrated into the bacterium's genome randomly because of the upstream and downstream homology to the recipient's genome of the exogenous DNA. This process is called allelic replacement and is the gold standard for gene knock-in techniques for *Staphylococcus.* This process occurs at a low frequency as it requires a double cross-over for proper integration of the gene of interest and removal of the plasmid. However, by manipulating the non-permissive temperature of the plasmid with use of an antibiotic selection marker, the first cross-over resulting in plasmid integration can be selected for. Then, the second cross-over is selected for by growing at a permissive temperature. Ultimately after the second cross-over, there are two possibilities that can form. The first is reversion to

wild-type and the second is the mutant containing our gene knock-in or gene knock-out. Creating a gene knock-in or knock-out follows a similar premise of having upstream and downstream homology to the recipient strain with the main difference being what is in-between the homology. For a gene knock-in, the gene-of-interest would be in-between the homology regions. While for a gene knockout, the upstream and downstream homology would be designed in a way that it would remove areas of the target gene resulting in loss-of-function. Additionally, loss-of-function can also be attained by adding in an insert that causes a frameshift mutation. For example, Bae and Schneewind, performed a dehydrogenase, *rocA,* knock-out in *S. aureus* using the pKOR1 plasmid[53]. This plasmid relies on the traditional double cross-over recombination events for its genetic knock-out but has antibiotic markers for selection, and a counter-selection marker, anti-sense *secY,* for curing[53]. The counter-selection marker works by inhibiting *secY*, a gene necessary for bacterial growth. *SecY* is needed for protein translocation across the bacterial inner membrane as it encodes for a subunit part of the SecYEG translocon complex[54]. This complex spans the bacterial inner membrane and helps with protein translocation[54] Essentially, if the transformant still has the plasmid, then the anti-sense *secY* will inhibit its growth to show smaller colonies compared to transformants that have lost the plasmid and are larger. This makes isolating the desired mutants easier as the recombination event occurs at a low frequency and eliminates the need for blind screening. In this case, when Dr. Bae picked 10 suspected mutants based on the larger colony size from the counter-selection plates; 80% of the mutants carried the *rocA* deletion[53]. This high frequency is possible due to permissive and non-permissive plasmid temperature manipulation. Furthermore, plasmid curing occurred at a 100% frequency because of the anti-sense *secY* counterselection method on the plasmid. Essentially, if the mutant contains the plasmid, then anti-sense *secY* will be produced, inhibiting the growth of the mutant. Thus, a size difference will be seen on plates that induce the production of anti-sense *secY.* Similarly, Schuster and colleagues performed a magnesium transporter gene, *mgtE,* knock-out in *S. aureus* using the piMAY* plasmid[55]. This plasmid

follows the same premise for the double cross-over recombination events as pKOR1 except for two differences. Instead of antisense *secY,* the plasmid contains *pheS*,* which allows for the incorporation of toxic phenylalanine amino acid analogue *para*-chlorophenylalanine (PCPA) into the mutant[55]. This ultimately causes a growth reduction in mutants to show a size difference between mutants that contain or lost the plasmid[55]. Secondly, it contains a different replication origin, pWV01ts, instead of the typical pE194ts in pKOR1[55]. This was to increase the temperature sensitivity so plasmid loss can occur at a lower non-permissive temperature (37 vs 43°C). This trend of replacing the replication origin or the counterselection methods adds to the large repertoire of methods for gene manipulation and curing[56,57]. It is also important to mention that despite the following methods being gene knock-outs, a gene knock-in can be performed similarly as the gene of interest will be flanked by the homology regions in the plasmid instead.

CRISPR-Cas9 is another powerful tool that can be used to edit DNA. This complex was identified in *E. coli* in 1987, but it was not until 2007 that it was discovered as a defense mechanism used by bacteria against viral invaders[58]. Successful cleavage of foreign DNAs in the system requires three components: crRNA (CRISPR RNA), tracrRNA (trans-activating RNA), and Cas9 protein. The crRNA acts as the specific target for the DNA locus, as it is the spacer in the CRISPR array[59]. The tracrRNA, a short RNA, works with the crRNA to form a complex and bring in the Cas9 protein for editing[59]. Doudna and Charpentier quickly realized that they could convert this bacterial defense mechanism into a novel genetic manipulation tool[59]. Chen and colleagues followed a similar approach as they adapted the *Streptococcus pyogenes* CRISPR-cas9 system to genetically modify *S. aureus*' genome[60]. By customizing the spacer to any region in *S. aureus*'s genome that is adjacent to a PAM sequence and providing homology and the gene of interest in the plasmid, it allows for specific genome editing[60]. The gene integration occurs after the double-strand DNA cleavage is repaired through homology-directed repair instead of the regular allelic replacement such as pKOR1, piMAY*, etc[60]. This proof-of-concept is

demonstrated through their *agrA* gene knock-out, alongside an *rfp* gene knock-in[60]. Their choice of the *agrA* gene knockout was to show a phenotypic change, specifically the loss of hemolytic ability in the mutant. In the same fashion, Liu and colleagues did a gene knock-out and knock-in of the *tgt* gene using CRISPR-Cas9 in *S. aureus*[61]. The main difference was that they tested different promoters to drive the expression of *cas9* to break the DNA more effectively. Interestingly, Penewit and colleagues approached the genetic editing of *S .aureus* differently. Instead of relying on the rare homologous recombination, they engineered a two-vector system that allows for conditional recombineering of single-stranded oligonucleotide and CRISPR-Cas9 as the counterselection method[62]. This contrasted the previous method by Bae and Schneewind where the oligonucleotide was part of the CRISPR-Cas9 plasmid. As part of their method, *S. aureus* was successively transformed with a CRISPR-Cas9 recombination vector to produce a conditionally recombinogenic strain[62]. Next, recombination was carried out with the addition of single-stranded oligonucleotides while using a CRISPR/Cas9-mediated counterselection vector, which resulted in the elimination of cells that are not altered[62]. This recombineering is possible because the modified *S. aureus* strain contained an endogenous bacteriophage recombinase, allowing for DNA modifications. Furthermore, successful recombineering of the oligonucleotide will confer rifampin resistance and immunity to Cas9-mediated counterselection[62]. Indeed, there are many ways to utilize and optimize CRISPR-Cas9 system to genetically modify DNA and as time progresses, the efficiency of gene integration will increase, and the time span of the experiments will shorten. However, a disadvantage of CRISPR-Cas9 is the large size being around 10Kb due to all of its components needed for genomic engineering. This larger plasmid size would lower transformation efficiency. However, this problem can be slightly alleviated by using different suitable Cas9s from different generas as they can differ in size.

Transposon mutagenesis is used to randomly insert mutations into the genome of bacteria to understand the role of specific genes. This works by having a transposable element delivered by one or

two plasmids into the bacterium. Transposons, commonly referred to as "jumping genes," can transfer from one region of the genome to another. Depending on the new location the transposon moved to, this may result in a loss-of-function mutation showing the function of a gene. For example, Bae and colleagues used a mariner-based transposon for random mutagenesis in *S. aureus*[63]. In short, the mariner-based transposon plasmid and the pBursa plasmid used for promoter tagging was transformed into *S. aureus.* This ultimately resulted in the creation of 10 325 *S. aureus variants* and 71 virulence genes being detected[63].

Genetic Manipulation in Staphylococcus

Genetic manipulation in *S. aureus* is difficult for a multitude of reasons. First and foremost, this genus has evolved defenses against foreign DNA, including restriction enzymes and restriction modification (RM) systems that break down foreign DNA based on methylation patterns[64-66]. The second reason is due to its thick Gram-positive cell wall, making the introduction of foreign DNA challenging.

S. aureus contains three types of RM systems (I, II, and IV) to protect itself well from foreign DNA, making genetic manipulation difficult[64-66]. The type III RM system is extremely rare in *S.* aureus and thus, will not be covered[67]. The type I RM system is a multifunctional enzyme that does both restriction and modifications[68]. The methylation status of the target sequence affects the type I RM enzyme's activity as an endonuclease or a methyltransferase after it binds to that region[68]. With unmethylated DNA, the type 1 RM system will bidirectionally translocate the DNA towards itself in an ATP-dependent manner and it is believed that through this translocation, collisions with enzymes occur resulting in DNA cleavage[68,69]. However, methylation on specific bases on the bacterial DNA will protect it from cleavage. DNA cleavage occurs at variable positions that are 100-10000s bp outside of the type I RM recognition sequences[70,71]. Cleavage requires several cofactors such as ATP, S-adenosylmethionine (AdoMet), and Mg^{2+} ions[68,72]. This system is composed of three subunits encoded by three genes: *hsdR, hsdM, and*

hsdS. Interestingly, these are acronyms that stand for "host specificity of DNA" and the R, M, and S represent their role of restriction, modification, and specificity[68]. To dive deeper into each subunit's role, hsdM and hsdS are needed for methyltransferase activity[68]. Methylation activity requires Mg^{2+} and AdoMet[68,73]. Two target recognition domains are found in hsdS that give the complex specificity in restriction and modifications of target sequences[68]. Finally, HsdR contains sequences necessary for DNA translocation and endonuclease activity, alongside having an active site for ATP hydrolysis. In contrast to type I, type II systems are generally homodimeric or homotetrameric and are either restriction enzymes or methyltransferase enzymes[74]. When Mg^{2+} is present, type II restriction enzymes can cleave DNA located within or near the recognition site, which typically consists of short palindromic sequences containing 4-8 bp[75]. Type II methyltransferases only need AdoMet as a donor for methyl groups so it can carry out modifications of cytosines or adenines[73]. The modifications of the cytosines occur at the N4 or C5 position, while it occurs at N6 in adenine[73]. Interestingly, these type II restriction enzymes are commonly used in recombinant DNA technology to manipulate DNA fragments of interest. Finally, the type IV RM system is composed of modification-dependent restriction enzymes, meaning they only cleave recognition sites that have been modified through methylation, hydroxymethylation, or glucosylhydroxymethylation[76]. To be more specific, this cleavage can only occur at the N4 or C5 position of cytosines or the N6 position of adenines[67]. The cofactors needed for this cleavage are similar to type I and II in that Mg^{2+} is needed but with addition of GTP[73].

S. aureus is a Gram-positive bacterium meaning it has a thick peptidoglycan cell wall. This wall can act as a barrier making the introduction of foreign DNA more difficult than gram-negative bacteria such as *E. coli.* Contrastingly, Gram-negative bacteria have a thinner peptidoglycan layer and an outer membrane containing specific proteins making the introduction of DNA easier. Transformation with a larger plasmid is more inefficient than with a smaller plasmid[77]. Chan et al. transformed *E. coli* with three different plasmid sizes ranging from 2.7 and 4.3kb to 16.1kb[77]. When plating 100 uL of the

transformants, there was a decreasing number of transformants as the plasmid size increased and the 16.1kb plasmid resulted in no transformants even when varying ranges of DNA were used[77].

Causes of Mastitis

Bacterial IMI is the primary cause of bovine mastitis. Bacteria that infect the mammary gland are classified into two major categories: contagious or environmental pathogens. Contagious pathogens include *S. aureus,* NAS, *Streptococcus agalactiae, Mycoplasma* species[78]. Environmental pathogens include *E. coli*, *Klebsiella* species, and some *Streptococcus* species[79]. These pathogens are typically found in bedding, soil, walkways, or any surface where the cow and their manure come into contact. One of the primary routes of infection is through the teat canal which allows for a direct entry into the mammary glands. This sheds light on the importance of having an effective pre and post sanitation routine during milking as the teats are exposed to a plethora of potential sources of infection such as the milking machine and manure.

Non-aureus Staphylococci

NAS are considered to be of lesser importance in dairy production compared to *S. aureus* in terms of the severity of the IMI [80]. Interestingly, they are frequently found in cows with subclinical mastitis [80]. The effect NAS has on udder health remains a topic of debate. Some authors consider NAS to be the main cause of subclinical and persistent mastitis milk with an increase in somatic cell count (SCC)[81,82]. On the opposite end, others suggest that NAS could have a protective effect against the mastitis-causing pathogen like *S. aureus.* Carson et al. (2017) demonstrated this in a study testing the inhibitory capability of 441 bovine NAS isolates against bovine *S. aureus* and human methicillin-resistant *S. aureus* (MRSA)[83]. Forty isolates from the 441 NAS isolates like *Staphylococcus capitis, Staphylococcus chromogenes, Staphylococcus epidermis, Staphylococcus pasteuri, Staphylococcus simulans,* and

Staphylococcus xylosus inhibited the two pathogens due to their ability to produce antimicrobial proteins called bacteriocins. Interestingly, in some NAS infected cows compared to NAS-negative cows, there was a slight increase in milk yield[81,84]. Multiparous cows and first parity heifers that developed clinical mastitis, produced more milk compared to uninfected controls before symptoms appeared[84,85]. However, the reasoning behind it is still unknown[84]. This is different from infections with major pathogens such as *S. aureus* or *S. agalactiae* where there was a significant loss in milk production[81]. This discrepancy in NAS' impact may be because over 25 NAS species have been isolated that caused bovine IMI. These species have varying numbers of virulence factors in a complex interplay with each other. It is important to note that there no clear link between VF and mastitis has been demonstrated. And the role NAS has on mastitis is multifactorial in that it depends on the NAS, host immunity, and environmental factors. Condas et al. (2017) determined the prevalence and distribution of NAS in milk samples from Canadian cows with IMI[80]. For example, *S. simulans, S. xylosus,* and *S. epidermidis* had a higher proportional prevalence in cows with a SCC greater than 250,000 cells/mL compared to cows with a SCC lower than 150,000 cells/mL. *Staphylococcus* species that had a lower prevalence in high SCC compared to low SCC cows are *Staphylococcus equorum, Staphylococcus devriesei, and Staphylococcus caprae.* It is important to note that these findings were derived from a specific collection of milk isolates from Canada and thus, different isolates collected from cows elsewhere may show varying results. Furthermore, Condas et al. (2017) assessed how the NAS distribution changes depending on the month of lactation in the cow and the cow's parity (number of calf births)[80]. *S. chromogenes* was the most prevalent from month 1 to the end of the lactation cycle. However, there is variation in the second, third, and fourth most prevalent NAS species as the month progresses between *S. simulans, S. xylosus*, and *Staphylococcus haemolyticus*. Interestingly, this distinct distribution of NAS species is also seen when compared to the cow's parity. *S. chromogenes* was the most prevalent NAS in parity 1, 2, 3, 4, and above 5 with the other NAS species varying in prevalence[27].

Using NAS as a Probiotic

Use of a probiotic instead of direct bacteriocin application to the cow's udder quarters can bring several benefits. These benefits are associated with induction of protective immune responses and persistence of the probiotic in the mammary grands which is mediated by functional properties of the probiotic strain such as adhesion to the epithelium, co-aggregation, biofilm production and inhibition, competition for nutrients, colonization, and production of antagonistic metabolites[23]. By adhering to the epithelium, the probiotic strain not only competes with pathogens for host cell-binding sites but can promote temporary colonization[86]. Colonization is important for a probiotic as it extends its benefits especially if it produces antagonistic metabolites. The longer the probiotic persists, the longer the protective effect lasts. During physical activities such as milking, if the probiotic can adhere to the epithelial cells, it can prevent the probiotic from being washed out. Another aspect of adhesion is the attachment to different bacterial species which is called co-aggregation[87]. Co-aggregation is presumed to be important for probiotics producing antagonistic metabolites like bacteriocins. By being in close proximity the antimicrobial concentration produced will be higher and thus, be more effective at inhibiting the pathogen. Factors such as electrostatic and hydrophobic interactions, proteins such as adhesins, target species, and incubation condition play a role in whether the probiotic is adhesive to the surface of interest[87-89]. Furthermore, colonization can allow for competition of nutrients that are consumed by the pathogens[90]. However, this can also be disadvantageous if the probiotic competes with the beneficial microbiota. Some NAS such as *S. chromogenes, S. haemolyticus, S. xylosus,* or *S. epidermidis* have the ability to produce biofilms[91,92]. Biofilms are communities of bacteria that adhere to the surface and form a protective layer of extracellular matrix. This matrix can help bacteria persist as it is protected from external stresses like antimicrobials, host immune defenses, and also physical disturbances. It is one of *S. aureus'* characteristics that makes it such a persistent pathogen to control.

Interestingly, another benefit of using NAS is that as weak biofilm producers, they can negatively affect the biofilm forming activity of other bacteria[93]. This could prove to be a synergistic interaction especially if the probiotic NAS produces antimicrobials, allowing the antimicrobials easier access to the weakened biofilm. Lastly, using a probiotic can induce an immune response in the cow that could be used as a potential protective mechanism. There has been several studies where after infusion of *Lactobacillus*¸ it induced a strong immune response as seen by a large spike in SCC[23,94,95]. This immune response in combination with the probiotic can potentially act synergistically to clear any mastitis pathogens currently inside the mammary glands. However, this does assume that the probiotic itself is not cleared out by the cow's immune response so a dose-dependent study is needed to find the optimal dose for persistence and immune response.

Phylogeny

Determining the phylogeny is useful to understand patterns and differences between closely related species, alongside discovering shared ancestry between species[96,97]. Comparing the genomics of different species can explain why certain morphological or chemical characteristics evolved because of the species' similarities or differences[97]. Naushad et al. (2016) created a well-resolved NAS phylogenetic tree based on whole-genome sequencing and comparison of several genes like *rpoB* (β-subunit of RNA polymerase), *tuf* (elongation factor Tu), hsp60 (heat shock protein 60), *dnaJ* (heat shock protein 40), and 16S rRNA[96]. Five distinct monophyletic clades were branched out for the 441 NAS isolates tested. It is important to note that this phylogenetic tree does not include all *Staphylococcus* species but only the species from the 441 NAS isolates tested. Clade A is considered the most ancient divergence of the NAS group and is composed of *Staphylococcus sciuri, Staphylococcus fleurettii,* and *Staphylococcus vitulinus*. Clade B consisted of *S. chromogenes, Staphylococcus hyicus,* and *Staphylococcus agnetis*. The next lineage of NAS to diverge was clade C, which only has one single species, *S. simulans.* Clade D contained

eight species and can be subdivided into three clusters. Cluster D1 was composed of *S. devriesei* and *S. haemolyticus* diverging from a common ancestor with *S. hominis* being the basal lineage of the cluster. Cluster D2 consisted of two NAS species, *S. pasteuri* and *Staphylococcus warneri*. Cluster D3 showed *S. caprae* and *S. capitis* diverging from a common ancestor with *S. epidermidis* appearing as the basal lineage of the cluster. The last clade, E, is the biggest with 10 species and is the most recently diverged clade. It is comprised of *S. xylosus, Staphylococcus auricularis, Staphylococcus saprophyticus, Staphylococcus kloosii, S. equorum, Staphylococcus arlettae, Staphylococcus cohnii, Staphylococcus gallinarum, Staphylococcus nepalensis,* and *Staphylococcus succinus.* However, a recently published paper suggested for the reclassification of five species, specifically *Staphylococcus sciuri, Staphylococcus fleurettii, Staphylococcus lentus, Staphylococcus stepanovicii,* and *Staphylococcus vitulinus* into a novel genus, *Mammaliicoccus*[98]. Furthermore, the author suggests that the genus *Nosocomiicoccus* be assigned to the genus of *Staphylococcus* instead[98].

The research for my thesis can be broken down into three objectives. The first objective was to find a recipient NAS strain for bacteriocin gene cluster integration. This strain must have high persistence and colonization in the cow's mammary glands alongside a low effect on inflammation. The second objective was to identify a bacteriocin gene cluster and integrate that into the recipient NAS strain. This integration was attempted through allelic replacement. Lastly, the third objective is characterization of the probiotic (recipient NAS after bacteriocin gene cluster integration), the wild-type NAS recipient strain, the donor bacteriocin gene cluster strain, and their respective controls. Overall, we hypothesize that by creating a NAS probiotic that produces bacteriocins, it could inhibit the growth of *S. aureus* intramammarily and be used as a preventative measure against mastitis.

Methods:

Objective 1: Identify a Recipient NAS Strain for Bacteriocin Gene Clusters Genomic Integration

Recipient Strain Selection Criteria

We used a NAS collection containing 6213 isolates that were obtained from a collection of cow milk samples from the Canadian Bovine Mastitis Research Network[80]. Potential recipient strains were selected based on morphology differences on CHROMagar Staphylococcus (CHROMagar *Staphylococcus*, Dalynn Biologicals, Calgary, AB, Canada) and having no antibiotic resistant (ABR) genes. CHROMagar is a selective chromogenic culture medium that shows different color colonies depending on the type of staphylococcus species. Antibiotic resistance was assessed based on a study by Nobrega et al. (2018) who investigated the drug-specific resistance of the same NAS isolates from the study above[99]. This study tested a series of antimicrobials commonly used in dairy cattle and humans at their minimum inhibitory concentrations. The NAS isolates were challenged on Sensititre plates containing antibiotics to determine their antimicrobial resistance. Potential strains that had no ABR were selected as potential recipient strains. After 16 NAS isolates with no ABR and distinguishable morphology in their respective mixtures on CHROMagar were found (Table 2.1.1), the next step was to see which strain can best colonize the cow's quarters and determine its effect on SCC. This was determined through the NAS mixture persistence experiment and SCC experiment explained below. Lastly, the NAS strain must be competent (i.e. transformability) as the recipient strain will be attempted to be genetically modified with bacteriocins through a plasmid containing the proper genetic material. This was tested by transforming a 6.6kb plasmid called pCDM29 containing a gene coding for a green fluorescent protein

inside the NAS strain[49]. This plasmid was gifted by Dr. Bas Surewaard of the University of Calgary.

Plating of NAS on CHROMagar

CHROMagar was used to distinguish between different *Staphylococcus* isolates through morphological differences such as color and size. All potential recipient strains were grown on CHROMagar separately to find their morphological characteristics by spread plating the isolate from a frozen glycerol stock onto CHROMagar followed by a 36-48 h incubation at 37°C.

NAS Mixture Characterization

NAS isolate mixtures were composed of 4 different NAS strains that are distinguishable from each other on CHROMagar. The mixtures were created based on growing all potential isolates on CHROMagar to determine their morphological characteristics. Furthermore, the mixtures had to have consistent and reproduceable number of colonies after a 75 µl plating of 10^{-6} diluted mixture as some NAS isolates inhibited other isolates. Lastly, the total number of cells per mixture was 1,000,000 with 250,000 cells coming from each isolate. Isolates were grown in a 15 mL round-bottom test tube for 16-18 h in a 37°C shaking incubator at 225 rpm. All isolates were diluted to an OD_{600} = 0.3 and the mixture combinations was formed by adding 200 µl of each isolate into their respective mixtures. Then the mixture was tenfold serially diluted to 10^{-6} and 75 µl of the dilution was plated on CHROMagar and spread using a L-shape rod. The plate was incubated for 36-48 h and then individual colonies of the different stains were counted to ensure there were repeatable numbers of colonies of the four separate isolates.

Determine CFU of NAS strains for NAS mixtures containing 250,000 cells per isolate

Frozen stocks of the isolates were streak plated onto agar plates to allow for single colony formation after 16 h incubation at 37°C. Single colonies were picked and grown in 5 mL LB broth inside a

tube for 16 h at 37°C in a shaking incubator. The OD_{600} after overnight culture was recorded using an EnSpire Multimode Plate Reader and then diluted to OD_{600} = 0.3. After dilution, the stocks were diluted 10-fold to the power of 6 to 8 and 100 µl of each dilution was spread on LB agar plates. The plates were incubated for 16 h at 37°C and colony forming units were counted the next day. The CFU was calculated by dividing the colonies on plate by the final dilution factor (FDF). The FDF was calculated by multiplying the total series dilution factor (TSDF) by the plating dilution factor (PDF). This CFU calculation was used to ensure each NAS mixture had 1,000,000 cells per infusion.

Pre-screening of the Cows for NAS Mixture Persistence and SCC experiment

Six Holstein cows were purchased from Alberta dairy farms. Inclusion criteria for the Holstein cows were: SCC < 150,000 cells/mL, the milk does not contain *S. aureus*, the cow has four functional quarters that produce at least 4 L of milk per lactation, and were not a 'kicker'. Before the purchase was finalized, the cow's milk was pre-screened to ensure the first two criteria are met as a healthy mammary gland microbiome was needed.

Two milk samples were collected from each quarter following the pre-cleaning teat protocol listed below. Sterile milk sample vials (Milk Sample Vials, Lactanet, Edmonton, AB, Canada) were used to collect the samples and then transported to the Foothill campus on ice inside a cooler for analysis. Then 100 µl of the sample was plated on blood-agar plates and incubated at 37°C for 24 h. Hemolysis on the blood-agar plates indicated that there might be *S. aureus* in the milk or if colonies were yellow and spherical. Colonies suspected to be *S. aureus* were confirmed by colony PCR targeting S*. aureus nuc* gene and sequencing. The *nuc* gene was chosen as it is a common diagnostic marker for the identification of *S. aureus*[100].

Housing of Cows *for NAS mixture persistence and SCC Experiment*

All animal use for this project was approved under protocol #AC20-0073 by the University of Calgary Veterinary Services Animal Care Committee (VSACC). Cows were housed at the Spy Hill campus in the quarantine barn. The cows were housed in free-stalls with careful attention to prevent cross-contamination between the cows tested. Cross-contamination risks were reduced by using appropriate PPE while milking the cows and by adhering to measures to ensure cleanliness and hygiene in the barn by power washing the housing pen daily and replacing the hay daily. In total, there were 6 cows, 3 for the NAS mixture persistence experiment and 3 for the SCC experiment. Transportation was done using Spyhill's horse trailers.

Feed for cows

In the general feeding bin, there was a 1:1 mixture of alfalfa hay and horse hay filled up to 75% of the bin. This was topped up daily after the milkings with total alfalfa and hay tracked on the bulletin board. During the AM and PM milkings, each cow would be fed a 1 L bucket of grains.

Pre-cleaning and Post-cleaning of Teat Ends

Before sampling, each teat was wiped with a 70% alcohol wipe to remove any dirt or feces, using a new wipe for each teat. The teats were sprayed with a pre-iodine solution allowing for 30 s of contact time. Once the contact time was achieved, each teat was wiped with a new 70% alcohol wipe. After the milking was done, each teat was cleaned with a new 70% alcohol wipe. Then, a post-iodine solution was sprayed ensuring complete coverage of the teats.

Information recorded during milkings

During the milkings, daily amounts of horse hay and alfalfa hay added was tracked alongside the hay left over from the previous milking. This was the same for grains fed and that was left over. Total

milk production per cow was tracked daily alongside the physical quality of the fore stripped milk and quarters. Any abnormalities to the norm were reported to the veterinarian.

Pre-cleaning of Milking Machine

Two pails were made containing 12 L hot water (70°C) with 0.5% hydrogen peroxide and 12 L hot water (70°C). Then, the cow's milking cluster was submerged into the hydrogen peroxide pail for 1 min before doing a rinse in the other hot water pail.

Milk Disposal

After all the milk was collected from the cows in the milking bucket, bleach was added for a final concentration of 500 ppm. Milk from each cow was measured individually and then poured into the communal milking bucket. Then, an average milk production was calculated to create a standardized amount of bleach to add per milking session.

Movement of Cows during Milking

All cows were moved into the catch pen once the milking machine was set up and ready. The catch pen had three separate bowls of grain to stimulate the movement of the cows. After all the cows are in the catch pen, one cow at a time was moved into the milking pen with a bowl of grain and hay outside the head lock gate. It was up to the milker's preference to choose which cow as some cows may be heavy milk producers and needed to be milked first. Once the first cow was done milking, she was moved into the housing pen and the process was repeated with the remaining cows.

Usage of Milking Machine

Before turning on the machine, the claw assembly unit was attached to the pulsator and milk hose. Once attached, the machine was turned on with both valves in the closed position for 10-20 s to

build up pressure in the main system. Then, the valve attached to the cluster was switched to the open position to build up pressure in the claw cluster. Pressure was finished building up when the claw cluster starts “clicking”. Finally, the claw cluster was attached to the cow’s respective quarter for milking.

Post-Cleaning of Milk Machine

Three sets of 4 individual pails containing pail #1: 12 L hot water (70°C) and 46 mL Chlorklenz, pail #2: 12L warm water (45°C), pail #3: 12L warm water (45°C) with 17 mL acid-55, and pail #4 12 L warm water (45°C) were prepared for the cleaning process. The entire cleaning process involved sucking up the pail #1 all the way through #4. Firstly, the claw part of the assembly was hung so the inflations face downward. The vacuum pump was turned on and the inflations were dunked all at once into pail #1 to suck up 12L Chlorklenz water. The first claw cluster was then detached and left to hang for 5 min. The dirty 12L Chlorklenz in the milk tank was poured back into the pail. This process was repeated with the remaining two dirty claw clusters. Secondly, the first inflation was then dunked into pail #2 to suck up 12 L of warm water. The first claw was then detached and the dirty water in the milk tank was poured back into the pail. This process was repeated with the two remaining dirty claw cluster. Thirdly, the first inflation was then dunked into pail #3 to suck up 12 L of acid wash. The first claw was then detached and the dirty acid wash in the milk tank is poured back into the pail. This process was repeated with the two remaining dirty claw cluster. Lastly, the first inflation was then dunked into pail #4 to suck up 12 L of warm water. The first claw was detached and the dirty warm water in the milk tank was poured back into the pail. This process was repeated with the two remaining dirty claw cluster. The three clean claw clusters, tubing, and milk tank was wiped with a disinfectant wipe and stored properly. The dirty pail #1-4 are emptied down the drains.

Milk Sampling

Milk samples were sterilely collected daily once a day at milking during the AM or PM milking. After fore stripping, quarter milk samples were collected by hand in sterile milk sample vials. The milk samples were stored in a 4°C fridge overnight until plating the next morning. Samples taken on a Friday were stored in a 4°C fridge and plated Monday morning. Milking after that was done using a Delux Portable Milker (Homesteader Supply, Coleman, MI, USA). The milk collected was discarded after addition of bleach as described in the paragraph below. The milking machine was electronic and has a bucket capacity of 30 L. Each cow had their own portable milking machine claw cluster to reduce the risk of cow cross-contamination. All this was performed following standard protocol and good milking techniques, including checking the milk and teat for any symptoms of mastitis, using a sanitized milking cluster unit, and having proper pre- and post-cleaning protocol.

NAS Mixture Persistence Experiment

Three lactating cows were used to assess persistence of NAS in the mammary glands of lactating cows. The NAS mixture preparation was prepared accordingly to the following steps. Frozen stocks of the isolates were streak plated onto agar plates to allow for single colony formation after 16 h incubation at 37°C. Single colonies were picked and grown in 5 mL LB broth inside a tube for 16 h at 37°C in a shaking incubator. The overnight stocks OD_{600} was recorded using a plate reader and then diluted to OD_{600} = 0.3. The OD_{600} = 0.3 was centrifuged at 4500g for 10 min. The LB supernatant was removed and was replaced with a 0.8% NaCl sterile saline solution. The pellet washed twice more before serially diluting down to 10^6 CFU/mL using the calculated CFU for each NAS strain. The 10^6 CFU/mL infusion was stored at 4°C until the infusion was performed on the same day. There were four different NAS mixtures with an intended concentration of 10^6 cfu/mL per strain for each quarter in the cows. In total, 1 mL was infused with an equal volume from each strain. Before infusion, the teat was cleaned following the pre-cleaning protocol described above. The NAS mixture was directly infused into the teat via the teat

canal using a sterilized syringe mount luer lock to prevent injury to the teat (Syringe Mount Luer Lock, SyrvetCanada, QC, Canada). The teat canal was then pinched off, and the inoculum was massaged upwards into the gland cistern. The person applying the inoculum wore clean latex gloves and new gloves were used for each inoculum infused into the quarter. After the infusion with the NAS mixture during the evening milking, milk samples were collected the next morning.

Milk Bacteriological Cultures

Plating was done by adding 100 µl of milk on CHROMagar and spread with a L-shape rod. The plate was incubated in a 37°C incubator for 36-48 h and individual colonies were recorded.

SCC Associated with Most Persistent Strain

After determining the three most persistent isolates from the colonization experiment, another experiment was done to investigate their individual effects on SCC in an IMI experiment. Three lactating Holstein cows were used. The cows had the same inclusion criteria and housing as in the NAS mixture persistence experiment. The bacterial inoculum was prepared similarly to the NAS mixture persistence experiment. Three quarters of each cow was infused with 1 mL of each of the three persistent isolates at an intended CFU of 10^6 with one control quarter infused with saline. After the infusion, the milk was tested to see the isolate's effect on the SCC. Milk samples for SCC was collected on day 0, 1-5, 7, 9, 11, 13, and 14. The SCC was determined through flow cytometry using a Fossomatic 7 DC which was done by Lactanet. Fresh milk samples were mixed with a preservation pill and stored in a milk container supplied by Lactanet. Preserved milk samples were stored in the 4°C fridge until the shipping date that was no later than 2 weeks and not on a Friday. Shipment was done by getting a shipping slip and shipping container from Lactanet. Furthermore, milk samples also be collected daily for bacteriology following the same collection and plating method as the NAS mixture persistence experiment.

After this experiment, the cows were euthanized, and mammary tissues collected following the same protocol as the NAS mixture persistence experiment.

Bacterial Identification using rpoB-based identification

To identify the most persistent isolate from the milk samples, 100 µl of milk was first directly plated on CHROMagar and incubated at 37°C for 36-48 h to detect which isolate remained. The number of different colonies formed was counted. Furthermore, different bacterial colonies were picked and suspended in 20 µl of lysis buffer (0.25% SDS, 0.05 M NaOH) and heated for 5 min at 95°C and diluted 10-fold in distilled water. The lysate was centrifuged, and the supernatant was used as the PCR template. The two primers used for amplification are rpoB_1418F and rpoB_3554R which was taken from a paper made by Mellmann et al[48]. Thermal cycling conditions were 5 min at 94°C for the first denaturation step, followed by 35 cycles of denaturating at 94°C for 45 s, annealing at 52°C for 60 s, and extension at 72°C for 90 s, with a final extension step at 72°C for 10 mins. The amplicon was confirmed through gel electrophoresis with an expected length of 899 bp. After confirmation, the amplicon was sent for DNA sequencing to University of Calgary's Core DNA services. The DNA sequence results were then imported into Geneious Prime and submitted to BlastN to confirm the species. The sequence was compared to database sequences and the highest identity percentage was used to identify the species if above the threshold for species identification. After completion of the 2-wk infection experiment, the cows were euthanized, and tissue samples were collected following euthanasia and homogenized for bacteriology following the protocol below.

Euthanasia of Cows

The cows were euthanized by a licensed veterinarian through sedation using 1 mL of 100mg/mL xylazine injected into the tail vein followed by captive bolt.

Tissue Collection

The udder was dissected from the ventral abdominal wall and isolated from the cow. After gentle hosing to remove surface skin debris, the udder was placed on a dissection table with the teats facing up, and the entire skin surface sprayed with 70% ethanol and allowed to dry. All subsequent procedures were performed using instruments that had been soaked in 70% ethanol and allowed to air dry. Instruments were changed for a fresh set between each quarter. One udder quarter was sampled completely, and then all steps were repeated for the remaining three quarters.

First, the skin was removed from one quarter using a knife. Next, a deep scalpel incision was made into the dorsal part of the udder parenchyma and a sterile swab was collected from the cut parenchymal surface. Then a new incision was made into the gland cistern and a second swab was collected. Finally, the teat canal was incised longitudinally and a third swab was collected from its lumen.

After swab collection of three parenchymal tissue samples, each approximately 10 x 10 x 10 mm parenchymal pieces were collected using sterile forceps and scalpels. These were collected from the dorsal, middle and ventral parts of the glandular parenchyma and placed into sterile Whirl-Pak bags or conical centrifuge tubes and stored on ice. During the 2nd tissue collection, four parenchymal pieces were collected instead ranging from dorsal, upper middle, lower middle, and ventral.

Finally, teat epithelial lining each approximately 5 x 5 x 5 mm were collected using sterile scissors during the 1st tissue collection. This step was removed during the 2nd tissue collection due to issues with homogenization of the lining.

Tissue Homogenization and Plating

Tissues was homogenized one-day after tissue collection with the fresh tissues stored in the 4°C refrigerator. After placing the tissues stored in Whirl-pak bags on ice, 1 g of the tissue was placed inside

a Gentlemacs purple C tube containing 4 mL PBS. The samples were homogenized by using the adipose tissue program of the Gentlemacs Octo Dissociator thrice and a customized program once for further homogenization. The customized program was another 60 s homogenization to remove any large chunks of tissues. After placing the homogenate on ice, 100 µl was spread on CHROMagar plates and incubated at 37°C in a non-shaking incubator for 48 h. The remaining homogenate was stored at 80°C with glycerol for a final glycerol concentration of 25%.

Competence (transformability) of the Recipient Strain

Having a competent recipient strain was another important criterion for the goal to introduce 1 to 3 gene clusters into the recipient strain on a plasmid. Competence was tested by electroporation of the recipient strain with a plasmid, pcdm29, that contained a green fluorescent protein gene. The green fluorescent protein allowed for easier secondary confirmation of transformants through detection on a plate reader. First, NAS were made electrocompetent. The recipient strain was grown in 5 mL Todd Hewitt broth at 37°C during the day. In the evening, 120 µl of the day culture was added to 25 mL of medium A in a 50 mL falcon tube and grown overnight in a 37°C shaking incubator at 225 rpm. Media A was made with 2.5 mL 20% glycine, 5 mL 1 M sucrose, and 42.5 mL brain and heart infusion (BHI) broth. Then, the overnight culture was spun down at 4500 rpm for 5 min and resuspended with new 25 mL pre-warmed at 37°C medium A. This mixture was incubated in a 37°C shaking incubator at 225 rpm. After 1 h, the cells are spun down at 4500 rpm for 5 min and resuspended in 10 mL ice-cold wash buffer. The wash buffer contains a 1:1 ratio of 1 M sucrose and 20% glycerol in double distilled H_2O. The 10 mL suspension was divided over five 2-mL sterile ice-cold tubes and then spun down at 13,500 rpm for 1 min. The supernatant was discarded, and the pellet was resuspended in 2 mL ice-cold washing buffer. This step was repeated two more times. Finally, the cells were spun down at 13,500 rpm for 1 min and the supernatant was discarded. The pellet was resuspended in 200 µl ice-cold wash buffer and the five

tubes are combined into one. The cells were stored at -80°C until needed. For the transformation, a mix 5 µl of 1 µg/µl plasmid with 100 µl electrocompetent cells was left in ice for 30 min. The plasmid was extracted from *E. coli* DC10B which has been modified to allow for easier acceptance of the plasmid into *Staphylococcus* species*. E. coli* DC10B has a mutation in the *dcm* system that no longer causes cytosine methylation. Thus, when the plasmid was passed through *E. coli* DC10B, the restriction modification system In the *Staphylococcus* species does not recognize the foreign DNA as foreign. After 30 min, the mixture was transferred into a 1 mm ice-cold electroporation cuvette ensuring no bubbles were formed. The cells were electroporated using a gene pulser at 2500 voltage, 200 ohms resistance, and 25 µF capacitance. Immediately after, the electroporated cells were resuspended in 1 mL of recovery media containing a 1:1 ratio of 2x BHI broth and 1 M sucrose. This mixture was transferred into a sterile Eppendorf tube and grown in a 37°C shaking incubator at 225 rpm for 2 h. Then, the cells were spun down at 13,000 rpm for 1 min and 800 µl of the supernatant was removed from the tube. The pellet was resuspended with the remaining supernatant and 75 µl of the mixture was plated on 10 µg/mL chloramphenicol Todd Hewitt agar plates and incubated for 36-48 h at 37°C. Finally, successful transformants were grown in 10 µg/mL chloramphenicol broth overnight. For secondary confirmation of transformation, 150 µl of the overnight culture was placed in a 96-well plate and put through a plate reader to detect the green fluorescent protein signal. Fluorescent microscopy can also be used to detect the GFP signal.

Table 2.1.1: Strains and plasmids associated with Objective 1

Strain/Primer	Description	Reference
S. chromogenes 5987	Mixture 1 Potential NAS Probiotic	[99]
S. xylosus 5435	Mixture 1 Potential NAS Probiotic	[99]
S. warneri 1052	Mixture 1 Potential NAS Probiotic	[99]
S. devriesei 1316	Mixture 1 Potential NAS Probiotic	[99]
S. arlettae 5134	Mixture 2 Potential NAS Probiotic	[99]
S. hominis 2694	Mixture 2 Potential NAS Probiotic	[99]
S. devriesei 4143	Mixture 2 Potential NAS Probiotic	[99]
S. pasteuri 2657	Mixture 2 Potential NAS Probiotic	[99]
S. warneri 2140	Mixture 3 Potential NAS Probiotic	[99]
S. caprae 4023	Mixture 3 Potential NAS Probiotic	[99]
S. equorum 2121	Mixture 3 Potential NAS Probiotic	[99]
S. warneri 2993	Mixture 3 Potential NAS Probiotic	[99]
S. cohnii 1091	Mixture 4 Potential NAS Probiotic	[99]
S. devriesei 4438	Mixture 4 Potential NAS Probiotic	[99]
S. warneri 4140	Mixture 4 Potential NAS Probiotic	[99]
S. pasteuri 2044	Mixture 4 Potential NAS Probiotic	[99]
Pcdm29	GFP Plasmid used for transformation practice	This study
E. coli DC10B	*E. coli* strain for plasmid preparation	This study

Objective 2: Integration of a Bacteriocin Gene Cluster into the Recipient NAS Strain

Criteria for Bacteriocin Selection

Carson et al. (2017) studied the inhibitory effects of 441 bovine NAS isolates on *S. aureus*[83]. It was discovered that 40 isolates inhibited *S. aureus* presumably through the production of bacteriocins. Some bacteriocins had partial inhibition of *S. aureus* which means not complete inhibition of *S. aureus.* The bacteriocins produced were also classified into their respective classes. The selection of the bacteriocin gene cluster to be added into the recipient strain is derived from these 40 isolates.

As a proof of principle, one bacteriocin gene cluster was attempted to be introduced into the recipient strain. There were two main criteria used to find the most appropriate bacteriocin. The first criterium was that it inhibits *S. aureus in vitro*. The second criterium was that the entire cluster is as small as possible. This is an important consideration to increase the likelihood of transformation with the bacteriocin plasmid.

Bacteriocin in-vitro Inhibition of S. aureus via a Modified Cross-Streak Method

The bacteriocin-producing isolate was grown on blood agar plates as a center streak for roughly 36-48 h. Thereafter, the blood agar was flipped and then challenged with 100 µl *of S. aureus*, 1/1000 diluted from a suspension with an OD600 of 0.3. The blood agar plate was then incubated at 37°C overnight and checked the next day to see if there was a zone of inhibition of *S. aureus* because of the bacteriocin production.

Gibson Assembly

The total reagents for this reaction 3:1 ratio of vector to insert for a total volume of 10 µl,

alongside 10 µl of the 2x Gibson Assembly master mix. The sample was incubated in a heat block (Thermomixer, Eppendorf) at 50°C for 1 h. After 1 h, the sample was placed on ice in preparation for chemical transformation into *E. coli* (NEB® 10-beta Competent *E. coli*, Whitby, ON, Canada)

Creation of pKOR1 plasmid with the RFP g-block for Genetic Editing in S. warneri 2993

The purpose of first transforming *S. warneri* 2993 with an red fluorescent protein (*RFP*) gene instead of the bacteriocin gene cluster was to gain familiarity with the molecular biology techniques and optimize the protocol since the gene was 6 KB smaller than the bacteriocin gene cluster. If the bacteriocin gene cluster had been used initially, failure in producing results would have been either due to the bacteriocin gene cluster being too large or incorrect technique. The empty allelic replacement plasmid (pKOR1) had been generously gifted by Dr. Taeok Bae from the University of Chicago. The first step was to choose a target locus where the *RFP* gene would be knocked into. The target gene coded for a mannitol-specific phosphotransferase enzyme, mtlF, that is used to transport mannitol across the cell membrane. This is a non-essential gene that could cause an observable phenotypic change through the loss of mannitol metabolism in *S. warneri* once *RFP* was knocked in. From there, a synthetic double-stranded DNA fragment was ordered from IDT containing homology that was 1KB upstream and 1KB downstream of mtlF with three important elements. These three elements were to prepare it for Gibson assembly that would be used further downstream. The first addition was homology to EcoRV and the pKOR1 plasmid on the 5' end (CTGCAGCTGCTAGCTAGCTAGAGAT) as the pKOR1 plasmid would be linearized with EcoRV and KpnI for Gibson assembly. The second addition was homology to KpnI and the pKOR1 plasmid on the 3' end (GTACCGGTTCCGAGGCTCAACGTCA). The third addition was the actual *RFP* gene between the upper and lower mtlF homology. The g-block was received in a dry tube and needed to be resuspended. This was achieved by spinning the tube in a microcentrifuge for 3-5 s to ensure DNA was in the bottom of the tube. Then, molecular-grade water was added to reach a final concentration of

10 ng/µl and vortexed to dissolve the DNA. The tube was then incubated at 50°C for 15 min to ensure the solvent dissolved the DNA. Finally, the tube was vortexed once last time and centrifuged. Then, 10.4 µg of pKOR1 plasmid was double digested with 100 units each of EcoRV and KpnI for 1 h at 37°C. The digest was purified using a PCR purification kit (QIAquick PCR Purification, Qiagen, Toronto, ON, Canada) and then gel electrophoresis was run with the purified digest and undigested plasmid for confirmation. The gel was made 1% agarose and 70 mL of 1x TAE buffer and ran for 40 min at 120 V. After confirmation of the digest, the two linearized fragments: digested pKOR1 and g-block were annealed using Gibson assembly. Finally, the plasmid was confirmed through gel electrophoresis by running the empty backbone plasmid and the transformant plasmid to detect a size increase.

Conversion of S. aureus secY counter-selection method into S. warneri 2993 secY counter-selection

The original counterselection method in the *pKOR1* plasmid is through the production of anti-sense *secY* that inhibits *secY*, a gene important for bacterial growth. However, as this plasmid was designed for *S. aureus* and not *S. warneri*, the anti-sense *secY* is specific to *S. aureus* and not *S. warneri.* This may result in the anti-sense *secY* not inhibiting growth in *S. warneri* due to imperfect homology, and thus, we replaced the anti-sense *secY* with a version that is 100% homologous to *S. warneri 2993's secY*. Since *S. warneri* 2993 was whole genome sequenced, we found *secY* and created a g-block that reverse complemented 554 bp from the 3' end of *S. warneri* 2993's *secY.* The g-block also contained SmaI homology on the 5' end (TAGATATGAATCATTAGATCACCCC), alongside pKOR1 and xhoI homology on the 3' end (TTATACTCTATCAATGATAGAGTGTCAATATTTTTTTTAGTTTTTCATGAACTCGAGGGGATCCAAA TAAAAAACTA). This was because the original *secY* in the pKOR1 plasmid is flanked by two restriction enzymes, SmaI and xhoI. After digesting the plasmid, the two linearized fragments, digested pKOR1 and g-block, were annealed using Gibson assembly. Confirmation of the *S. warneri* 2993 *secY* replacement was through PCR targeting a 271 bp region in the new *secY*. The forward primer was FSecY and the

reverse primer was RSecY. The PCR conditions were 95°C for 1.5 min, then 30 cycles of 95°C for 30 s, 46.5°C for 30 s, and 72°C for 30 s, followed by a final extension of 72°C for 5 min and a holding temperature of 10°C. Finally, the reagents included 12.5 µl 2x Master mix, 2.5 µl 10x CoraLoad buffer, 7.5 µl H_2O, 1 µl 10 uM forward and reverse primer, and 0.5 µl template for a total volume of 25 µl.

Genetic modification of S. warneri 2993 with pKOR1 containing RFP g-block

The *E. coli* strain containing pKOR1 and the *RFP* g-block was grown in 200 mL BHI broth containing 100 µg/mL ampicillin to do a large plasmid extraction using a maxiprep kit (PureLink HiPure Plasmid Maxiprep Kit, ThermoFisher, Mississauga, ON, Canada). After obtaining a plasmid yield that was 3 µg/µl or higher, electro-transformation was performed. From the -80°C freezer, 100 µl electro-competent *S. warneri* 2993 was thawed on ice and then 5 µl 3 µg/µl pKOR1-*RFP* was added to the cells and mixed gently by pipetting. The solution was kept on ice for 30 min and then transferred to a 1 mm electroporation cuvette (Electroporation Cuvettes, ThermoFisher, Mississauga, ON, Canada) and added to the Bio-rad electroporation machine. The settings were: potential 2.5kV, capacity 25 µF, and a shunt resistance of 200 Ω. After the cuvette was electroporated, 1 mL of medium B (1:1 ratio of 2x BHI and 1M sucrose) was added for recovery and then transferred into a new 2 mL microcentrifuge tube. This tube was incubated in a 30°C 225 rpm incubator for 2 h on its side. In the meantime, 10 µg/µl chloramphenicol THA plates were pre-warmed in the 30°C incubator. Once the 2-h incubation was complete, the cells were spun down at 13 000 rpm for 1 min and 800 µl of the supernatant was removed. The pellet was resuspended in the remaining supernatant and plated on the chloramphenicol plates. The plates were incubated for 48-72 h until individual transformants appeared. To integrate the plasmid into *S. warneri* 2993's genome, the transformant was inoculated in 10 mL of tryptic soy broth (TSB) with 10 µg/ml chloramphenicol in a 250 mL flask at 30°C with vigorous shaking. The resulting

culture was streaked on 43°C pre-warmed tryptic soy agar (TSA) with 10 µg/µl chloramphenicol and then incubated at 43°C overnight. This step helped select the first cross-over integration event. Then, a big and well-isolated colony was inoculated in 5 mL TSB with 10 µg/mL chloramphenicol and incubated at 30°C overnight to facilitate the second cross-over integration event or the plasmid excision. The resulting culture was 10^4-fold diluted with sterile water and 100 µl aliquots were spread on TSA with 50 ng/mL of anhydrotetracycline (Atc). These plates were incubated at 30°C for 2 days for the counter-selection with antisense secY. Finally, 40-50 big colonies were chosen and streaked on both TSA with 10 µg/mL chloramphenicol and plain TSA and incubated at 30°C. Colonies that had lost the plasmid only grew on plain TSA and not TSA with chloramphenicol. These colonies were confirmed of their *RFP* gene knock-in through PCR targeting the *RFP* gene and plating on mannitol agar plates.

Making Electrocompetent Staphylococcus species

Todd-Hewitt broth (5 ml) was added to a 50 mL centrifuge tube containing a single colony of *S. warneri* 2993 to grow during the day in a 37°C 225 rpm incubator. Media A, containing 2.5 mL of 20% glycine, 5 mL of 1M sucrose, and 42.5 mL of brain and heart infusion broth, was divided into two 50 mL centrifuge tubes containing 25 mL of the mixture. After 6-8 h, 30 µl of the THB culture was added to media A and grown overnight in a 37°C 225 rpm incubator. Following the overnight incubation, the cells were then spun down at 4500 rpm for 5 min, resuspended with 25 ml of pre-warmed medium A, and incubated in a 37°C 225 rpm incubator for 1 h. Then, the cells were spun down at 4500 rpm for 5 min and resuspended in 10 mL of ice-cold washing buffer (1:1 ratio of 1M sucrose and 20% glycerol). The 10 mL suspension was divided over 6 x 2 mL microcentrifuge tubes and constantly kept on ice. Then, the cells were centrifuged at 13 500 rpm for 1 min. The supernatant was discarded, and the cells were resuspended in a washing buffer. This process was repeated twice more. Finally, the cells were centrifuged one last time at 13 500 rpm for 1 min and resuspended in 200 µl of ice-cold washing buffer.

The six tubes were combined and then 100 µl aliquots were made and stored in the -80°C freezer.

RFP gene knock-in confirmation in S. warneri 2993

Primers were made to target a region overlapping *mtlF* and *RFP* gene that was 905 bp long. The forward primer, FRfpMtlf, was in the *mtlf* gene while the reverse primer, RRfpMtlf, was in the *RFP* gene. The PCR conditions were 95°C for 1.5 min, 95°C for 30 s, 50°C for 30 s, 72°C for 1 min (steps 2-4 were cycled 35 times), 72°C for 5 min, and then 10°C forever. The total volume of the reaction was 25 µl and contained 12.5 µl 2x Master mix, 2.5 µl 10x CoraLoad buffer, 7.5 µl H_2O, 1 µl 10 uM forward and reverse primer, and then finally 0.5 µl template. The PCR product size was confirmed through gel electrophoresis alongside DNA sequencing.

Plating on mannitol agar plates was another confirmation technique as the *RFP* gene was knocked into the *mtlF* gene, which was responsible for the metabolism of mannitol. The suspected modified *S. warneri* 2993, *S. warneri* 2993 Δ*mtlf,* was plated on mannitol plates alongside the wild-type to see if there was a difference in the strain's ability to metabolism mannitol. This was seen as no change in the pH indicator of pink to yellow on the plates.

The gene that was knocked into *S. warneri* 2993 was the *RFP* gene, which codes for a red fluorophore that fluoresces red-orange when excited. Unfortunately, despite using a fluorescent microscope (Zeiss Axio Imager M2 Fluorescent Microscope) with an excitation laser line of 488 nm and 532 nm, no emission was detected at 584 nm. Furthermore, no fluorescence was detected from the transformants on agar plates when placed under an UV illuminator (Spectroline Ultraviolent Transilluminator).

Conversion of pKOR1-RFP into pKOR1-Bacteriocin plasmid

The plan to convert the pKOR1-*RFP* plasmid into a pKOR1-Bacteriocin plasmid was to replace the

RFP portion of the plasmid with the bacteriocin gene cluster. To achieve this, PCR primers were created that amplify the entire plasmid except for the *RFP* gene cluster. The forward and reverse primer each contained an overhang that was complementary to the 5' and 3' end of the bacteriocin gene cluster. These overhangs were CACACGTCGTCGAAATGCATTCTTG and TAAATGCAGAAAACAACGGGGTAAG respectively and were important for downstream Gibson assembly to ligate the two linearized DNA fragments together. The forward primer, FRfpSC803, was located on the 3' end of the *RFP* gene while the reverse primer, RRfpSC803, was located on the 5' end of the *RFP* gene. PCR conditions were as follows: 98°C for 3 min, then 30 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 5.5 min, followed by a final extension of 72°C for 2 min and a holding temperature of 4°C. The PCR reagents included 12.5 µl Master Mix, 1.25 µl of 10 uM forward and reverse primer, 5 ng of pKOR1-*RFP* plasmid, and 9 µl of nuclease-free water for a total reaction. This experiment was also carried out with a lower amount of pKOR1-*RFP* plasmid, 50 pg, to increase the specificity of the reaction. Another set of forward primer, FRfpSC803v2, and reverse primer, RRfpSC803v2, were made targeting slightly different regions of the *RFP* gene. Primer set 1 and 2 were used in a gradient PCR with annealing temperatures of 60°C, 57.7°C, 55.5°C, 52.7°C, 49.1°C, and 48°C to find the optimal temperature. Once PCR products were amplified, their base pair length was confirmed with gel electrophoresis with the regular pKOR1-*RFP* plasmid for comparison.

Creation of pKOR1 plasmid with the Bacteriocin Gene Cluster for Genetic Editing in S. warneri 2993

The creation of the pKOR1 plasmid containing the bacteriocin gene cluster was very similar to the pKOR1-RFP plasmid except for a few extra steps. The first difference is in the g-block, as in-between the upper and lower *mtlF* homology there are two restriction enzyme sites added so the bacteriocin gene cluster can be added through Gibson assembly. These two restriction enzymes were SbfI and DraIII with the following sequence: CCTGCAGGACTCACGGCGTGCGTTGAGGATCGACGCGT. From there, the

primers for the bacteriocin gene cluster were made with consideration to these two sites. The forward primer, FSC803, had a 5' overhang that is homologous to the SbfI restriction site in the g-block and a section to amplify the bacteriocin gene cluster. The reverse primer, RSC803, also had a 5' overhang that is homologous to the DraIII restriction site and a section to amplify the bacteriocin gene cluster. Furthermore, the forward primer was designed 150 bp away from the start of the bacteriocin gene cluster and the reverse was 50 bp downstream of the last gene. This was to ensure the entirety of the promoter sequence was captured by the PCR primers. To lyse the bacteriocin strain donor, *S. chromogenes* 803, one colony was added to 20 µl of lysis buffer (0.25% SDS, 0.05 N NaOH) and heated for 5 min at 95°C and diluted 10-fold in distilled water. Then, 1 µl of the diluted solution, 1 µl of 20 µM forward primer, 1 µl of 20 µM reverse primer, 45 µl platinum-taq PCR supermix high fidelity, and 2 µl of 4% DMSO were combined into a PCR tube. The cycling conditions were a hot start for 2 min at 94°C, followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 54.1°C for 30 s, extension 47°C for 7 min, with a final extension step at 68°C for 10 min. The PCR product was confirmed on an agarose gel by checking for the correct band size. After successful confirmation, the PCR product was sent off for sequencing (Sanger DNA Sequencing, University of Calgary, Calgary, Alberta, Canada). Then, 10.4 µg of pKOR1 plasmid was double digested with 100 units each of EcoRV and KpnI for 1 h at 37°C. The digest was purified using a PCR purification kit (QIAquick PCR Purification) and then gel electrophoresis was run with the purified digest and undigested plasmid for confirmation. Finally, all the reagents were ready for Gibson assembly to create the completed bacteriocin-pKOR1 plasmid. Finally, the plasmid was confirmed through gel electrophoresis by running the empty backbone plasmid and the transformant plasmid to see for a size increase. It is also important to note that this Gibson assembly was re-attempted with different restriction enzymes to digest pKOR1 as DraIII leaves a GCG overhang that is prone to self-ligation. Instead, it was digested with BseRI as it leaves a TA overhang that is less likely to self-ligate. The downside of BseRI is that it is found in the lower *mtlF* region of the g-block inside pKOR1.

As a result, 222 bp out of the total 979 bp lower *mtlF* homology will be lost which may lower the probability of homologous recombination. Since the downstream restriction enzyme has changed, this means the overhang on the reverse bacteriocin gene cluster primer must be homologous to the region. Therefore, the new reverse primer, RSC803v2, overhang is new while the region amplifying the bacteriocin gene cluster is still the same. The resulting Gibson assembly mix followed the same protocol as above.

*Genomic Editing in S. warneri 2993 with RFP Using an Allelic Exchange Plasmid piMAY**

The purpose of first transforming *S. warneri* 2993 with a red fluorescent protein gene instead of the bacteriocin gene cluster was to gain familiarity with the molecular biology techniques and optimize the protocol since the gene was 6 KB smaller than the bacteriocin gene cluster. The main difference between the piMAY* plasmid and the pKOR1 plasmid was a different counter-selection marker as this used pheS*, a mutated subunit of the phenylalanine tRNA synthetase that allowed for the incorporation of toxic phenylalanine amino acid analogue para-chlorphenyalanine (PCPA)[55]. The plasmid, deposited by Dr. Angelika Grundling, was purchased from Addgene. The empty piMAY* backbone plasmid was successfully transformed into *S. warneri* 2993 according to the same protocol as the pKOR1 plasmid. The piMAY* plasmid has also been successfully cured by growing the transformants in 5 mL TSB with 10 µg/mL chloramphenicol overnight in a 28°C 225 rpm incubator. After incubation, the culture was 6 times ten-fold diluted and 100 µl was plated on 20mM PCPA agar plates for 2 days at 28°C. After the incubation, the colonies were plated on regular LB and 10 µg/mL chloramphenicol plates to see which colonies have lost their plasmid. The *RFP* gene was added into the piMAY* plasmid by converting the pCasSA g-block into the piMAY* g-block. This conversion was done by creating new PCR primers containing homology to two restriction sites in the piMAY* plasmid, smaI and xhoI, alongside homology to the upper and lower mtlF gene. The forward primer was FCaspiMAY and the reverse primer was

RCaspiMAY. Thus, the PCR product would contain homology to the piMAY* plasmid and would allow Gibson assembly to create the completed piMAY*-*RFP* plasmid. The PCR conditions were: 98°C for 30 s, then 30 cycles of these three temperatures of 98°C for 10 s, 67.2°C for 30 s, and 72°C for 1.5 min, then a final extension of 72°C for 2 min and a holding temperature of 4°C. The PCR reagents included: 25 µl Master Mix (Q5® High-Fidelity 2X Master Mix, Whitby, ON, Canada), 2.5 µl 10 uM forward and reverse primer, 0.5 µl 20 ng/µl template, and 19 µl nuclease-free water. The PCR product was verified through gel electrophoresis and DNA sequencing (Sanger DNA Sequencing). After confirmation, the piMAY* plasmid was double digested to become linearized and have the homology needed for Gibson assembly at its ends. The double digest contained 10 µg piMAY* plasmid, 5 µl of smaI and xhoI, 10 µl of 10x rCutSmart, and nuclease-free water was added for a total volume of 100 µl. The digest was run at 37°C for 1 h. The digest was then purified using a PCR purification kit (QIAquick PCR Purification) and then gel electrophoresis was run with the purified digest and undigested plasmid for confirmation. Finally, the linearized piMAY* plasmid and the piMAY* g-block were ligated together using Gibson assembly. Finally, the plasmid was confirmed through gel electrophoresis by running the empty backbone plasmid and the transformant plasmid to see for a size increase. The piMAY*-*RFP* transformant was grown in 200 mL BHI broth containing 100 µg/mL ampicillin to do a large plasmid extraction using a maxiprep kit (PureLink HiPure Plasmid Maxiprep Kit). The PCR product had been verified through gel electrophoresis and DNA sequencing (Sanger DNA Sequencing). After confirmation, the piMAY* plasmid was double digested to become linearized and have the exposed homology needed for Gibson assembly. The double digest contained 10 µg piMAY* plasmid, 5 µl of smaI and xhoI, 10 µl of 10x rCutSmart, and nuclease-free water was added for a total volume of 100 µl. The digest was run at 37°C for 1 h. The digest was then purified using a PCR purification kit (QIAquick PCR Purification) and then gel electrophoresis was run with the purified digest and undigested plasmid for confirmation. Finally, the linearized piMAY* plasmid and the piMAY* g-block were ligated together using Gibson assembly. Finally, the plasmid was confirmed

through gel electrophoresis by running the empty backbone plasmid and the transformant plasmid to see for a size increase.

The piMAY*-*RFP* transformant was grown 200 mL BHI broth containing 100 µg/mL ampicillin to do a large plasmid extraction using a maxiprep kit (PureLink HiPure Plasmid Maxiprep Kit). After obtaining a plasmid yield that was 3 µg/µl or higher, electro-transformation was performed using two different plasmid concentrations: 1.5 µg/µl and 3 µg/µl.

Genomic Editing in S. aureus (USA300) & NAS Using an Engineered CRISPR/Cas9 System

The purpose of first transforming *S. aureus* instead of NAS was to build technical knowledge on genomic editing with CRISPR-Cas9[60]. If NAS had been used initially, failure in producing results would have either been due to the NAS being incompatible with the CRISPR-plasmid or incorrect technique. An empty backbone CRISPR-plasmid (pCasSA) for *S. aureus* was purchased from Addgene [Figure 2.1.0]. To create the specific CRISPR-plasmid for *S. aureus*, a 20 bp-spacer sequence was needed that was adjacent to a PAM sequence. This spacer needed to be phosphorylated and then annealed for it to be inserted into with pCasSA through Golden Gate cloning. Once assembled, pCasSA-spacer was transformed into a special *E. coli* strain called DH10B. This strain provided the methylation pattern needed to bypass the S*. aureus* restriction modification system. The transformant was verified through PCR targeting the cap1A promoter region directly above the spacer sequence and sequencing to ensure the plasmid was inside *E. coli* DH10B. The verified transformant was then grown in BHI media and a maxi-prep was performed to isolate the plasmid. The plasmid was then digested as preparation for Gibson assembly. From there, the bacteriocin gene cluster was amplified and verified through gel electrophoresis and sequencing. The 1 KB upstream and 1 KB downstream region of the target gene for homologous recombination in *S. aureus* USA300 was amplified with a 40 bp homology to the pCasSA plasmid and a 30 bp homology to the bacteriocin gene cluster [Figure 2.1.1]. This homology was a requirement for Gibson assembly as DNA

attaches through overlapping regions through the help of the T5 exonuclease and Phusion polymerase in the Gibson assembly master mix. The target gene was a non-essential gene that could cause an observable phenotypic change. The agrA gene was used because it is indirectly involved in the production of several hemolysins. If the recombination was successful, the transformant should no longer be hemolytic[61]. The homology arms were verified through gel electrophoresis and then purified using a gel purification kit. An alternative approach was having the homology arms ordered as g-blocks (IDT). The upstream and downstream homology arms, digested pCasSA plasmid, and bacteriocin gene cluster were then added to a Gibson assembly mixture to create the complete CRISPR-plasmid (Figure 2.1.1).

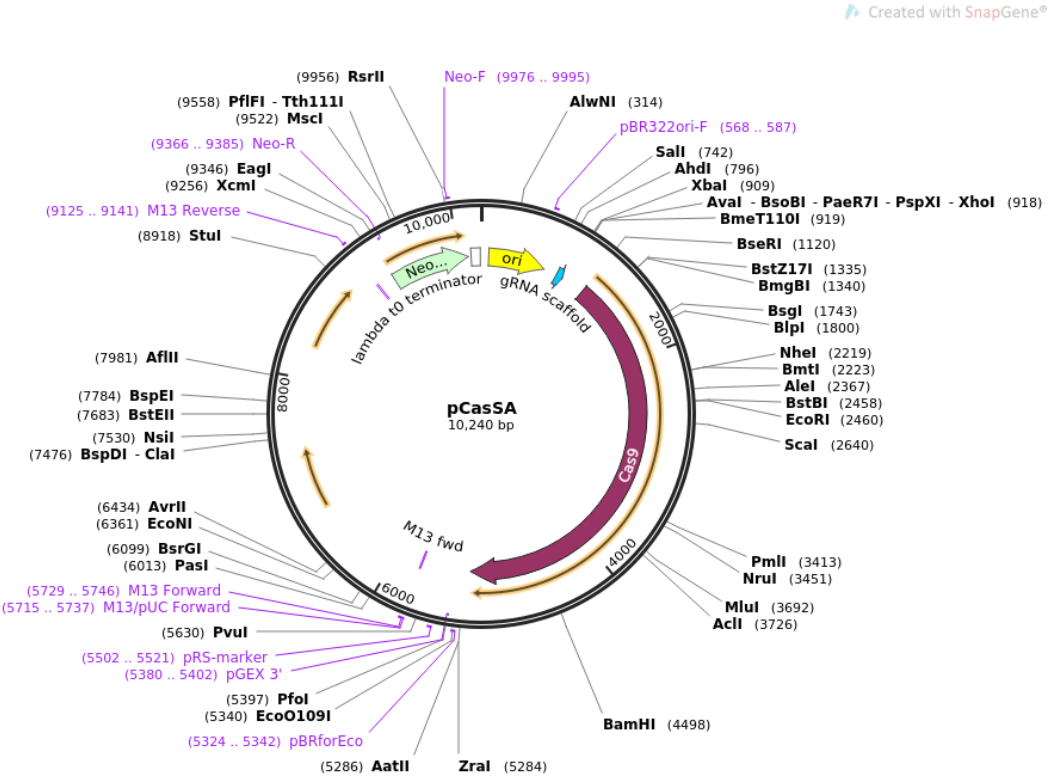

Figure 2.1.0. Empty backbone CRISPR-plasmid for genomic editing in *S. aureus*[1].

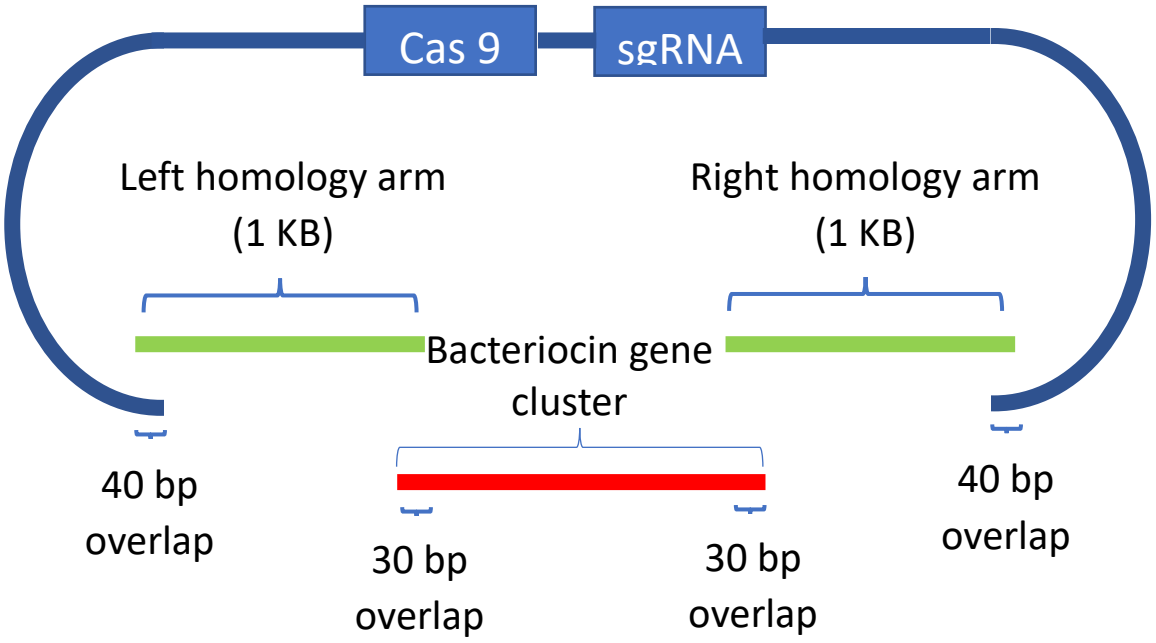

Figure 2.1.1. Diagram for Gibson assembly showing the overlap between DNA fragments. There are four linear fragments of DNA: left homology.

Blunt-end ligation of pKOR1 and Bacteriocin Gene Cluster

Blunt-end ligation of pKOR1 and the bacteriocin gene cluster was used as an alternative to joining the linearized pKOR1 with the bacteriocin gene cluster because Gibson assembly was giving difficulties due to self-ligation of the linearized plasmid. To achieve this, both the DNA fragments were blunted. For both the linearized pKOR1 plasmid and the bacteriocin gene cluster, they were blunted and phosphorylated using a kit (NEB Quick Blunting Kit, Whitby, ON, Canada). The mixture contained 3-5 µg DNA template, 2.5 µl 10x blunting buffer, 2.5 µl of 1 mM dNTP mix, 1 µl blunting enzyme mix, and nuclease-free water for a total volume of 25 µl. The mixture was added to a thermocycler and heated up to 23°C for 30 min, and then an inactivation step of 70°C for 10 min. Both products were purified using a kit (QIAquick PCR Purification). Since the pKOR1 plasmid was restriction enzyme digested, it still contains the 5’ phosphate which may allow for self-ligation. Thus, the purified blunted pKOR1 plasmid was

dephosphorylated to prevent non-directional ligation and the lower the chances of self-ligation. This was done using a dephosphorylation kit (NEB Quick CIP, Whitby, ON, Canada). The mixture contained 3.2 µg of plasmid, 20 µl 10X rCutSmart buffer, 10 µl quick CIP, and water for a total volume of 200 µl. This mixture was incubated at 37°C for 10 min and then heat-inactivated at 80°C for 2 min. Finally, the dephosphorylated blunted pKOR1 plasmid was purified using a kit (QIAquick PCR Purification).
Next, the two fragments were ligated together using a quick ligation kit (NEB Quick Ligation Kit, Whitby, ON, Canada). The ligation components included 0.027 pmol of pKOR1, 0.08 pmol of the bacteriocin gene cluster, 10 µl 2X quick ligase reaction buffer, 1 µl quick ligase, and nuclease-free water for a total volume of 20 µl. A control ligation buffer containing everything except for the bacteriocin gene cluster was also performed to see the rate of plasmid self-ligation. The reaction was gently mixed and incubated at room temperature for 5 min. The reaction was then chilled on ice in preparation for chemical transformation to *E. coli* (NEB® 10-beta Competent *E. coli*). The competent *E. coli* was thawed on ice and then 2 µl of the ligation mixture was added to 50 µl of cells. The mixture was pipetted gently up and down and then placed on ice for 30 min without mixing. Then, a 42°C heat shock was performed for 30 s and then placed on ice once again for 5 min. After that, 950 µl of NEB 10-beta/stable outgrowth medium was added into the mixture and incubated at 30°C for 1 h in an incubator shaking at 225 rpm. Finally, 100 µl cells were plated on pre-warmed 30°C 100 µg/mL ampicillin THA plates and incubated at 30°C for 24 h. Transformants were grown in 5 mL 100 µg/mL ampicillin broth overnight at 30°C and then plasmid was extracted with a QIAprep Spin Miniprep Kit. Once the plasmid was extracted, gel electrophoresis was performed with the transformant plasmids and the original pKOR1 plasmid to allow for size comparison.

Sticky-end ligation of pKOR1 and Bacteriocin Gene Cluster

The last alternative, in lieu of the blunt-end ligation and Gibson assembly approaches, was to try sticky-end ligation. This was achieved by digesting both the pKOR1 plasmid and the bacteriocin gene

cluster with the same restriction enzymes (Sbfl-HF and BseRI) to expose complementary overhangs that could be used to ligate the pieces together. Firstly, pKOR1 was digested with Sbfl-HF and BseRI and then purified using a kit (QIAquick PCR Purification). The purified product was placed on a gel and then only the digested plasmid was purified using a gel purification kit (QIAquick Gel Extraction Kit, Qiagen, Toronto, ON, Canada). Then, the bacteriocin gene cluster was amplified using specific primers containing an overhang with the Sbfl-HF and BseRI restriction sites. The forward primer was FSC803sticky and the reverse primer was RSC803sticky. The cycling conditions were a hot start for 2 min at 94°C, followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 30 s, extension 47°C for 7 min, with a final extension step at 68°C for 10 min. The PCR components consisted of 1 µl of the lysed *S. Chromogenes* 803 solution, 1 µl of 20 µM forward primer, 1 µl of 20 µM reverse primer, 45 µl platinum-taq PCR supermix high fidelity, and 2 µl of 4% DMSO were combined into a PCR tube. The PCR product was confirmed on an agarose gel by checking for the correct band size. Then, the PCR product was digested using Sbfl-HF and BseRI and then purified using the same PCR purification kit as above. Finally, the two fragments were ligated together using a quick ligation kit (NEB Quick Ligation Kit). There was 0.021 pmol of digested plasmid and 0.064 pmol of digested bacteriocin gene cluster. Furthermore, a negative control of just digested plasmid was performed to check for self-ligation. The reaction was then chilled on ice in preparation for chemical transformation to *E. coli* (NEB® 10-beta Competent *E. coli*). The competent *E. coli* was thawed on ice and then 1 µl of the ligation mixture was added to 50 µl of cells. The mixture was pipetted gently up and down and then placed on ice for 30 min without mixing. Then, a 42°C heat shock was performed for 30 s and then placed on ice once again for 5 min. After that, 950 µl of NEB 10-beta/stable outgrowth medium was added into the mixture and incubated at 30°C for 1 h in an incubator shaking at 225 rpm. Finally, the cells were 10-fold diluted and 100 µl diluted cells were plated on pre-warmed 30°C 100 µg/mL ampicillin THA plates and incubated at 30°C for 24 h.

Table 2.2.1: Primers associated with Objective 2

Primer	**Sequence (5'-3')**	**Target**
FSecY	TGGTCACCAAGCCATATCAA	*S. warneri* 2993
RSecY	CCGCATCTATCGTAATGCAA	*S. warneri* 2993
FRfpMtlf	TGAAGATGGTCAGTCATTCGC	*S. warneri* 2993
RRfpMtlf	CGCCGTCCTCAAAGTTCATT	*S. warneri* 2993
FRfpSC803	CACACGTCGTCGAAATGCATTCTTGCACCACCATCACCACTGAAC	pKOR1-*RFP* plasmid
RRfpSC803	TAAATGCAGAAAACAACGGGGTAAGTCATACGGACGACCTTCACC	pKOR1-*RFP* plasmid
FRfpSC803v2	CACACGTCGTCGAAATGCATTCTTGCACTGAACACGATGGATGCA	pKOR1-*RFP* plasmid
RRfpSC803v2	TAAATGCAGAAAACAACGGGGTAAGTCCTTCATACGGACGACCTT	pKOR1-*RFP* plasmid
FSC803	TGGGCAATGGCTTAGCTACCTGCACTTACCCCGTTGTTTTCTGC	*S. chromogenes* 803
RSC803	ACGCGTCGATCCTCAACGCACGCCCAAGAATGCATTTCGACGAC	*S. chromogenes* 803
RSC803v2	CACCAAAATGTACTGCTTTCATTATCAAGAATGCATTTCGACGAC	*S. chromogenes* 803
FCaspiMAY	CCGCTCTAGAACTAGTGGATCCCCCTGTTTGTAGTAGCGGTATTGGG	pCasSA G-block
RCaspiMAY	AGCTGGGTACCGGGCCCCCCCCTCGAGCTAATACACGTCTTAAACCTGC	pCasSA G-block
FSC803sticky	GATTACCTGCAGGCTTACCCCGTTGTTTTCTGC	*S. chromogenes* 803
RSC803sticky	GTCATGTTACGTAATCTCCTCCAAGAATGCATTTCGACGAC	*S. chromogenes* 803

Table 2.2.2: Strains and plasmids associated with Objective 2

Strain/Primer	Description	Reference
pKOR1	Traditional allelic replacement for *Staphylococcus* genetic editing	[53]
E. coli	NEB® 10-beta Competent	This study
S. warneri 2993 Δ*mtlf*	Mutant *S. warneri* 2993 with *mtlf* knockout	This study
S. chromogenes 803	Bacteriocin gene cluster donor	[83]
piMAY*	Allelic replacement plasmid for *Staphylococcus* genetic editing	[55]
pCasSA	CRISPR/Cas9 plasmid for *S. aureus*	[60]

Objective 3: Bacteriocin Gene Expression Characterization

RNA Extraction for Staphylococcus species

An overnight culture containing 10^{8-9} cells was centrifuged at 5000 g for 5 min. The supernatant was then removed and 1 mL of trizol was added and mixed to break up the cell wall and pellet. The cells were homogenized by vortexing for 1 min at maximum speed. After homogenizing the Trizol samples, 100 µL 1–bromo–3–chloropropane (BCP) was added to the sample in the fume hood due to its toxicity. The tubes were shaken vigorously for 15 s and then incubated for 3 min at room temperature. The tubes were then centrifuged for 15 min at 12,000 g at 4°C. The upper aqueous phase (300 to 500 µL) was then transferred to a new 1.5 mL tube, without touching the white DNA, until 50 uL was left. The aqueous phase was centrifuged for 15 min at 12,000 g at 4°C and the top 450 µL was removed and placed in a new tube, leaving behind 50 µL at the bottom of the tube, which consisted of carryover phenol or interphase DNA, for higher RNA purity. Then, 675 µL of 100% EtOH was added to the aqueous phase sample and mixed via pipetting. The sample was transferred to a Rneasy Mini column or minRNeasy

column. The column was centrifuged twice for 1 min at 14,000 g, discarding the flow-through between spins. The column was then transferred to a new collection tube and washed twice with 500 µL RPE buffer, using a 1-min centrifugation step first, then a 2-min step, both at 14,000 g. After the washes, the column was moved to a new collection tube and was centrifuged for 3 min at 14,000 g to remove residual EtOH. After that, the column was transferred to a 1.5 mL tube and the column tube was uncapped to air dry the silica membrane for 3 min. Then, 30 µL of Rnase-free water was applied directly onto the membrane and incubated for 5 min. The column was spun for 1 min at 14,000 g to elute the RNA. RNA was then measured in a NanoDrop spectrophotometer to determine RNA concentration and quality as judged by A260/230 ratios. The purified RNA was then frozen and stored at -80°C until future use.

DNase Treatment to Deplete gDNA Carryover in RNA Samples

After the extraction of RNA, 10 µL of 10X TURBO DNase buffer and 2 µL of TURBO DNase (2 units/µL) were added to a new 1.5 mL tube. Next, 5-10 µg of RNA were added to the reaction tube and the volume was then adjusted to 100 µL using RNase-free water. The sample was pulse-centrifuged and incubated at 37°C for 30 min. After this, an additional 2 µL of DNase was added to the reaction tube and the mixture was incubated for another 30 min.

RNA Cleanup and Concentration following DNase Digestion

After DNase digestion, 350 µL of RLT-2-mercaptoethanol solution was added to the sample and mixed by pipetting. To prepare fresh RLT-2-mEtOH, 10 µL of 2-mercaptoethanol was added for every 1 mL of RLT buffer. The solution was made fresh immediately before use, as it expires in one month. Afterward, 675 µL of 100% EtOH was added to the sample and mixed via pipetting once more. Half of the sample was applied to a RNeasy MinElute column and centrifuged for 1 min at 14,000 g. After

discarding the flow-through, the other half of the sample was applied and underwent the same centrifugation step. The column was then transferred to a new collection tube. It was then washed with 500 µL RPE buffer and centrifuged for 1 min at 14,000 g. The flow-through was discarded and 500 µL Rnase-free 75% EtOH was added to the column, which was centrifuged for 2 min at 14,000 g. The column was then transferred to a new collection tube and centrifuged for 6 min to remove residual EtOH. The column was moved to a new 1.5 mL tube and was uncapped for 3 min to air dry the membrane. Then, 15 µL of Rnase-free water was added directly onto the membrane and left to sit for 1 min. RNA was then eluted by centrifuging for 1 min at 14,000 g. RNA concentration and purity were estimated using A260/230 and A260/280 ratios, determined by NanoDrop spectrophotometry. Samples were frozen at -80°C until further processing.

cDNA Synthesis from DNase-treated RNA

To convert RNA into cDNA, a cDNA synthesis kit (BioRad iScript gDNA Clear cDNA Synthesis Kit, Saint-Laurent, Quebec, Canada) was used. The reagents included 5 ng of RNA, 4 µL of iScript reverse transcription supermix, and nuclease-free water up to 20 µL. There were three negative controls which included the no-reverse transcriptase control, a nuclease-free water control, and RNA from wild-type *S. warneri* 2993. The mixture is then added into PCR tubes and placed into a thermocycler with the following conditions: 25°C for 5 min, 46°C for 20 min, 95°C for 1 min, and a holding temperature of 4°C.

RT-qPCR for Bacteriocin Gene Cluster Characterization

Three sets of primers were designed targeting different sections of the bacteriocin gene cluster. The first set of primers QFLanP and QRLanP targeted an area overlapping *LanP* and the methyltransferase gene. The second set of primers QFLanA1 and QRLanA1 targeted *LanA1* which encodes the first bacteriocin gene. The final set of primers QFLanA2 and QRLanA2 targeted *LanA2* which

encodes the second bacteriocin gene. The primers were designed based on the following three criteria: a primer size of 20 bp, a GC content of 50%, and amplicons of 200 bp or smaller. The following master mix was for 1 samples: 10 µL supermix, 1 µL 10 uM forward and reverse primer, and 7 µL nuclease-free water. Then, 19 µL of master mix was pipetted into a 96-well PCR plate. Then, 1 µL of cDNA or negative control sample was placed in its respective well. The 96-well plate was gently mixed and centrifuged at 300 g for 2 min. Then, the plate was placed inside a CFX96 Touch System with the following RT-qPCR conditions: 94°C for 3 min, then 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 20 s, and then a melt curve from 65 to 95°C with 0.5°C increments every 5 s. The Cq values were recorded to allow comparison of gene expression between the samples.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) for Bacteriocin Gene Cluster

The target bacteriocin gene cluster came from *S. chromogenes* 803 and is composed of 6 different genes. These genes included LanA1, LanA2, LanM, NADPH-dependent FMN reductase, LanP, and finally a methyltransferase. To confirm that the genes were being expressed, RT-qPCR was used as it can accurately measure gene expression levels since it is highly sensitive and specific. Three genes in total were measured and they were LanA1, LanA2, and a section overlapping both LanP and the methyltransferase gene. Three replicates were performed on *S. chromogenes* 803, *S. chromogenes* 803 no reverse transcriptase (NRT), *S. Warneri* 2993, *S. warneri* 2993 NRT, and water.

In RT-qPCR, there is a detection point at which your reaction exceeds a fluorescent intensity above the background threshold. The Cq value refers to the quantification cycle of which the fluorescent signal from the amplified sample crosses that threshold. It essentially measures the relative abundance of specific RNA molecules in your sample. A lower Cq value means a higher abundance of target RNA while a higher CQ value refers to a lower abundance of target DNA. For the RT-qPCR detecting LanA1,

the average Cq value for *S. chromogenes* 803 was 22.96 while for the NRT, the average was 39.82. *S. warneri* 2993 had two cq values of 39.89 and 39.93 with the last replicate being N/A. The NRT *S. warneri* 2993 had one cq value of 40.36 with the remaining two replicates being N/A. Lastly, for the water negative control, all three values were N/A.

For the RT-qPCR detecting LanA2, the average Cq value for *S. chromogenes* 803 was 22.54 while for the NRT, the average was 39.78. *S. warneri* 2993 had an average Cq of 37.98 while the NRT's cq was 38.16. Lastly, similar to LanA1, the water negative control had three Cq values of N/A.

For the RT-qPCR detecting the region spanning across LanP and the methyltransferase, the average Cq value for *S. chromogenes* 803 was 23.42. The cq values for *S. chromogenes* 803 NRT, *S. warneri* 2993, *S. warneri* 2993 NRT, and water were all N/A.

Table 2.3.1: Primers associated with Objective 3

Primer	**Sequence (5'-3')**	**Target**
QFLanP	CTGGGAGCACCTCTACATAATC	*S. chromogenes* 803
QRLanP	AGCAAAGTCACTGGGTGTAA	*S. chromogenes* 803
QFLanA1	CGCCTGTACGACGAATTAAGA	*S. chromogenes* 803
QRLanA1	CACAAGCAGCACAACATCAA	*S. chromogenes* 803
QFLanA2	GCAACACGCTCAACGATTTC	*S. chromogenes* 803
QRLanA2	GAAGGTGACTCAGGTCTTCTAAC	*S. chromogenes* 803

Results

Objective 1: Identify a Recipient NAS Strain for Bacteriocin Gene Clusters Genomic Integration

Persistent NAS from Mixture 1 Infusion into Rear Left Quarter

Isolates of four different NAS species were infused into the rear left quarter of three lactating dairy cows, specifically *S. chromogenes* 5978, *S. xylosus* 5435, *S. warneri* 1052, and *S. devriesei* 1316. The

actual inoculum of cells for each NAS isolate was 2,660, 388, 6,658, and 150,079 cells, respectively. The first three NAS isolates were detected on CHROMagar plates after milk culturing only once throughout the 14-day trial on day 6, 4, and 5, respectively (Figure 3.1.0). In contrast, *S. devriesei* 1316 was positive 22 out of 42 milk sample cultures from the three cows' milk samples (Figure 3.1.0). Throughout the entire trial for cows 716, 1021, and 1088, the average log (CFU)/mL was 0.71, 1.76, and 0.95, respectively. Furthermore, the last day *S. devriesei* 1316 grew from the milk cultures on CHROMagar from those three cows was on day 6, 14, and 14 with a log (CFU) of 1.30, 2.73, and 2.79, respectively. The total bacterial count without discriminating between species was 3.70 log (CFU)/mL. This quarter had the highest total bacterial count with an average 14-d log (CFU)/mL of 3.70 in comparison to the other quarters. Additionally, it also had the highest total bacterial count on day 14 with a log (CFU)/mL of 5.52.

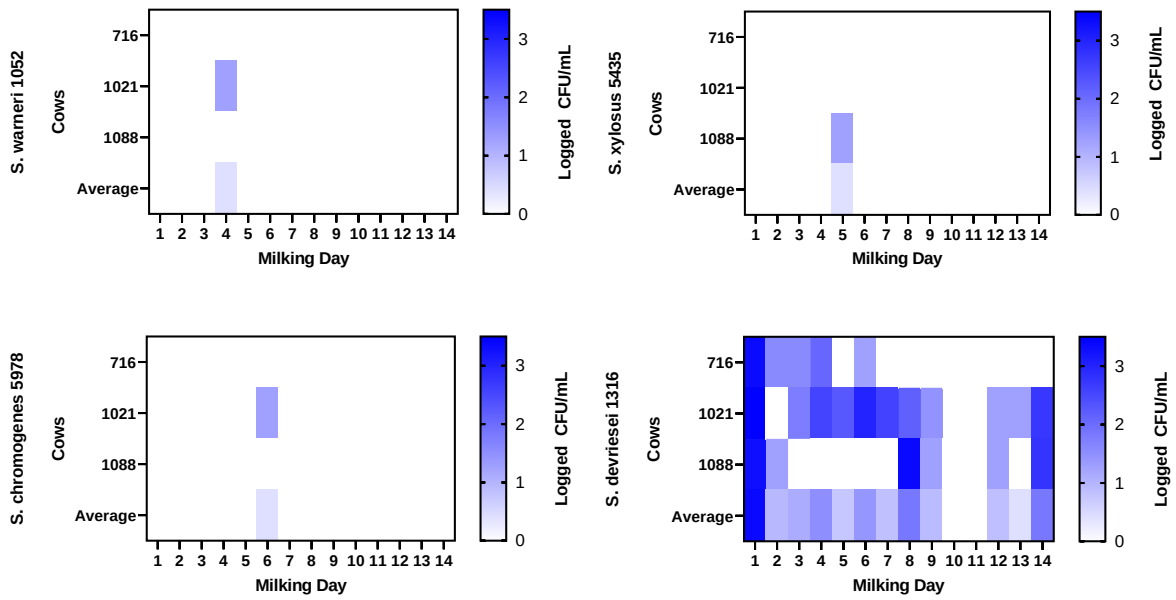

Figure 3.1.0: Heatmap of the quantities of *S. chromogenes* strain 5978, *S. warneri* strain 1052, *S. xylosus* strain 5435, and *S. devriesei* strain 1316 (log (CFU)/ml) in milk samples of 3 cows (IDs 716, 1021 and 1088) intramammary infused at day 0 with a mixture of NAS species (*S. chromogenes* 5978, *S. xylosus* 5435, *S. warneri* 1052, and *S. devriesei* 1316) for 14 days after inoculation. Quarter milk (100

μL) was plated on CHROMagar and incubated at 37°C for 2 days (n=1).

Differences in virulence genes, growth rate, NAS interactions, and initial cells of NAS in Mixture 1

Mixture 1 contained *S. chromogenes* 5978, *S. xylosus* 5435, *S. warneri* 1052, and *S. devriesei* 1316, and after the 14-day trial, *S. devriesei* 1316 was concluded to be the most persistent strain in this mixture. Naushad et al. compared the virulence gene profile of 441 NAS isolates based on their whole-genome sequence and use of virulence databases to find differences in virulence factor (VF) genes between these 4 NAS isolates[101]. It was important to note that the NAS isolates used in the study were the same as the ones in the mixture. There was a total of 191 different VFs analysed distributed into different functional categories such as adherence (n = 28), exoenzymes (n = 21), immune evasion (n = 20), iron metabolism (n = 29), and secretion/toxins (n = 93). *S. chromogenes* 5978, *S. xylosus* 5435, *S. warneri* 1052, and *S. devriesei* 1316 had a total of 35, 29, 36, and 37 VF genes (Table 3.1.0). In terms of adherence VFs for *S. chromogenes* 5978, *S. xylosus* 5435, *S. warneri* 1052, and *S. devriesei* 1316, they had 4, 6, 5, and 7, respectively. In terms of exoenzyme VFs for the four isolates in the same order, they had 5, 5, 10, and 5, respectively. In terms of immune evasion VFs for the four isolates in the same order, they had 9, 6, 5, and 14, respectively. For the iron uptake VFs, the four isolates in the same order had 13, 12, 13, and 13 respectively. Finally, for secretion/toxin VFs, they had 4, 0, 1, and 0, respectively.

When the density of cells between the four NAS isolates was studied, it was found that S. devriesei did have the largest concentration of cells when grown at an OD_{600} of 0.3. For instance, when comparing *S. chromogenes* 5978, *S. xylosus* 5435, *S. warneri* 1052, and *S. devriesei* 1316, the CFU/mL were 3×10^8, 1.8×10^8, 2.2×10^8, and 3.8×10^8, respectively. However, this difference was considered negligible as it was only a 2.1x fold when comparing *S. devriesei* 1316 to the slowest-growing isolate.

When all four isolates were grown together on CHROMagar at an OD600 of 0.3 after a 10^{-5} dilution, the proportion of colonies was not equal. When comparing *S. chromogenes* 5978, *S. xylosus*

5435, *S. warneri* 1052, and *S. devriesei* 1316, the average percentages of colonies were 12.2, 6.1, 59.9, and 21.9%, respectively. The dominant NAS isolate was *S. warneri* 1052.

Due to a pipetting mistake and judgement error that affected the correct quantification of CFU/mL, each NAS isolate did not contain 250 000 cells as planned. This mistake was realized after the end of both trial 1 and 2, unfortunately. When comparing the initial cell counts of *S. chromogenes* 5978, *S. xylosus* 5435, *S. warneri* 1052, and *S. devriesei* 1316, their number of cells were 2,660, 388, 6,658, and 150,079, respectively. *S. devriesei* 1316 had the highest initial cell count and when directly compared to *S. chromogenes* 5978, *S. xylosus* 5435, and *S. warneri* 1052, it was 56-fold, 387-fold, and 23-fold higher, respectively. Given that no other marked differences between the strains were identified, this difference in initial amount in the inoculum was the likely reason why *S. devriesei* had better persistence than the other strains.

Table 3.1.0. The VFs genes from each NAS isolate in mixture 1

Mixture 1	*S. chromogenes* 5978	*S. devriesei* 1316	*S. warneri* 1052	*S. xylosus* 5435
Adherence	*capC, hysA, cap5H, capJ*	*hysA, cap5H, ebp, cap8N, sbnI*	*scn, sspA, geh, coa, icaA, icaC, icaR*	*scn, sspA, sdrD, sdrD, set5, tsst-1*
Exoenzyme	*sspA, atl, splF, cap5A, capC*	*fnbA, capM, cap8H, cap8I, SSP0069*	*sspA, splC, cap8J, cap8K, capF, capG, esaA, cap8K, nuc, capI*	*coa, ebh, sdrE, capP, splD*

Host Immune Evasion	*capO, splE, cap8P, capC, htsC, I, ebh, icaB, icaB*	*SAOUHSC_00129, capF, scn, spa, spa, spa, isdG, lukS.I, ebh, clfA, clfA, clfA, eap/map*	*ebp, geh, cap8O, sbnC, sfaA*	*splF, isdH, cap8I, capB, adsA, sbnB*
Iron Uptake	*sdrD, sdrD, sdrD, sspA, hysA, lip, geh, geh, coa, coa, coa, coa, sak*	*fnbA, fnbA, icaC, sdrC, sdrC, sspA, sspA, hysA, hysA, lip, lip, geh*	*ebh, ebh, ebh, fnbB, icaB, icaD, icaR, sdrC, sspA, sspA, sspB, sspC, sspC*	*clfB, clfB, clfB, map, ebhA, efb, efb, icaB, icaC, sdrC, sdrE, sdrE*
Secretion System/Toxin	*eta, lukF-PV, set5, tsst-1*		*lukE*	

Persistent NAS from Mixture 2 Infusion into Rear Right Quarter

Isolates of four different NAS species were infused into the rear left quarter of three lactating dairy cows, specifically *S. arlettae* 5134, *S. hominis* 2694, *S. devriesei* 4143, and *S. pasteuri* 2657. The actual inoculum of cells for each NAS isolate was 185,980, 7,916, 25, and 8,000 cells respectively. *S. hominis* 2694 and *S. devriesei* 4143 grew only once during CHROMagar milk culturing throughout the 14-d trial at day 5 (Figure 3.1.1). *S. arlettae* 5134 had more persistence in the mammary gland as it grew 4 out of 42 times when culturing milk samples on CHROMagar (Figure 3.1.1). *S. pasteuri* 2657 was the most persistent strain in this mixture with 9 out of 42 positive milk sample cultures (Figure 3.1.1). More importantly, it grew on CHROMagar during day 14 for cow 1021 and 1088 with log (CFU)/mL of 2.18 and 1.30 respectively. The average log (CFU)/mL throughout the trial for the cow 716, 1021, and 1,088 was

0, 0.51, and 0.54, respectively. This quarter had the lowest total bacterial count with an average 14-day log (CFU)/mL of 1.76. However, it had the second highest total bacterial count on day 14 with a log (CFU)/mL of 3.48.

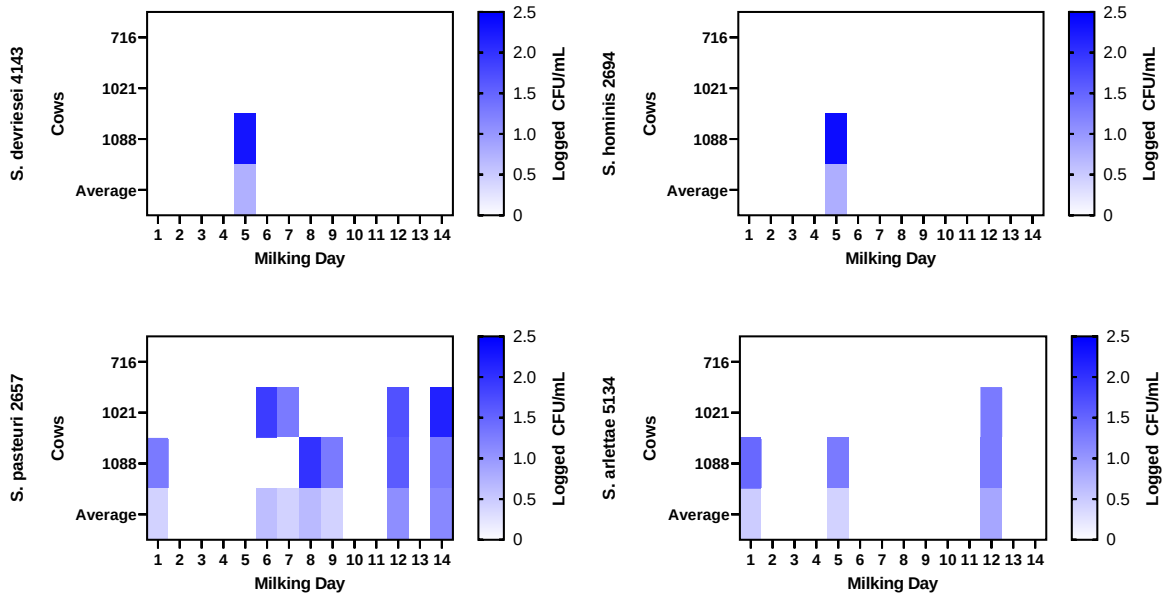

Figure 3.1.1: Heatmap of the quantities of *S. hominis* strain 2694, *S. devriesei* strain 4143, *S. arlettae* strain 5134, *S. pasteuri* strain 2657 (log (CFU)/ml) in milk samples of 3 cows (IDs 716, 1021 and 1088) intramammary infused at day 0 with a mixture of NAS species (S. *arlettae* 5134, *S. hominis* 2694, *S. devriesei* 4143, and *S. pasteuri* 2657) for 14 days after inoculation. Quarter milk (100 μL) was plated on CHROMagar and incubated at 37°C for 2 days (n=1).

Differences in virulence genes, concentration of cells, NAS interactions, and initial cells of NAS in Mixture 2

Mixture 2 contained *S. arlettae* 5134, *S. hominis* 2694, *S. devriesei* 4143, and *S. pasteuri* 2657 and after the 14-day trial, *S. pasteuri* 2657 was found to be the most persistent strain in the mixture.

Based on Naushad et al., *S. pasteuri* 2657 had the most virulence genes[101]. When comparing the total number of virulence genes for *S. arlettae* 5134, *S. hominis* 2694, *S. devriesei* 4143, and *S. pasteuri* 2657, they were 55, 31, 35, and 58 respectively (Table 3.1.1). In terms of adherence VFs for *S. arlettae* 5134, *S. hominis* 2694, *S. devriesei* 4143, and *S. pasteuri* 2657, they had 4, 4, 6, and 11 respectively. For the exoenzymes, there were 4, 4, 4, and 7 VFs following the same order as above. For the immune evasion VFs, there were 23, 13, 13, and 18 respectively. For iron uptake and secretion VFs, there were 18, 10, 12, and 22, respectively.

When looking at the density of cells between the four NAS isolates, *S. pasteuri* 2657 was found to have the highest concentration of cells when grown at an OD600 of 0.3. When comparing *S. arlettae* 5134, *S. hominis* 2694, *S. devriesei* 4143, and *S. pasteuri* 2657, the CFU/mL was 1.7×10^8, 6.3×10^7, 7.2×10^7, and 8.8×10^8 respectively. There was a 14-fold difference in the concentration of cells when comparing the highest, *S. pasteuri* 2657, with *S. hominis* 2694, the isolate that had the lowest concentration of cells.

Growing all four isolates together at an OD_{600} of 0.3 after a 10^{-5} dilution did not result in an equal proportion of colonies on CHROMagar plates. When comparing *S. arlettae* 5134, *S. hominis* 2694, *S. devriesei* 4143, and *S. pasteuri* 2657, their percentages of colonies was 25.1%, 3.2%, 56.6%, and 15.1% respectively. The dominant NAS in terms of proportion was *S. hominis* 2694.

Lastly, another big difference was the initial amount of NAS cells per isolate in the mixture. When comparing the true initial cell counts of *S. arlettae* 5134, *S. hominis* 2694, *S. devriesei* 4143, and *S. pasteuri* 2657, their number of cells were 185,980, 7,916, 26, and 8,000. Surprisingly, *S. pasteuri* 2657 did not have the highest initial number of cells as it was 23-fold lower than *S. arlettae* 5134. For this reason, the initial number of cells was not the reason *S. pasteuri* 2657 was the most persistent strain.

Overall, it was difficult to point to a specific difference that caused *S. pasteuri* 2657 to be the most persistent strain. It was likely multifactorial, coming from the combination of differences and

interactions inside the cow's microbiome leading to this outcome.

Table 3.1.1. The VFs genes from each NAS isolate in mixture 2

Mixture 2	*S. devriesei* 4143	*S. hominis* 2694	*S. arlettae* 5134	*S. pasteuri* 2657
Adherence	*ebh, cap8K, capK, spa, sdrF, sspC*	*cap8K, capK, esaA, spa*	*cap8K, capK, sspB, lip*	*icaA, SSP0068, ebp, fnbB, sspA, splF, cap8H, sdrG, spa, isdA, essC*
Exoenzyme	*SSP0065, ebp, fnbB, icaA*	*isdA, capE, clfB, ebp*	*cap8G, SSP0071, isdB, clfB*	*splB, coa, capC, capD, sbi, capL, sasC*
Host Immune Evasion	*sdrE, sspA, sspA, sspA, sspB, hysA, lip, lip, splB, splD, splF, nuc, nuc*	*fnbA, icaD, sdrC, sdrC, sdrC, sdrD, sdrD, sdrE, sdrF, gehD, splA, splD, splE*	*ebh, ebh, ebp, icaA, icaA, icaA, icaA, icaB, icaB, icaC, icaC, icaD, icaR, sdrE, sasG, sspA, hysA, hysA, lip, geh, geh, splA, splC*	*sbnA, atl, atl, atl, clfA, eap/map, ebp, efb, fnbA, fnbA, fnbA, fnbB, fnbB, fnbB, icaC, sdrE, sdrG, sraP*
Iron Uptake	*cap8C, cap8H, cap8H, capB, capD, capF, capF, capL, capL, capN, capO, capO*	*cap5C, cap5E, cap5G, cap8K, capA, capA, capG, capH, capI, capJ*	*cap5H, cap5M, cap5N, cap5O, capP, cap8J, cap8K, cap8L, cap8M, cap8N, capA, capB, capC, capE, capF, capG*	*geh, geh, geh, geh, splA, splB, splB, splC, splC, splC, splD, splF, coa, coa, sak, nuc, nuc, nuc, nuc, nuc*

Secretion System/Toxin			*SSP0063, SSP0068, esaA, esaC, essB, essB, eta, sbnI*	*lukM, uafA*

Persistent NAS from Mixture 3 Infusion into Front Left Quarter

Isolates of four different NAS species were infused into the rear left quarter of three lactating dairy cows, specifically *S. warneri* 2140, *S. caprae* 4023, *S. equorum* 2121, and *S. warneri* 2993. The actual inoculum of cells for each NAS isolate was 6038, 1678, 34833, and 2330 cells respectively. The first two NAS isolates grew only once during CHROMagar milk culturing throughout the 14-day trial at day 3 and 12 respectively (Figure 3.1.2). *S. caprae* 4023 grew twice from the CHROMagar milk culturing throughout all the milk samples specifically at day 1 for cow 716 and 1088 (Figure 3.1.2). The most persistent strain in mixture 3 was *S. warneri* 2993 as it grew 10 out of 42 times from CHROMagar milk culturing. The latest milk culturing containing *S. warneri* 2993 was on day 9 and 14 for cow 1021 and 1088 respectively (Figure 3.1.2). Its log (CFU)/mL in those milk samples were both 1.30. Lastly, the average CFU/mL throughout the trial for cows 716, 1021, and 1088 were 0, 0.74, and 0.57, respectively. This quarter had the second highest total bacterial count with an average 14-day log (CFU)/mL of 1.99. Interestingly, this quarter had the highest total bacterial count on day 1 with a log (CFU)/mL of 14.58.

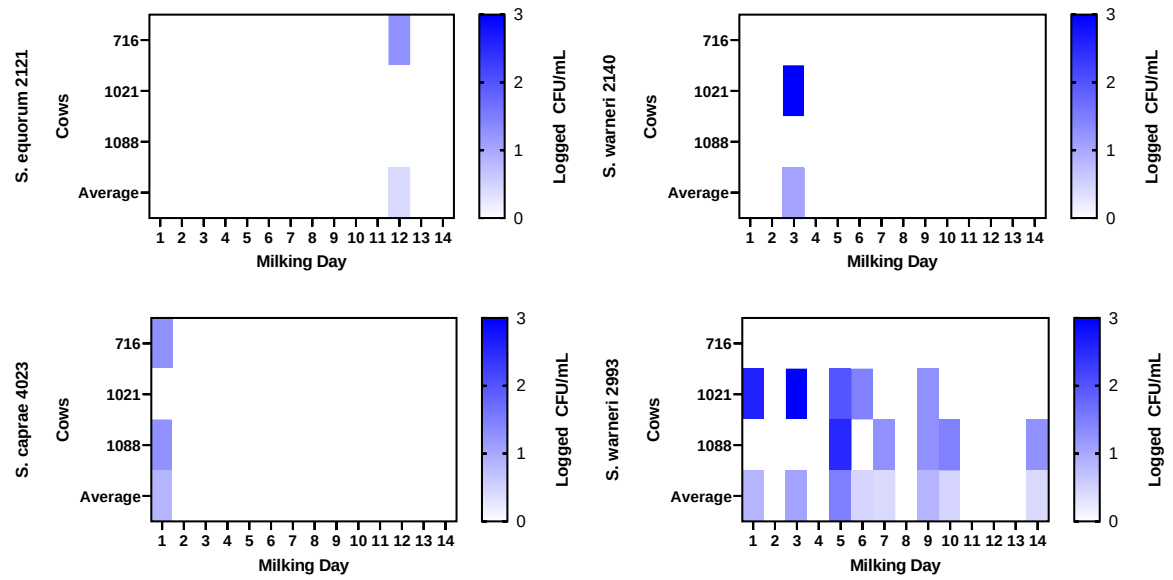

Figure 3.1.2: Heatmap of the quantities of *S. warneri* strain 2140, *S. equorum* strain 2121, *S. caprae* strain 4023, and *S. warneri* strain 2993 (log (CFU)/ml) in milk samples of 3 cows (IDs 716, 1021 and 1088) intramammary infused at day 0 with a mixture of NAS species (*S. warneri* 2140, *S. caprae* 4023, *S. equorum* 2121, and *S. warneri* 2993) for 14 days after inoculation. Quarter milk (100 μL) was plated on CHROMagar and incubated at 37°C for 2 days (n=1).

Differences in virulence genes, concentration of cells, NAS interactions, and initial cells of NAS in Mixture 3

Mixture 3 contained *S. warneri* 2140, *S. caprae* 4023, *S. equorum* 2121, and *S. warneri* 2993 and after the 14-day trial, *S. warneri* 2993 was found to be the most persistent strain in the mixture. Following the same virulence gene study by Naushad et al., it was discovered that *S. warneri* 2993 did not have the highest number of virulence genes or many unique virulence genes[101]. When comparing the total number of virulence genes between *S. warneri* 2140, *S. caprae* 4023, *S. equorum* 2121, and *S.*

warneri 2993, they had 36, 52, 47, and 37, respectively (Table 3.1.2). For the adherence VFs, there were 6, 12, 5, and 8 following the same order of isolates as above. For the exoenzyme VFs, there were 11, 8, 3, and 10 respectively. For the immune evasion VFs, there were 5, 7, 17, and 6 respectively. Lastly for the iron uptake and secretion VFs, there was 14, 19, 22, and 13 respectively.

When examining the density of cells between the four NAS isolates, *S. caprae* 4023 was found to have the highest concentration of cells when grown at an OD600 of 0.3. When comparing *S. warneri* 2140, *S. caprae* 4023, *S. equorum* 2121, and *S. warneri* 2993, their CFU/mL were 1.7×10^8, 6.4×10^8, 9.3×10^8, and 3.3×10^8, respectively.

Growing all four isolates together at an OD_{600} of 0.3 after a 10^{-5} dilution did not result in an equal proportion of colonies on CHROMagar plates. When comparing *S. warneri* 2140, *S. caprae* 4023, *S. equorum* 2121, and *S. warneri* 2993, the average percentage of colonies were 9.6, 62.2, 8.4, and 19.9%. *S. caprae* 4023 had the highest proportion of colonies with *S. warneri* 2993 having the second highest.

Lastly, there was a difference in the initial amount of NAS cells per isolate in the mixture. When comparing the true initial cell counts of *S. warneri* 2140, *S. caprae* 4023, *S. equorum* 2121, and *S. warneri* 2993, their number of cells were 6,038, 1,678, 34,833, and 2,330, respectively.

Overall, it was difficult to point to a specific difference that caused *S. warneri* 2993 to be the most persistent strain. It was likely multifactorial coming from the combination of differences and interactions inside the cow's microbiome leading to this result.

Table 3.1.2. The VFs genes from each NAS isolate in mixture 3

Mixture 3	*S. warneri* 2140	*S. warneri* 2993	*S. equorum* 2121	*S. caprae* 4023

Adherence	*atlC, ebh, sdrC, sdrF, sdrH, sasC*	*atlC, ebh, ebh, sdrC, sdrF, sdrG, sdrH, sasC*	*atlC, ebh, clfA, ebp, icaC*	*ebh, cap8I, capJ, sbnI, sdrC, atl, atlE, clfA, clfB, clfB, ebp, edin-B*
Exoenzyme	*adsA, sspA, sspA, sspB, sspB, sspC, aur, lip, geh, splE, nuc*	*adsA, sspA, sspB, sspB, sspC, aur, lip, geh, splE ,nuc*	*sdrC, sdrE, splE*	*efb, fnbA, icaA, icaB, sdrC, sspA, lip, lip*
Host Immune Evasion	*cap8J, cap8P, capC, capM, SSP0065*	*cap8J, cap8P, capC, capE, capM, SSP0064*	*cap5L, cap5P, cap8A, cap8C, cap8D, cap8I, capD, SaurJH1_0143, capF, capG, capJ, capK ,capL, capL, capL, capL, capM*	*splF, cap5I, capP, cap8B, cap8L, cap8N, capC*
Iron Uptake	*htsA, htsB, htsC, isdA, isdI, sirB, sbnA, sbnB, sbnD, sfaA, sfaB, sfaC, sfaD*	*htsA, htsB, htsC, isdI, sirB, sbnA, sbnB, sbnD, sfaA, sfaB, sfaC, sfaD*	*SSP0071, SSP0072, sbi, spa, spa, isdA, isdB, isdC, isdD, isdF, isdF, isdG, isdH, sirA, sirC, srtB, srtB, sbnE, sbnF, sbnG*	*capI, capJ, capK, capN, capN, capO, capP, SSP0060, SSP0062, SSP0065, SSP0067, SSP0069, SSP0070, chp, spa, spa, isdA, isdD*
Secretion System/Toxin	*SACOL0507*	*SACOL0507*	*eta, sbnI*	*isdE, esaA, esaC, essA, essC, essC, etb*

Persistent NAS from Mixture 4 Infusion into Front Right Quarter

Isolates of four different NAS species were infused into the rear left quarter of three lactating dairy cows, specifically *S. cohnii* 1091, *S. devriesei* 4438, *S. pasteuri* 2044, and *S. warneri* 4140. The actual inoculum of cells for each NAS isolate was *S. pasteuri* 2044 was the least persistent only grew from 2 out of the 42 CHROMagar milk culturing samples (Figure 3.1.3). These two growths from milk were found at day 1 and day 6 for cow 1088 and 1021 respectively. *S. cohnii* 1091 and *S. devriesei* 4438 both grew 4 out of 42 times from CHROMagar milk culturing (Figure 3.1.3). Their latest growth from milk culturing were at day 6 in cow 1021 and day 11 in cow 1088 respectively. *S. warneri 4140* was the most persistent strain in this mixture but was less persistent compared to the NAS isolates from mixture 1, 2, and 3. It only grew 5 out of 42 times from the CHROMagar milk culturing with the latest growth on day 6 (Figure 3.1.3). Its log (CFU)/mL on day 6 was 1.78. This quarter had the third highest total bacterial count with an average 14-day log (CFU)/mL of 1.83.

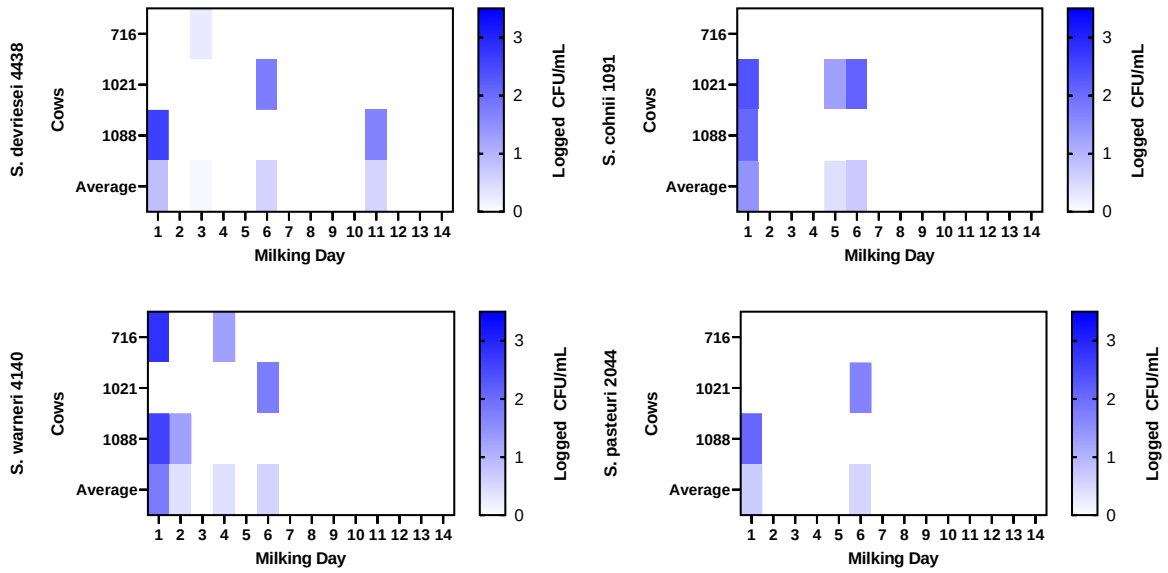

Figure 3.1.3: Heatmap of the quantities of *S. pasteuri* strain 2044, *S. cohnii* strain 1091, *S. devriesei* strain 4438, *S. warneri* strain 4140 (log (CFU)/ml) in milk samples of 3 cows (IDs 716, 1021 and 1088)

intramammary infused at day 0 with a mixture of NAS species (*S. cohnii* 1091, *S. devriesei* 4438, *S. pasteuri* 2044, and *S. warneri* 4140) for 14 days after inoculation. Quarter milk (100 μL) was plated on CHROMagar and incubated at 37°C for 2 days (n=1).

Differences in virulence genes, concentration of cells, NAS interactions, and initial cells of NAS in Mixture 4

Mixture 4 contained *S. cohnii* 1091, *S. devriesei* 4438, *S. pasteuri* 2044, and *S. warneri* 4140, and after the 14-day trial, it was found that *S. warneri* 4140 was the most persistent strain. According to a virulence gene study by Naushad et al., *S. warneri* 4140 had 38 out of 191 possible virulence factors (VFs), while S. cohnii 1091, S. devriesei 4438, and S. pasteuri 2044 had 34, 37, and 62 VFs, respectively (Table 3.1.3). For the adherence VFs for *S. warneri* 4140, *S. pasteuri* 2044, *S. devriesei* 4438, and *S. cohnii* 1091, there were 8, 8, 4, and 4, respectively. For the exoenzyme VFs, there were 10, 7, 5, and 5 following the same order as above. For the immune evasion VFs, there were 6, 26, 16, and 6 respectively. Lastly, for the iron uptake and secretion VFs, there were 14, 21, 12, and 13, respectively.

The density of cells of the four NAS isolates showed that *S. warneri* 4140 had the highest concentration of cells when grown at an OD_{600} of 0.3, with a CFU/mL of 3.9×10^8, compared to 2.0×10^8, 9.0×10^7, and 1.6×10^8 for *S. cohnii* 1091, *S. devriesei* 4438, and *S. pasteuri* 2044, respectively.

Growing all four isolates on CHROMagar at an OD_{600} of 0.3 after a 10^{-5} dilution did not result in an equal percentage of colonies, with 11.3, 26.8, 31.0, and 30.9% for *S. cohnii* 1091, *S. devriesei* 4438, *S. pasteuri* 2044, and *S. warneri* 4140, respectively.

Lastly, there was a difference in the initial amount of NAS cells per isolate in the mixture. When comparing the true initial cell counts of *S. cohnii* 1091, *S. devriesei* 4438, *S. pasteuri* 2044, and *S. warneri* 4140, their number of cells were 30,674, 18,292, 38,305, and 226,744 respectively.

Overall, the reason for *S. warneri* 4140 being the most persistent strain was likely due to its 10-

fold higher initial cell count in the infusion.

Table 3.1.3. The VFs genes from each NAS isolate in mixture 4

Mixture 4	*S. warneri* 4140	*S. pasteuri* 2044	*S. devriesei* 4438	*S. cohnii* 1091
Adherence	*atlC, ebh, ebh, ebh, sdrF, sdrG, sdrH, sasC*	*aur, atl, ebh, ebp, edin-B, efb, icaD, icaR*	*atl, ebh, sbnF, atl*	*atl, ebh, cap8K, sdrG*
Exoenzyme	*adsA, sspA, sspB, sspB, sspC, aur, lip, geh, splE, nuc*	*sdrC, sdrD, sdrE, sdrE, sdrI, geh, splE*	*ebh, sdrD, sdrE, sspB, sspB*	*clfA, clfA, eap/map, ebh, sdrC*
Host Immune Evasion	*cap8J, cap8P, capC, capE, capM, SSP0065*	*sak, vWbp, cap5A, cap5E, cap5K, cap8B, cap8D, cap8G, cap8I, cap8K, cap8O, capB, capC, capD, capD, SAOUHSC_00117, SAOUHSC_00129, SAS0139, SaurJH1_0143, capF, capG, capK, capL, capL, capN, capO*	*lip, splB, splC, splF, splF, coa, coa, sak, cap5J, cap8A, cap8H, cap8I, capB, capB, capC, capD*	*lip, splF, cap5F, cap5G, cap8E, cap8F*
Iron Uptake	*htsA, htsB, htsC, isdI, sirB, sbnA, sbnB, sbnD,*	*SSP0070, SSP0071, SSP0072, scn, spa, spa, spa, spa, spa, spa, isdA,*	*capJ, capK, capK, SSP0062, SSP0064,*	*SAOUHSC_00118, SAS0137, SaurJH9_0150,*

	sfaA, sfaB, sfaC, sfaD	*isdB, isdD, isdF, isdG, srtB, srtB, sbnC, sbnE, sbnF*	*SSP0069, sbi, isdD, isdE, isdF, isdH, sirA*	*capI, capM, capN, SSP0060, chp, spa, spa, isdA, isdC*
Secretion System/Toxin	*hld-II, SACOL0507*	*lukS.I*		*esaA, esaA, esaC, essA, essC, essC, eta*

Comparison of Three Persistent Strains from the NAS Mixtures

The three most persistent strains were *S. devriesei* 1316, *S. pasteuri* 2657, and *S. warneri* 2993. S. devriesei 1316 was the most persistent out of the three as it had the highest 14-day average bacterial count from CHROMagar milk culturing, alongside the highest day 1 and 14 bacterial counts on CHROMagar milk culturing. The respective bacterial counts were 3.43, 10.12, and 5.52 log (CFU)/mL. *S. warneri* 2993 had the second highest 14-day average bacterial count from the milk culturing with a log (CFU)/mL of 1.32. This leaves S. pasteuri 2657 with the lowest 14-day average bacterial count from milk culturing with a log (CFU)/mL of 1.04. Despite this, it had a higher day 14 bacterial count than *S. warneri* 2993 with a log (CFU)/mL difference of 2.1.

Tracking the growth of the three persistent strains on CHROMagar from day 1 to 14 showed a difference between strains. *S. devriesei* 1316 and *S. warneri* 2993 showed a slight decrease in growth as the data points were trending smaller in terms of log (CFU)/ml (Figure 3.1.4). Interestingly, *S. pasteuri* 2657 showed a slight increase in growth over the course of the 14 days (Figure 3.1.4). However, these data points were extrapolated and do not show significance.

The number of cells per volume (or concentration of cells) obtained from cultures with an OD600 of 0.3 were similar for the three strains. At that specific OD, the CFU/mL for *S. devriesei* 1316, *S. pasteuri* 2657, and *S. warneri* 2993 was 3.8 x 10^8, 8.8 x 10^8, and 3.3 x 10^8 respectively.

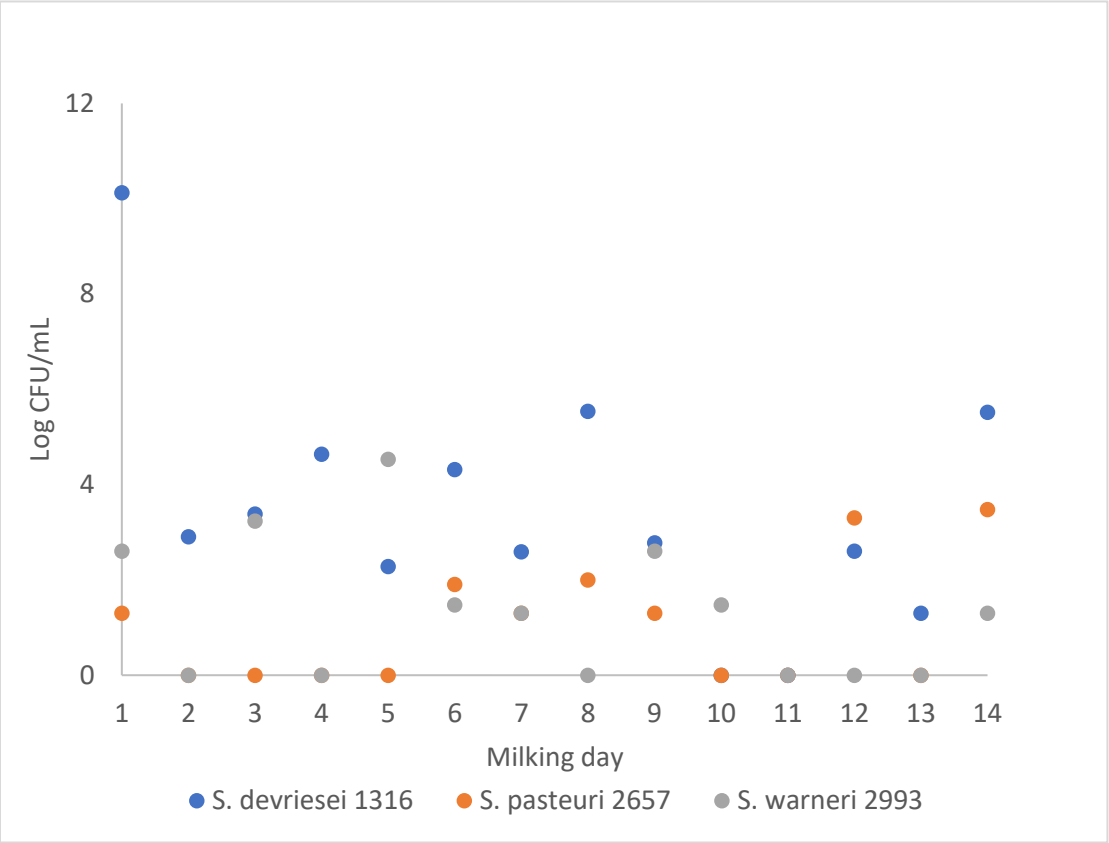

Figure 3.1.4: Scatter plot of the quantities (log (CFU)/mL) of the three persistent strains: *S. devriesei* 1316, *S. pasteuri* 2657, and *S. warneri* 2993, in milk samples of 3 cows infused at day 0 with a mixture of NAS species for 14 days after inoculation. Quarter milk (100 µL) was plated on CHROMagar and incubated at 37°C for 2 days (n=1).

NAS Mixture Effect on Somatic Cell Count in Milk

The NAS mixture inoculated in the rear left quarter of 3 cows resulted in an overall increase in

somatic cell count comparing day 0 to day 14 (Figure 3.1.5). When comparing day 0 to 7, all three cows' SCC increased but more importantly, two of the cows exceeded the subclinical mastitis threshold of 200 000 cells. The two cows, 716 and 1021, had a SCC of 1,178,000 and 796,000 cells, respectively. From day 7 to 14, only cow 1088 increased in SCC past the subclinical mastitis threshold. Interestingly, the other two cows' SCC dropped. Cow 716 had the largest drop, bringing it below the subclinical mastitis threshold while cow 1021 remained above the threshold.

The NAS mixture inoculated in the rear right quarter of 3 cows resulted in an overall increase in SCC comparing day 0 to day 14 (Figure 3.1.6). However, despite this increase, all three cows' SCC remained below the subclinical mastitis threshold for the entirety of the trial. Cows 716 and 1088 had an upward trend in SCC from day 0, 7, and 14. Cow 1021 had an upward SCC trend from day 0 to 7 but from 7 to 14, the SCC dropped instead.

The NAS mixture inoculated in the front left quarter of 3 cows resulted in an overall increase in SCC comparing day 0 to day 14 (Figure 3.1.7). Cows 716 and 1088 had an upward SCC trend from day 0, 7, and 14 but none of the values exceeded the subclinical mastitis threshold. This was not the case for cow 1021 as it had an upward SCC trend from day 0 to 7, where its SCC exceeded the subclinical mastitis threshold. However, that same cow's SCC from day 7 to 14 dropped below the subclinical mastitis threshold by the end of the trial. The highest peak in SCC from this quarter came at day 7 cow for 1021, with a SCC of 742 000 cells.

The NAS mixture inoculated in the front right quarter of 3 cows resulted in an overall increase in somatic cell count comparing day 0 to day 14 only in cow 716 (Figure 3.1.8). Cows 1021 and 1088 had an upward SCC trend from day 0 to 7, but both dropped by day 14. More importantly, all the SCCs' recorded in the front right quarter never exceeded the subclinical mastitis threshold.

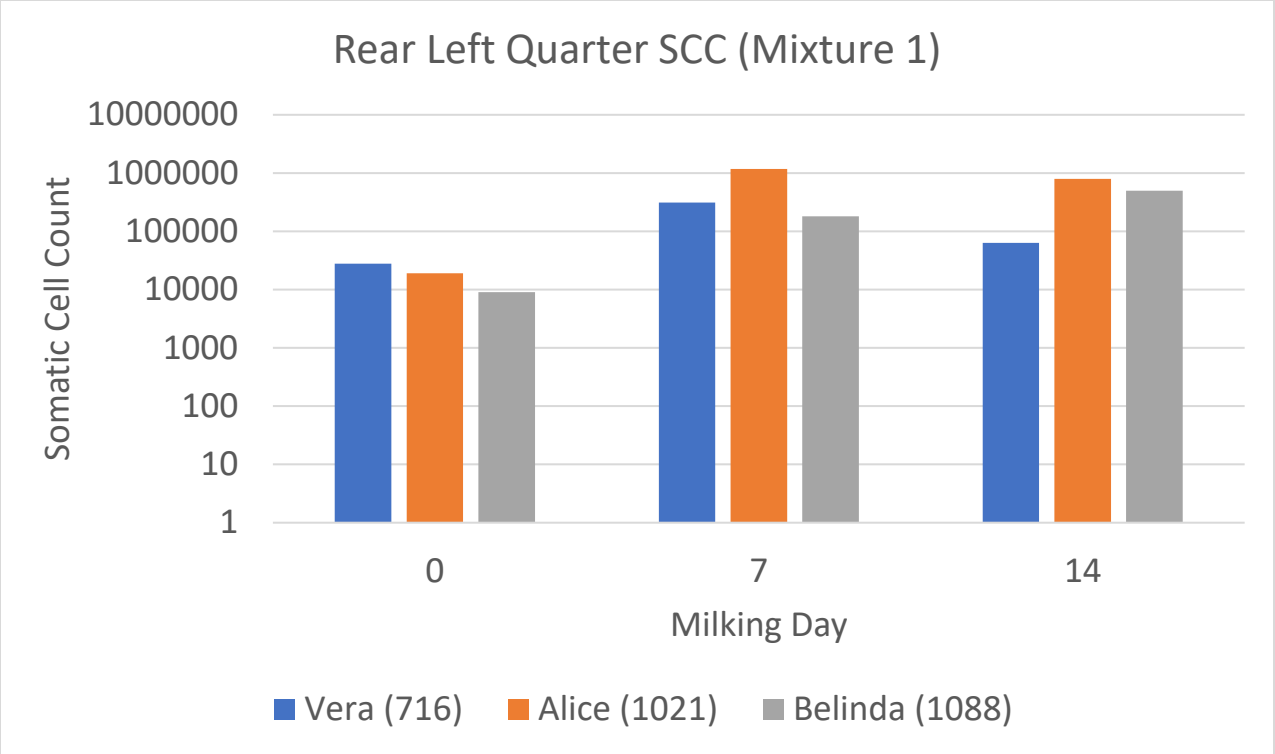

Figure 3.1.5: Somatic cell count in milk samples of three cows at day 0 (pre-infusion), day 7 (post-infusion), and day 14 (post-infusion) after intramammary infusion with *S. chromogenes* 5978, *S. xylosus* 5435, *S. warneri* 1052, and *S. devriesei* 1316 at day 0 (n=1).

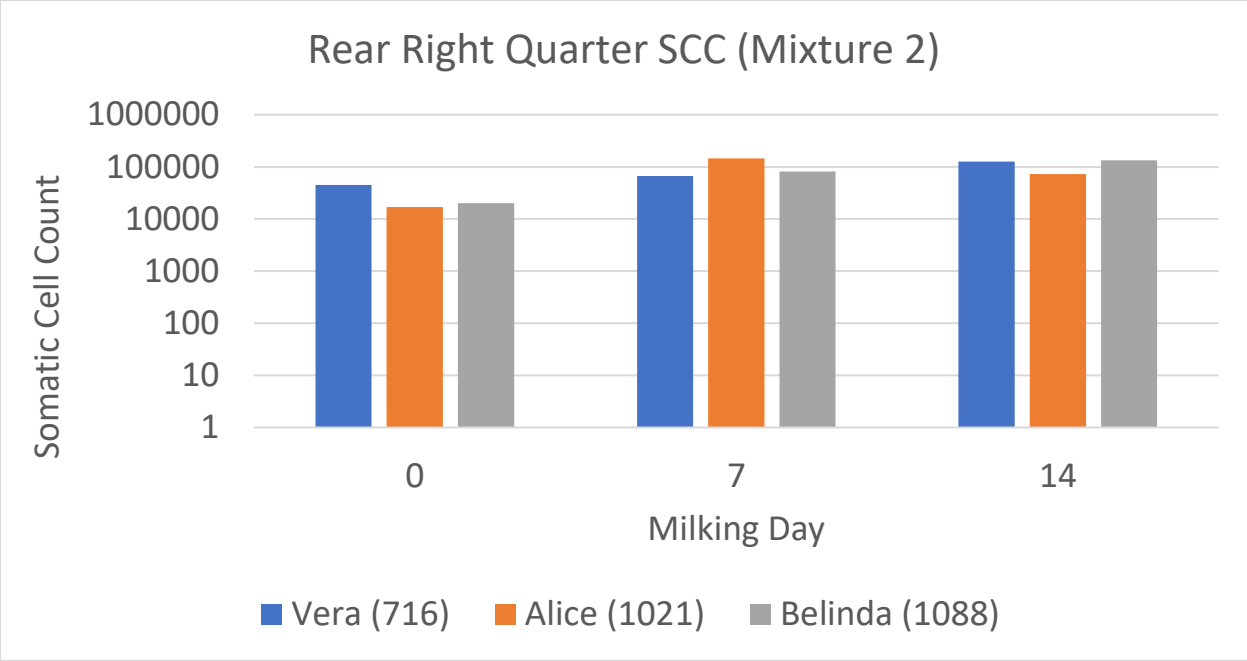

Figure 3.1.6: Somatic cell count in milk samples of three cows at day 0 (pre-infusion), day 7 (post-infusion), and day 14 (post-infusion) after intramammary infusion with *S. arlettae* 5134*, S. hominis* 2694*, S. devriesei* 4143*, and S. pasteuri* 2657 at day 0 (n=1)..

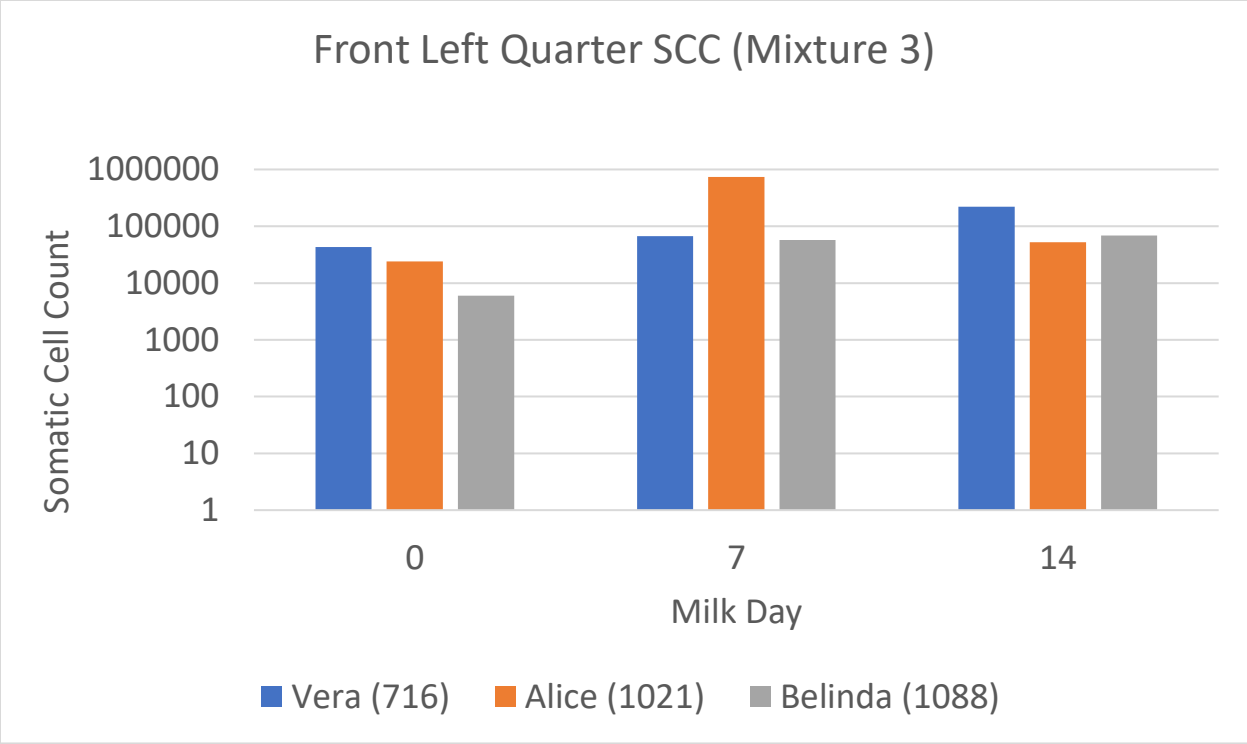

Figure 3.1.7: Somatic cell count in milk samples of three cows at day 0 (pre-infusion), day 7 (post-infusion), and day 14 (post-infusion) after intramammary infusion with *S. warneri* 2140*, S. caprae* 4023*, S. equorum* 2121*, and S. warneri* 2993 at day 0 (n=1)..

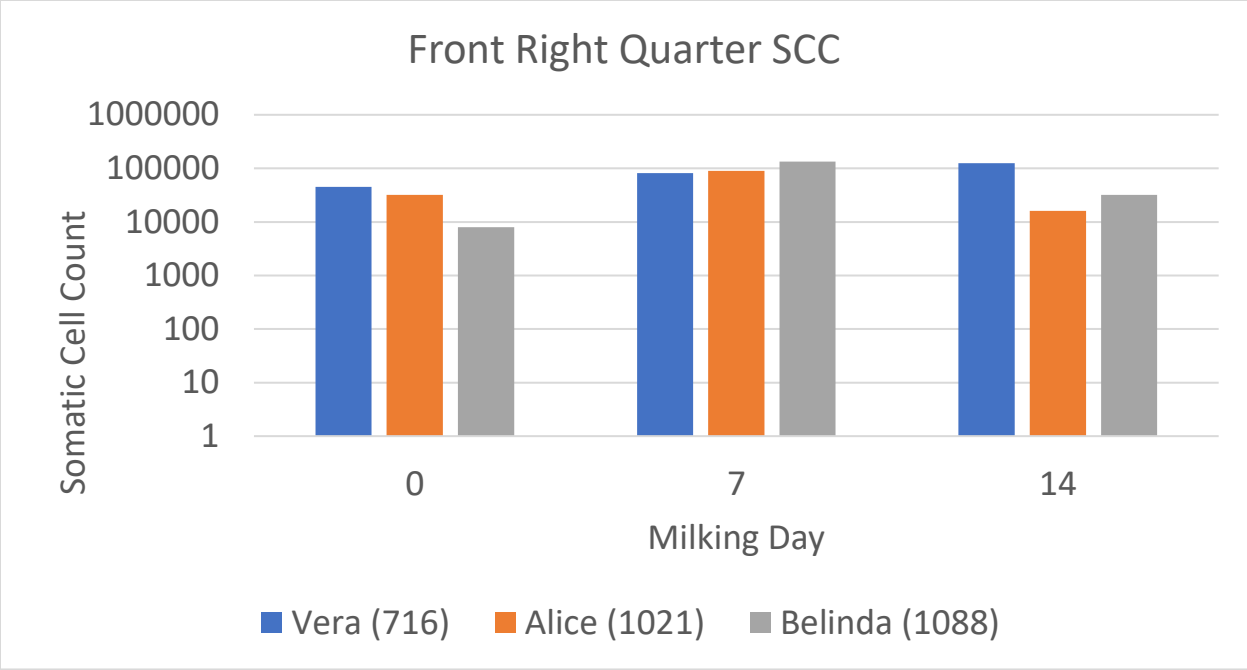

Figure 3.1.8: Somatic cell count in milk samples of three cows at day 0 (pre-infusion), day 7 (post-infusion), and day 14 (post-infusion) after intramammary infusion with *S. cohnii* 1091, *S. devriesei* 4438, *S. pasteuri* 2044, *and S. warneri* 4140 at day 0 (n=1).

Detection of Non-aureus Staphylococci Colonization of Mammary Gland Tissues by Bacterial Culture of Tissue Swabs

For each quarter, swabs were taken from the parenchyma, cistern, and teat canal and streaked on culture plates. Out of the resulting 36 plates, only 4 plates yielded colonies, all coming from cow 1088 (Table 3.1.4). There were 6 colonies found in the rear left parenchyma tissue and 1 colony in the canal both corresponding to *S. pasteuri 2657* and *S. devriesei* 1316 respectively. It is important to note that these NAS were the most persistent strains from their respective NAS mixtures. In the rear right quarter of cow 1088, 7 *S. pasteuri* 2657 colonies grew from the CHROMagar swabbing of the in the parenchyma. Furthermore, the rear right canal swabbing on CHROMagar yielded 34 *S. pasteuri* 2657 colonies. For the total bacterial count, 73% of all colonies that grew on the culture plates originated from the teat canal

while the remaining 27% from the parenchyma.

Table 3.1.4: Trial 1 non-*aureus* staphylococci CFU from swabs of different mammary tissues and spreading on CHROMagar after a 2-day incubation at 37°C. Quarters that did not have colonies were not shown.

	Rear Left			**Rear Right**		
Cow	Parenchyma	Cistern	Canal	Parenchyma	Cistern	Canal
716	0	0	0	0	0	0
1021	0	0	0	0	0	0
1088	6	0	1	7	0	34

Detection of Non-aureus Staphylococci Colonization of Mammary Gland Tissues by Bacterial Culture of Tissue Biopsy

For each quarter, four tissues samples were collected and homogenized coming from the ventral parenchyma, middle parenchyma, dorsal parenchyma, and the teat mucosal surface. The homogenized tissue was cultured on CHROMagar and there were 7 out of 64 plates that had growth, with the majority coming from the parenchyma tissue (Table 3.1.5). There were no colonies originating from cow 716. For cow 1021, the rear left quarter yielded a bacterial count of 9450 and 4300 CFU/g from the middle and dorsal parenchyma tissue culturing on CHROmagar. The same cow also had 200 CFU/g from the rear right ventral parenchyma tissue culturing on CHROMagar. For cow 1088, the front left quarter had bacterial growth from tissue homogenization and plating of the ventral parenchyma, middle

parenchyma, and teat mucosal surface. Their bacterial counts were 50, 100, and 50 CFU/g, respectively. Lastly, the same cow also had a bacterial growth of 50 CFU/g from the rear left middle parenchyma tissue plating.

Table 3.1.5: Trial 1 non-*aureus* staphylococci CFU per gram of homogenized tissue samples plated on CHROMagar. The tissues were 5x diluted in PBS and 100uL was plated on CHROMagar for 2-days at 37C. Quarters that did not have colonies were not shown.

CFU /g	Front Left				Rear Left				Rear Right			
Cow	Parenchyma (Ventral)	Parenchyma (Middle)	Parenchyma (Dorsal)	Teat Mucosal	Parenchyma (Ventral)	Parenchyma (Middle)	Parenchyma (Dorsal)	Teat Mucosal	Parenchyma (Ventral)	Parenchyma (Middle)	Parenchyma (Dorsal)	Teat Mucosal
716	0	0	0	0	0	0	0	0	0	0	0	0
1021	0	0	0	0	0	9450	4300	0	200	0	0	0
1088	50	100	0	50	0	50	0	0	0	0	0	0

Infection of Front Left Quarter with S. warneri 2993

A single NAS isolate, *S. warneri* 2993, was infused into the front left quarter of three lactating dairy cows. The actual inoculum of cells for this NAS isolate was 9,321 cells. This strain grew 28 out of 42 times from CHROMagar milk culturing (Figure 3.1.9). Interestingly, all the growth came from cow 1120 and 1179 as cow 1062 did not have any growth during the 14 d. Throughout the entire trial for cow 1120 and 1179, the average log (CFU)/mL was 3.07 (95% CI: ± 0.32) and 3.18 (95% CI: ± 0.21), respectively. This average was similar to the bacterial growth on day 14 for the two cows which was 3.23 and 3.18, respectively. When tracking the growth of *S. warneri* 2993 from day 1 to 14 for cow 1120 and 1179,

there was not a clear trend in growth (Figure 3.1.10). Unexpectedly, there were also three NAS species found during the milk culturation that were not in the initial infusion. These species were S. *xylosus, S. haemolyticus,* and *S. succinus* with log (CFU)/mL ranging from 0.3 to 0.6.

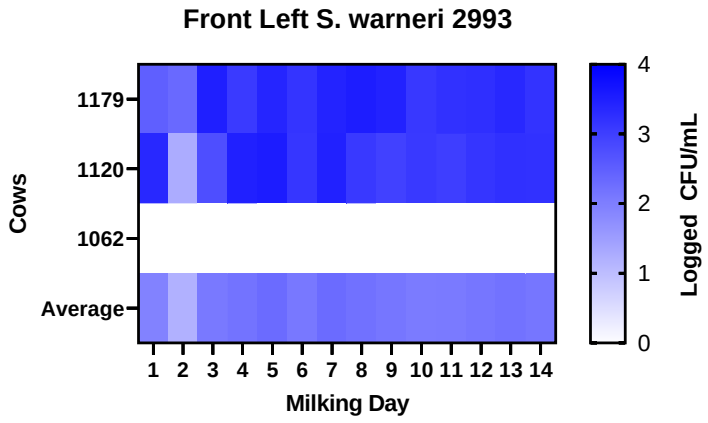

Figure 3.1.9: Heatmap of the quantities of *S. warneri* strain 2993 (log (CFU)/ml) in milk samples of 3 cows (with IDs 1062, 1120 and 1179) intramammary infused at day 0 with *S. warneri* 2993 for a period of 14 days post-inoculation. Quarter milk (100 µL) was plated on CHROMagar and incubated at 37°C for 2 days (n=1).

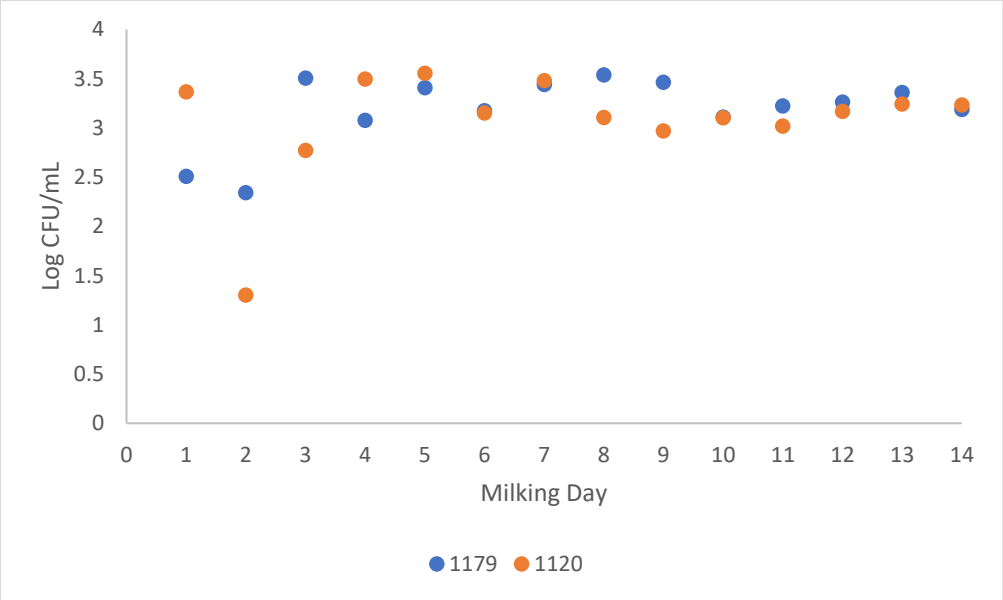

Figure 3.1.10: Scatter plot of the quantity (log (CFU)/mL) of *S. warneri* 2993 in milk samples of 3 cows infused at day 0 with 9 321 cells of *S. warneri* 2993 for 14 days after inoculation. Quarter milk (100 μL) was plated on CHROMagar and incubated at 37°C for 2 days (n=1). Cow 1062 was not shown as there were no growth of *S. warneri* 2993 on CHROMagar throughout the trial.

Infection of Rear Left Quarter with S. devriesei 1316

A single NAS isolate, *S. devriesei* 1316, was infused into the front left quarter of three lactating dairy cows. The actual inoculum of cells for this NAS isolate was 600 316 cells. This strain only grew 4 out of 42 times from CHROMagar milk culturing (Figure 3.1.11). The latest day *S. devriesei* 1316 grew from milk cultures on CHROMagar was day 3 with a log (CFU)/mL of 1.30. Unexpectedly, there were three different species of bacteria found during the milk culturation that were not in the initial infusion. These species were *S. haemolyticus, S. chromogenes*, and *Corynebacterium stationis* with a log (CFU)/mL ranging from 0.3 to 1.30. This strain was not considered a persistent strain due to its lack of growth from CHROMagar milk culturing.

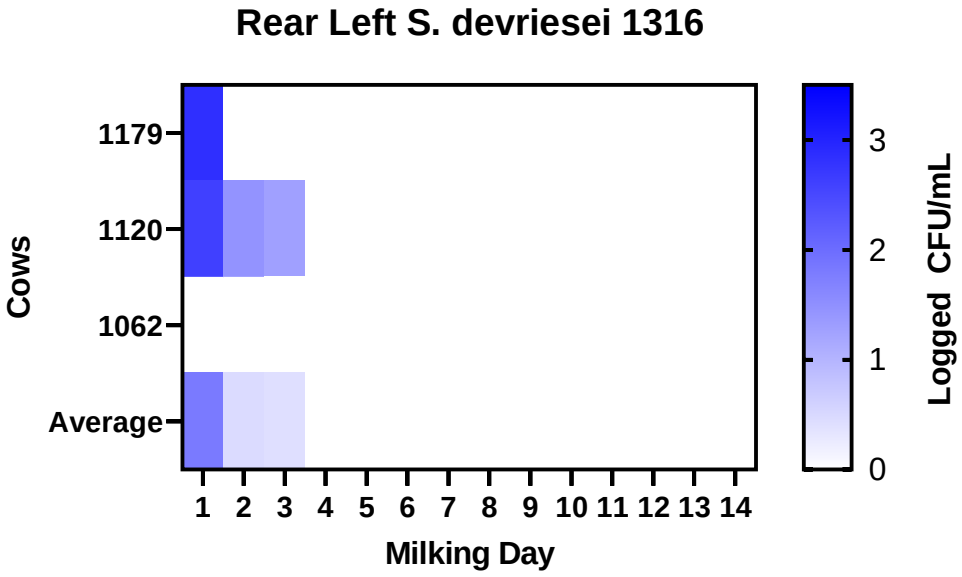

Figure 3.1.11: Heatmap of the quantities of *S. devriesei* strain 1316 (log (CFU)/ml) in milk samples of 3 cows (with IDs 1062, 1120 and 1179) intramammary infused at day 0 with *S. devriesei* 1316 for 14 days after inoculation. Quarter milk (100 μL) was plated on CHROMagar and incubated at 37°C for 2 days (n=1).

Infection of Rear Right Quarter with S. pasteuri 2657

A single NAS isolate, *S. pasteuri* 2657, was infused into the front left quarter of three lactating dairy cows. The actual inoculum of cells for this NAS isolate was 32 000 cells. This strain only grew 3 out of 42 times from CHROMagar milk culturing (Figure 3.1.12). The latest day *S. pasteuri* 2657 grew from milk cultures on CHROMagar was day 2 with a log (CFU)/mL of 1.85 from cow 1120. The average log (CFU)/mL from the three days of growth on CHROMagar was 1.74. Since there were only three data points, tracking the growth of *S. pasteuri* 2657 over that time frame did not give reliable insight into temporal trends. Unexpectedly, there were six different bacterial species found that were not in the initial infusion. These species *were Bacillus paralicheniformis, S. haemolyticus, S. auricularis, S. chromogenes, S. arlettae,* and *S. saprophyticus.* Their log (CFU)/mL ranged from 0.3 to 0.78. This strain was not the considered a persistent strain out of the three due to its lack of growth from CHROMagar milk culturing.

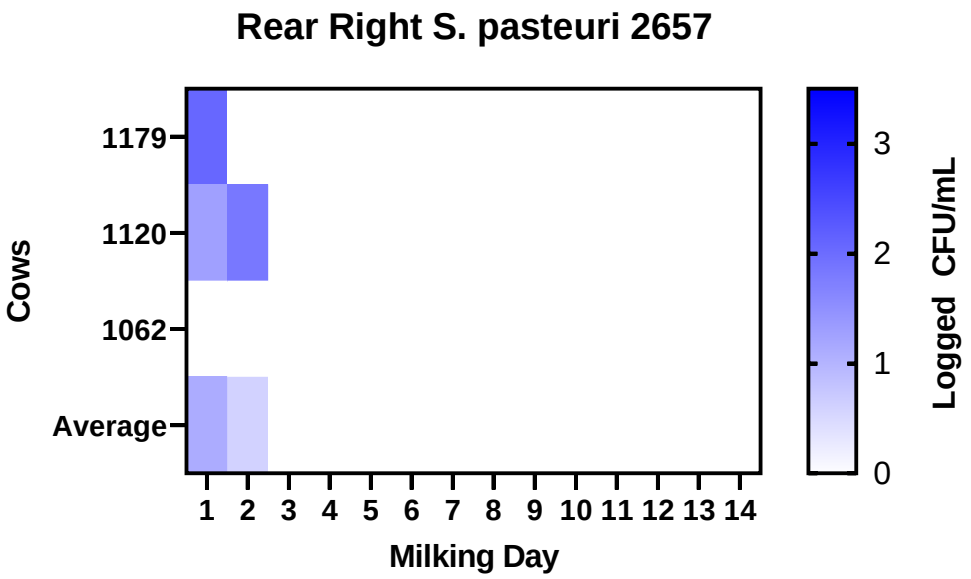

Figure 3.1.12: Heatmap of the quantities of *S. pasteuri* strain 2657 (log (CFU)/ml) in milk samples of 3 cows (with IDs 1062, 1120 and 1179) intramammary infused at day 0 with *S. devriesei* 1316 for 14 days after inoculation. Quarter milk (100 μL) was plated on CHROMagar and incubated at 37°C for 2 days (n=1).

Infusion of Front Right Quarter with Saline

The front right quarter was infused with a 0.8% NaCl saline solution. Despite being a negative control, there were 8 out of 42 times bacterial species grown by culturing the milk. There were six different bacterial species found during the milk culturing. These species include *S. haemolyticus, S. auricularis, Staphylococcus petrasii, S. chromogenes, S. succinus*, and *Bacillus pumilus*. Their log (CFU)/mL ranged from 0.3 to 0.48.

Individual NAS Effect on Somatic Cell Count in Milk

S. warneri 2993 was infused into the front left quarter of 3 cows and resulted in an overall increase in SCC comparing day 0 to 14 for cow 1179 and 1120 (Figure 3.1.13). The overall trend for cow

1179 and 1120 is a peak increase in SCC past the subclinical mastitis threshold at day 2. From day 2-5, there was a gradual decrease in SCC where both cows dropped below the subclinical mastitis threshold. Then, from day 5 until the end of the trial, all the cows remained below the subclinical mastitis threshold except for cow 1120 when its SCC went above the threshold at day 9. By the end of the trial, the SCC for both cow 1179 and 1120 was below the subclinical mastitis threshold. For cow 1062, its SCC was below the subclinical mastitis threshold for the entirety of the trial with no clear trend in SCC. Interestingly, its SCC at day 14 was below its initial SCC at day 0.

S. devriesei 1316 was infused into the rear left quarter of 3 cows and resulted in an overall increase in SCC comparing day 0 to 14 for cow 1179 and 1120 (Figure 3.1.14). The trend for cow 1179's SCC was a peak at day 1 that exceeded the subclinical mastitis threshold. From day 1-7, there was a gradual decrease in SCC until it dropped below the subclinical mastitis threshold at day 7. From day 7 until the end of the trial, the SCC remained below the subclinical mastitis threshold. Cow 1120's SCC peaked at day 2 where it exceeded the subclinical mastitis threshold. The SCC peak at day 2 was the highest for the entirety of the trial with 22 million cells/ml. Following the same trend as cow 1179, its SCC continued to decline from day 1 to 9 until it dropped below the subclinical mastitis threshold. It is important to note that in-between those days, the SCC exceeded the subclinical mastitis threshold. Lastly, for cow 1062, its SCC peaked to a similar range which exceeded the subclinical mastitis threshold from day 1-3. Then from day 3-7, its SCC gradually dropped below the subclinical mastitis threshold by day 7. From day 7 until the end of the trial, the SCC remained below the subclinical mastitis threshold.

S. pasteuri 2657 was infused into the rear right quarter of 3 cows and resulted in an overall increase in SCC comparing day 0 to 14 for cow 1179 and 1120 (Figure 3.1.15). For cow 1179, its SCC remained below the subclinical mastitis threshold for the entirety of the trial. However, its SCC did peak at day 2 with 83 000 cells. For the rest of the trial, its SCC would fluctuate up and down randomly. Cow 1120's SCC exceeded the subclinical mastitis threshold at day 2, 3, and 4 with its peak SCC at day 3. Then

from day 5 and onwards, its SCC remained below the subclinical mastitis threshold. Lastly, for cow 1062, its SCC remained below the subclinical mastitis threshold for the entry of the trial as well. The highest peak in SCC came from day 3 with 69 000 cells. Interestingly, its SCC at day 14 was lower than its day 0 SCC by 25 000 cells.

A 0.8% NaCl saline solution was infuse into the front right quarter of 3 cows and resulted in no notable increase or decrease in SCC. Throughout the entirety of the trial, all three cows remained below the subclinical mastitis threshold. Furthermore, when comparing day 0 to day 14, two of the cows had a lower SCC than the beginning of the trial. For cow 1179, 1120, and 1062, their highest SCC recorded from the trial was 40 000, 26 000, and 108 000 cells respectively.

When comparing the 14-day average natural log (SCC) of the infected and non-infected quarters, there is a difference of 1.63. More importantly, at day 2-4, there is a spike in the infected quarters where the SEM bars extend past the non-infected quarters (Figure 3.1.16). During this period, the ln (SCC) of infected and non-infected quarter was 12.96 and 10.30 respectively. When looking at the trend of the infected quarters, the ln (SCC) peaked at day 2. From day 2 to day 5, the ln (SCC) gradually dropped until it fell below the subclinical mastitis threshold. Then from day 5 until the end of the trial, it stayed near the same value below the subclinical mastitis threshold.

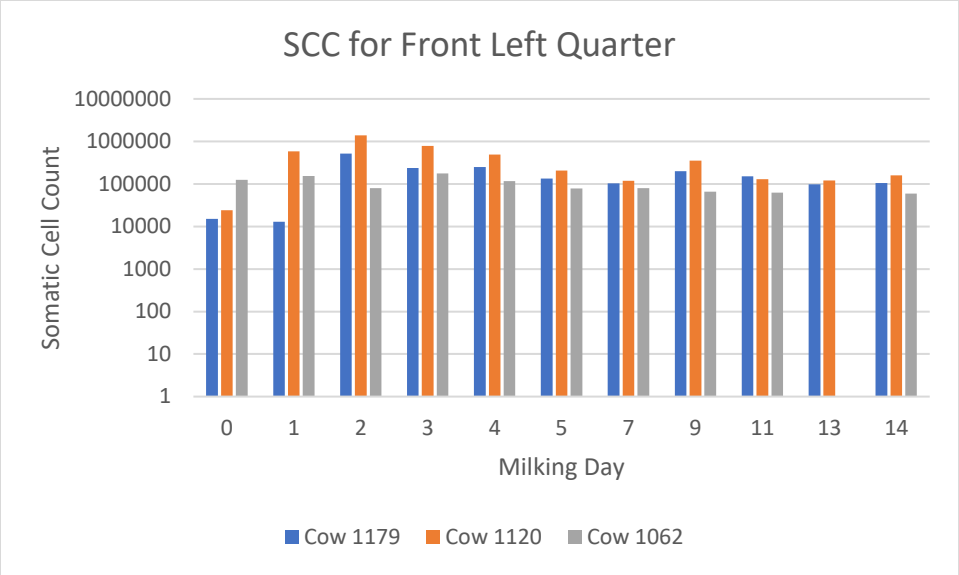

Figure 3.1.13 Somatic cell count of milk samples of three cows' front left quarter at day 0 (pre-infusion), 1-5, 7, 9, 11, 13, and 14 after intramammary infusion with *S. warneri* 2993 at day 0 (n=1).

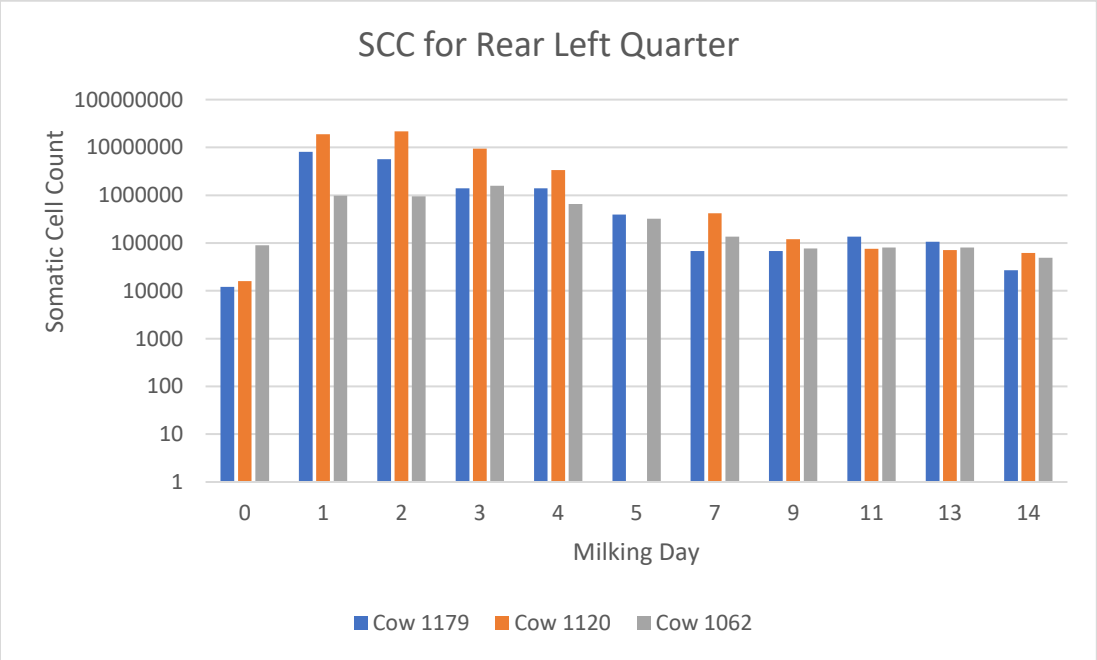

Figure 3.1.14: Somatic cell count in milk samples of three cows' rear left quarter at day 0 (pre-infusion), 1-5, 7, 9, 11, 13, and 14 after intramammary infusion with *S. devriesei* 1316 at day 0 (n=1).

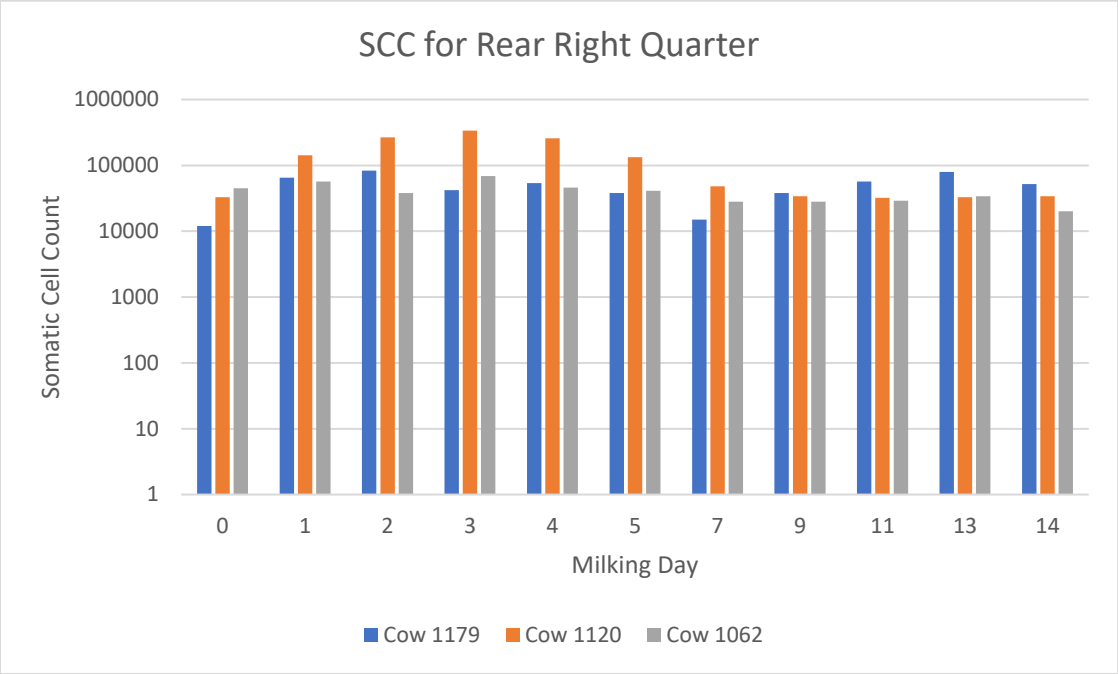

Figure 3.1.15: Somatic cell count in milk samples of three cows' rear right quarter at day 0 (pre-infusion), 1-5, 7, 9, 11, 13, and 14 after intramammary infusion with *S. pasteuri* 2657 at day 0 (n=1).

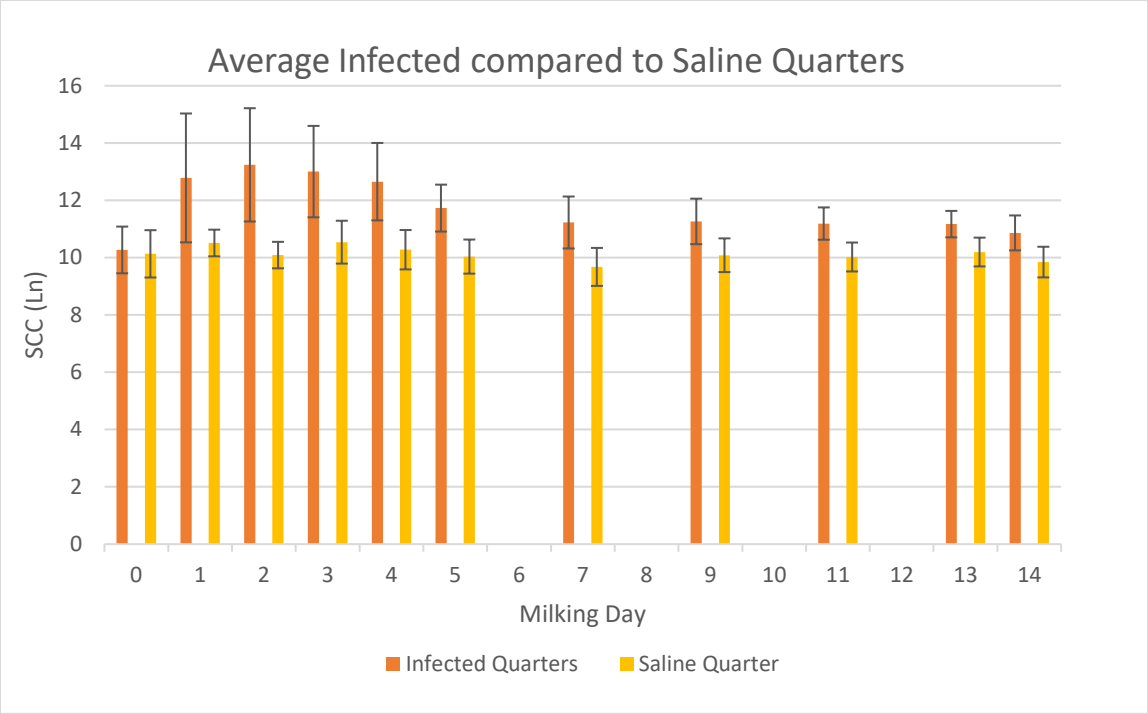

Figure 3.1.16: Somatic cell count in milk samples of three cows comparing their infected and non-infected quarters at day 0 (pre-infusion), 1-5, 7, 9, 11, 13, and 14 after intramammary infusion with individual NAS (n=1). The error bars represent the variation between the three different cows (1062, 1120, and 1179).

Detection of Individual Non-aureus Staphylococci Colonization of Mammary Gland Tissues by Bacterial Culture of Tissue Swabs

Each quarter had three swabs coming from the parenchyma, gland cistern, and teat canal. Out of the 36 possible CHROMagar plates that were swabbed, only 5 plates yielded colony growth (Table 3.1.6). Most of the colonies came from cow 1179, specifically the teat canal and parenchyma with 212 and 38 colonies respectively. Cow 1120 had 81 colonies coming from the teat canal and a single colony from the parenchyma and gland cistern. Lastly, no colonies grew from swabbing cow 1062.

Table 3.1.6: Trial 2 non-*aureus* staphylococci CFU per gram of homogenized tissue samples on

CHROMagar. The tissues were 5x diluted in PBS and 100uL was plated on CHROMagar for 2-days at 37C. Quarters that did not have colonies were not shown.

CFU/g	Front left (S. warneri infusion)			
Cow	**Parenchyma (Ventral)**	**Parenchyma (Ventral Middle)**	**Parenchyma (Dorsal Middle)**	**Parenchyma (Dorsal)**
1179	**1100**	**1100**	**400**	**300**
1120	**50**	**0**	**0**	**0**
1062	**0**	**0**	**0**	**0**

Detection of Individual Non-aureus Staphylococci Colonization of Mammary Gland Tissues by Bacterial Culture of Tissue Biopsy

Each quarter had four tissues that were homogenized, and they all came from different areas of the parenchyma (ventral, ventral middle, dorsal middle, and dorsal). The homogenized tissue was cultured on CHROMagar and there were 5 out of 64 plates that had growth, with 4 of them coming from cow 1179 (Table 3.1.7). For cow 1179, there were 1100 CFU/g of *S. warneri* 2993 colonies coming from the ventral and ventral middle parenchyma. The dorsal middle and dorsal parenchyma had roughly a third of those numbers with 400 CFU/g and 300 CFU/g respectively. The remaining sample that yielded 50 CFU/g came from cow 1120's ventral parenchyma tissue.

Table 3.1.7: Trial 2 non-*aureus* staphylococci CFU from swabs of different mammary tissues and spreading on CHROMagar after a 2-day incubation at 37°C. Quarters that did not have colonies were not shown.

	Front left (*S. warneri* infusion)		
Cow	**Parenchyma**	**Gland Cistern**	**Teat Canal**
1179	**38**	**0**	**212**
1120	**1**	**1**	**81**
1062	**0**	**0**	**0**

Comparison of the Three Persistent Strains Growth from Trial 1 and Trial 2

In trial 1, the most persistent strain was *S. devriesei* 1316 as it was recovered 22 out of the 42 times from the milk sample cultures (Figure 3.1.0). This contrasted its recovery rate from trial 2 where it was only recovered 4 out of the 42 times from the milk samples. *S. pasteuri 2657* was recovered 9 out of 42 times from the milk culture samples in trial 1. This frequency of appearance from milk samples dropped further in trial 2 down to only 3 out of 42 milk samples. Lastly, *S. warneri 2993* was recovered 10 out of 42 times from the milk culturing in trial 1. This number increased roughly 3-times to 28 out of the 42 milk samples in trial 2.

Comparison of the Three Persistent Strains from the Individual Infusions into Bovine Mammary Glands

The three persistent strains were *S. devriesei* 1316, *S. pasteuri* 2657, and *S. warneri* 2993 was

infused individually into the rear left, rear right, and front left quarters of 3 cows with the remaining quarter as the saline control. For the total number of the respective NAS growing from CHROMagar milk culturing, *S. warneri* 2993 was the most persistent as it grew 28 out of 42 times. *S. devriesei* 1316 was the second most persistent as it grew 4 out of 42 times from CHROMagar milk culturing. This leaves *S. pasteuri* 2657 as the last persistent since it grew 3 out of 42 times from CHROMagar milk culturation (Table 3.1.8). When comparing the SCC between the three persistent strains, *S. devriesei* 1316 has the largest increase in SCC. Furthermore, there were 15 out of 42 time points during the trial where a cow exceeded the subclinical mastitis threshold. *S. warneri* 2993 had the second largest increase in SCC with 9 out of 42 time points where a cow exceeded the subclinical mastitis threshold. *S. pasteuri* 2657 had the lowest effect on SCC as there were only 3 out of 42 time points where a cow exceeded the subclinical mastitis threshold.

Ultimately, *S. warneri* 2993 was the best persistent strain out of the three tested due to its extremely high prevalence from CHROMagar milk culturing alongside a moderate increase in SCC. *S. devriesei* 1316 had the highest increase in SCC alongside only 4 out of 42 growths from milk culturation making it a weaker candidate compared to *S. warneri* 2993. Lastly, despite *S. pasteuri* 2657 having the least effect on SCC, it also had the lowest persistence in milk making it a weak candidate as well

Table 3.1.8: Summary of Persistent Strain milk culture frequency and effect on SCC from trial 1 and 2.

	Milk Culturation Frequency (Trial 1)	Milk Culturation Frequency (Trial 2)	Effect on SCC (Trial 2 only)
S. devriesei 1316	22/42	4/42	High
S. pasteuri 2657	9/42	3/42	Low
S. warneri 2993	10/42	28/42	Moderate

Objective 2: Integration of a Bacteriocin Gene Cluster into the Recipient NAS Strain

Genetic editing of S. warneri 2993 with pKOR-RFP Plasmid

There were 11 total steps needed for the RFP gene knock-in into *S. warneri* 2993 using pKOR1 (Figure 3.2.0).

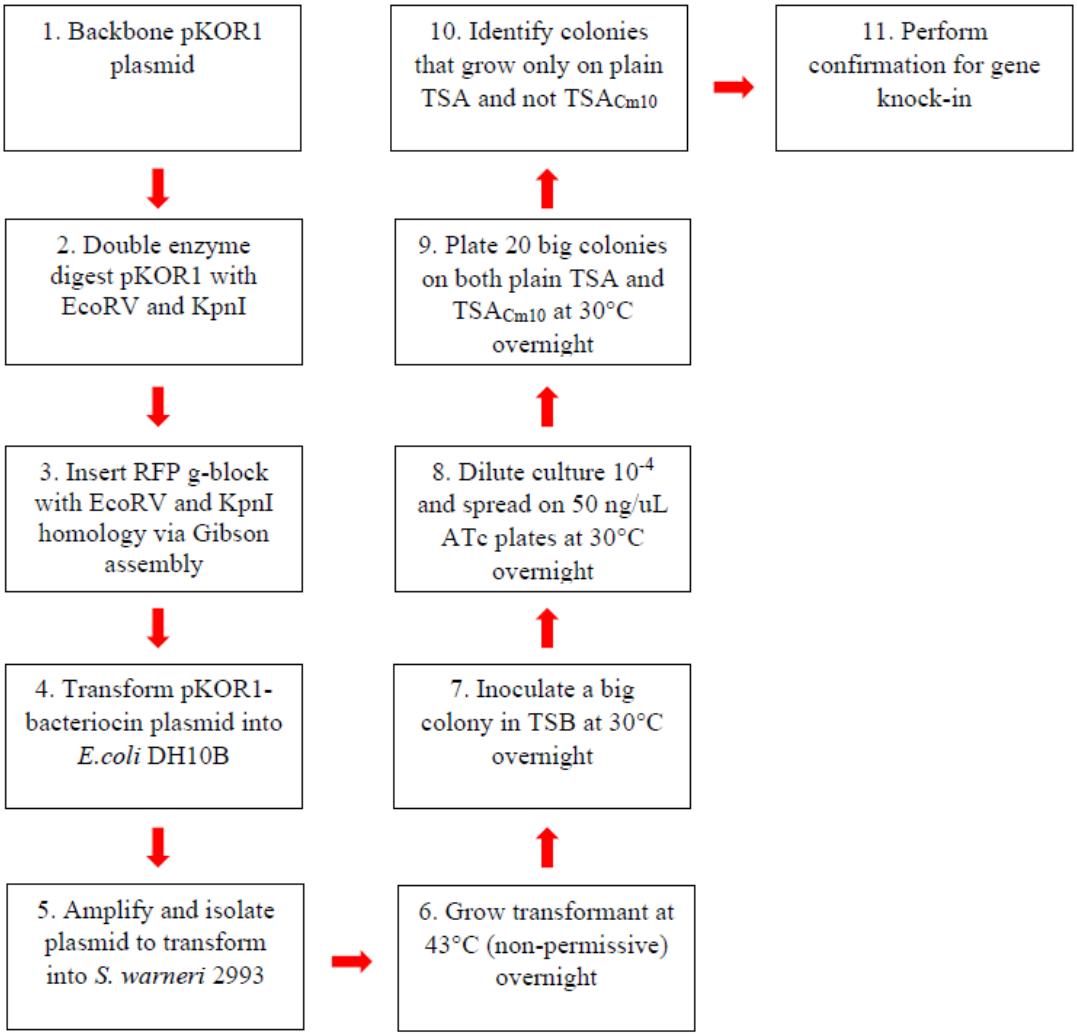

Figure 3.2.0: Flowchart for RFP gene knock-in with pKOR1 into *S. warneri* 2993

Transformation of S. warneri 2993 with the pKOR1-RFP plasmid

Six different pKOR1-RFP plasmid concentrations were used to find the optimal concentration for the electroporation (Figure 3.2.0 Step 5). The six concentrations were 1.7 µg/µl, 3.5 µg/µl, 5.5 µg/µl, 8 µg/µl, 10.7 µg/µl, and 13 µg/µl. From these various transformation concentrations, there were 1, 0, 0, 2, 2, and 1 transformants on the chloramphenicol selection plates, respectively. This indicates that the varying plasmid concentrations have no effect on transformation efficiency.

Curing of the pKOR1-RFP plasmid from S. warneri 2993

From the 20 colonies that were taken from the 50 ng/mL ATc plates and transferred to both regular agar plates and chloramphenicol plates, 2 out of the 20 colonies lost the plasmid (Figure 3.2.0 Step 9). This frequency is lower than the expected as when the donors of this plasmid used this plasmid, all 10 out of 10 mutants did not carry pKOR1.

Confirmation of RFP knock-in into S. warneri 2993

The first method to detect the RFP gene knock-in was by designing primers that amplified a region of the genetically modified *S. warneri* 2993's genome that overlaps with the mtlF and RFP gene. These primers were used on wild-type *S. warneri* 2993, *S. warneri* 2993 Δ*mtlf*, and the pKOR1-RFP plasmid as a positive control. After gel electrophoresis of these 3 samples, there was no band for the first sample, a 905 bp band for the second, and a 905 bp band for the third sample.

The second method to confirm that the RFP gene was knocked into the mtlF gene was through the loss of *S. warneri* 2993's ability to ferment mannitol. When the wild-type S. warneri 2993 was grown on mannitol plates, the plates became yellow after overnight incubation (Figure 3.2.1). In contrast, when *S. warneri* 2993 Δ*mtlf* was grown on mannitol plates, the plates remained pink even after the overnight

incubation (Figure 3.2.1).

The third method of confirmation was to see if *S. warneri* 2993 Δ*mtlf* fluoresced under UV light or through fluorescence microscopy. Unfortunately, no fluorescence could be observed under the microscope or on the UV transilluminator.

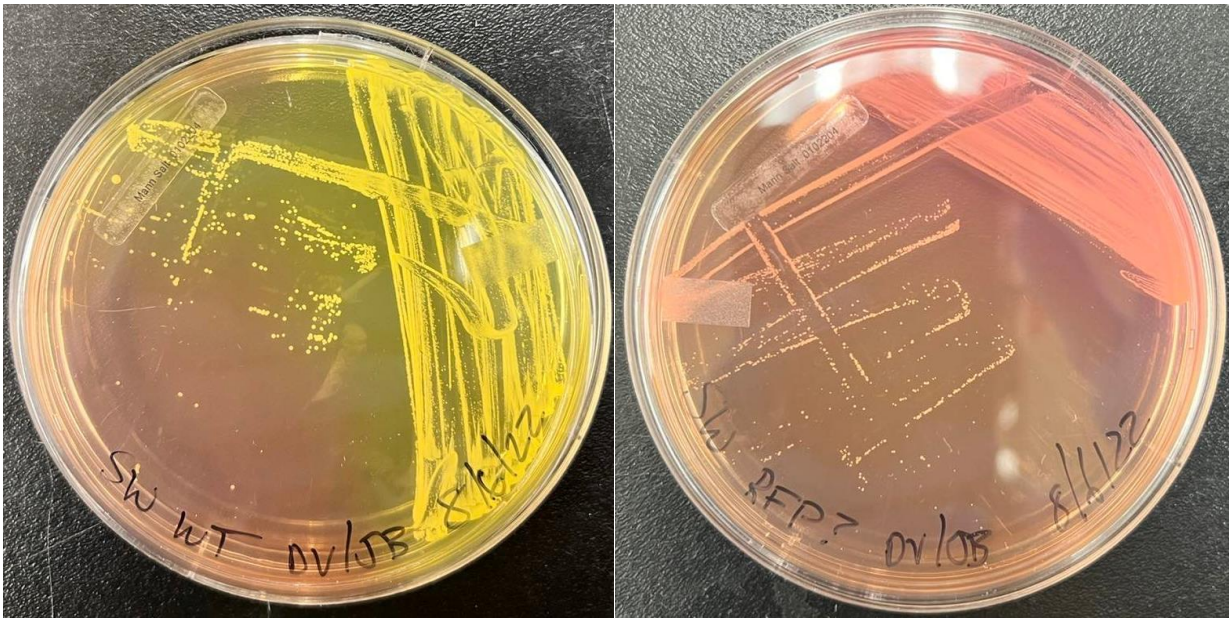

Figure 3.2.1. Wild type *S. warneri* 2993 (left) and *S. warneri* 2993 Δ*mtlf* (right) grown on mannitol salt agar plates after 24 h in a 37°C incubator.

Genetic editing of S. warneri 2993 with piMAY plasmid*

There were 11 total steps needed for the RFP gene knock-in into *S. warneri* 2993 using pIMAY* (Figure 3.2.2). Unfortunately, we were unable to get past step 5 and transform *S. warneri* 2993 with the piMAY*-RFP plasmid. However, this was not the case for the empty backbone plasmid as we reached step 10 which was the curing step.

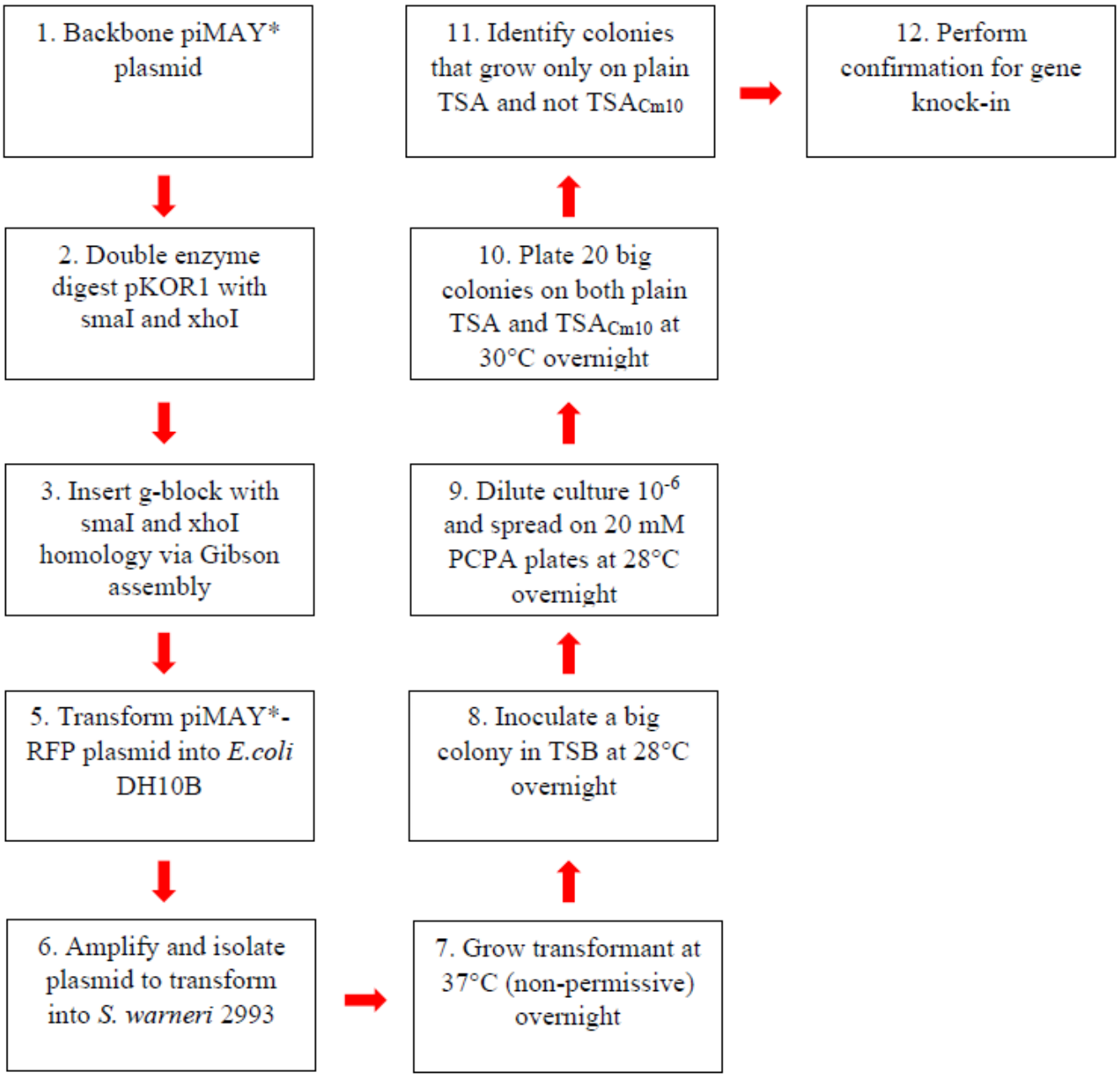

Figure 3.2.2. Flowchart for RFP gene knock-in with piMAY* into *S. warneri* 2993

Transformation of S. warneri 2993 with the piMAY plasmid*

One singular piMAY* plasmid concentration was for *S. warneri* 2993 transformation. From a plasmid concentration of 1.5 µg/µl, 2 transformants appeared on the chloramphenicol selection plate.

Curing of the piMAY plasmid from S. warneri 2993*

From the 20 colonies that were taken from the 20mM PCPA plates and transferred to both regular agar plates and chloramphenicol plates, only 1 out of the 20 colonies lost the plasmid.

Transformation of S. warneri 2993 with the piMAY-RFP plasmid*

Three different piMAY-RFP plasmid concentrations were used to find the optimal concentration for the electroporation (Figure 3.2.2 Step 6). The three concentrations were 1.5 µg/µl, 3 µg/µl, and lastly 3 µg/µl but with a double electric pulse instead of a single pulse. Unfortunately, none of these transformation attempts resulted in any transformants on the chloramphenicol selection plates.

Genetic editing of S. warneri 2993 with pCasSA plasmid

There were total of 15 steps needed for the gene knock-in into *S. warneri* 2993 using pCasSA (Figure 3.2.3). Unfortunately, there were two steps where we encountered difficulties. The first was transforming the empty pCasSA plasmid into *S. warneri* 2993. At a concentration of 1 µg/µl and 1.5 µg/µl, no transformants appeared. The second was the creation of the pCasSA plasmid with the bacteriocin gene cluster and homology arms using Gibson assembly. Despite having the correct ratios of vector to insert, no transformants would appear.

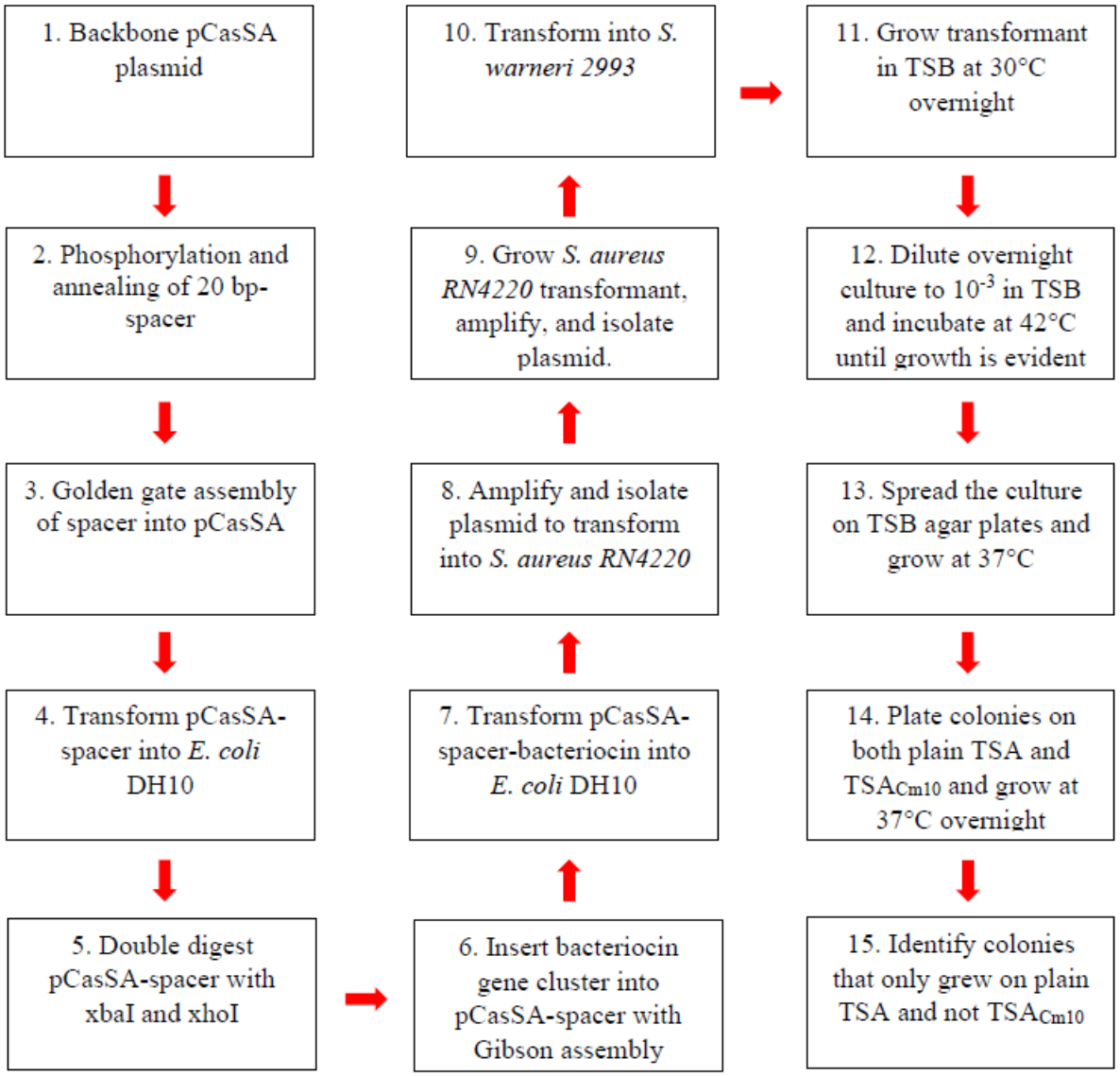

Figure 3.2.3. Flowchart for pCasSA gene knock-in into *S. warneri* 2993

Roadblocks in Genetic Engineering with S. warneri 2993

In total, three different genetic manipulation techniques were tested on *S. warneri* 2993 to determine the best technique for the gene knock-in. Unfortunately, all three methods did not lead to the introduction of the bacteriocin gene cluster (Figure 3.2.4). However, it is interesting to note that the roadblock in each method was different in comparison to each other.

For the piMAY* plasmid, the empty backbone was successfully introduced and cured from *S. warneri* 2993. However, after the introduction of the RFP g-block into the plasmid, transformation attempts were no longer successful. Since the bacteriocin gene cluster will be 6 Kb longer than the RFP gene, the likelihood that the larger plasmid transformation will be successful is improbable.

For the pKOR1 plasmid, the RFP gene was successfully knocked into *S. warneri* and cured. However, this was not the case for the bacteriocin gene cluster as we were unable to ligate the cluster into the plasmid. Three different attempts were made through Gibson assembly, Gibson assembly with a lower G/C content overhang, and blunt-end ligation but none were successful.

Lastly, the pCasSA plasmid gave us difficulties from the beginning. We were unable to transform the empty backbone into *S. warneri* 2993. Thus, if we can't transform the backbone, there is an even lower likelihood of us transforming pCasSA containing the bacteriocin gene cluster.

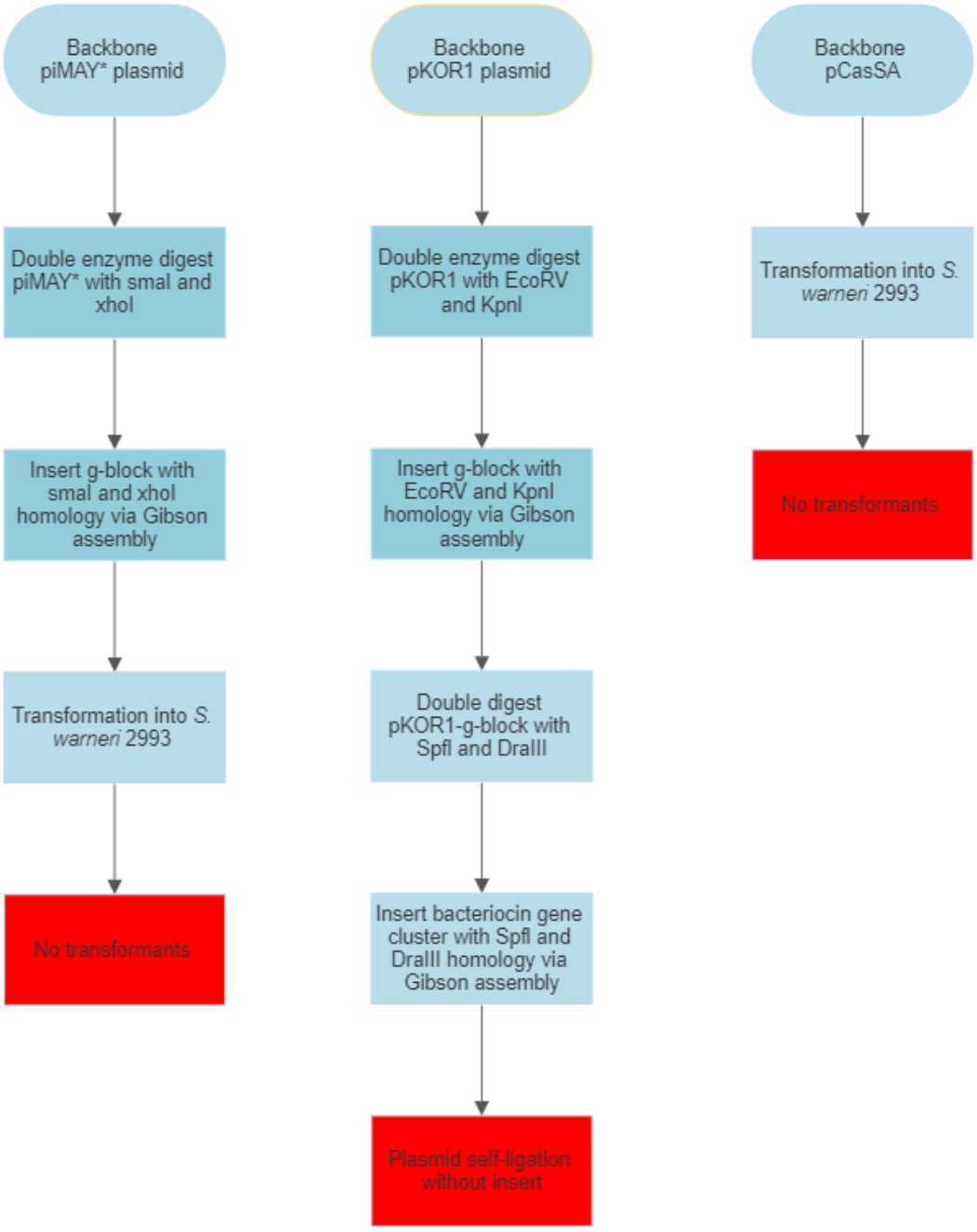

Figure 3.2.4. Roadblocks in the various genetic editing techniques in *S. warneri* 2993

Objective 3: Bacteriocin Gene Expression Characterization

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) for Bacteriocin Gene Cluster

The target bacteriocin gene cluster came from *S. chromogenes* 803 and is composed of 6 different genes. These genes included LanA1, LanA2, LanM, NADPH-dependent FMN reductase, LanP, and finally a methyltransferase. To confirm that the genes were being expressed, RT-qPCR was used as it can accurately measure gene expression levels since it is highly sensitive and specific. Three genes in total were measured and they were LanA1, LanA2, and a section overlapping both LanP and the methyltransferase gene. Three replicates were performed on *S. chromogenes* 803, *S. chromogenes* 803 no reverse transcriptase (NRT), *S. warneri* 2993, *S. warneri* 2993 NRT, and water (Table 3.3.0).

In RT-qPCR, there is a detection point at which your reaction exceeds a fluorescent intensity above the background threshold. The Cq value refers to the quantification cycle of which the fluorescent signal from the amplified sample crosses that threshold. It essentially measures the relative abundance of specific RNA molecules in your sample. A lower Cq value means a higher abundance of target RNA while a higher CQ value refers to a lower abundance of target DNA. For the RT-qPCR detecting LanA1, the average Cq value for *S. chromogenes* 803 was 22.96 while for the NRT, the average was 39.82. *S. warneri* 2993 had two cq values of 39.89 and 39.93 with the last replicate being N/A. The NRT *S. warneri* 2993 had one cq value of 40.36 with the remaining two replicates being N/A. Lastly, for the water negative control, all three values were N/A.

For the RT-qPCR detecting LanA2, the average Cq value for *S. chromogenes* 803 was 22.54 while for the NRT, the average was 39.78. *S. warneri* 2993 had an average Cq of 37.98 while the NRT's cq was 38.16. Lastly, similar to LanA1, the water negative control had three Cq values of N/A.

For the RT-qPCR detecting the region spanning across LanP and the methyltransferase, the average Cq value for *S. chromogenes* 803 was 23.42. The cq values for *S. chromogenes* 803 NRT, *S.*

warneri 2993, *S. warneri* 2993 NRT, and water were all N/A.

Table 3.3.0: Cq values from RT-qPCR targeting LanA1, LanA2, and LanP/Methyltransferase with five different samples: *S. chromogenes* 803, *S. chromogenes* 803 NRT, *S. warneri* 2993, *S. warneri* 2993 NRT, and water. Three replicates were performed.

	LanA1			LanA2			LanP/Methytransferase		
	Cq			Cq			Cq		
S. chromogenes 803	24.23	23.08	22.87	22.59	22.57	22.46	23.50	23.55	23.21
S. chromogenes 803 NRT	39.47	40.36	39.63	38.40	40.86	40.09	N/A	N/A	N/A
S. warneri 2993	N/A	39.89	39.93	38.58	36.88	38.47	N/A	N/A	N/A
S. warneri 2993 NRT	N/A	40.36	N/A	37.78	38.39	38.30	N/A	N/A	N/A
Water	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Discussion

Objective 1: Identify a Recipient NAS Strain for Bacteriocin Gene Clusters Genomic Integration

In this study, we developed a lactating cow model to test the persistence of non-aureus *staphylococci* inside the mammary glands. In a first 2-week NAS persistence experiment (trial 1), we cultured the milk samples from 3 cows on a daily period to have a total of 42 milk sample. There was one NAS strain per mixture that stood out in terms of frequency of isolation and persistence. They were *S. devriesei* 1316 which grew from 22/42 milk samples, *S. pasteuri* which grew from 9/42 milk samples, and *S. warneri* 2993 which grew from 10/42 milk samples. The persistence of the NAS was expected as these isolates were isolated from the infected milk of cows meaning they have natural persistence and

colonization[80]. Furthermore, NAS are the most frequently isolated pathogens from bovine milk, thus, it would have been surprising for none of the NAS isolates to persistent. Having these three species be the most persistent was unexpected as usually *S. chromogenes, S. simulans, S. xylosus, S. haemolyticus, and S. cohnii* are seen at a higher prevalence than *S. warneri, S. devriesei,* and *S. pasteuri*[80,102,103]. However, this is a generalization for species and isolates can sometimes differ dramatically from other isolates of the same species.

In a second 2-week NAS persistence experiment to test the inflammation associated with the persistent strain (trial 2), the most persistent strain was *S. warneri* 2993 which grew from 25/42 milk samples, *S. devriesei* 1316 only grew from 4/42 samples, and *S. pasteuri 2657* only grew from 3/42 samples. This decrease in milk frequency from *S. devriesei* 1316 in trial 1 and increase in milk frequency from *S. warneri* 2993 in trial 2 is particularly interesting and requires further discussion. The lower milk frequency from *S. devriesei* 1316 is hypothesized to be because of its inoculation amount purposely quadrupling from 150 079 in trial 1 to 600 316 cells in trial 2. This higher amount may have triggered an immune response to eliminate the NAS as there was a substantial surge in SCC by day 2. When comparing the SCC of day 2 and day 0 (pre-infusion), there was a 1 362-fold increase in SCC (Figure 3.1.14). Furthermore, there were some physical changes to the milk but these changes disappeared 48 h post-inoculation. This trend of having a SCC peak at day 2 and physical changes in milk corresponds to a study testing the effects of varying inoculation amounts of *Lactobacillus perolens* (10^3, 10^6, and 10^9) in lactating dairy cows[23]. The inoculation with 10^9 bacteria resulted in physical changes to milk in terms of clots and lumps but disappeared 48 h post-inoculation[23]. However, it's important to note that the increase to 600 316 cells is still 3 orders of magnitude smaller than the 10^9 infusion, but potentially *S. devriesei 1316* may be more virulent than *L. perolens*.

Similarly, in a different study that infused *Lactobacillus lactis* into the mammary gland of cows to trigger an immune response, it invoked a 20 000-fold increase in polymorphonucleocytes (PMN) and

lymphocytes[94]. For *S. warneri* 2993, the increase in milk frequency is possibly due to the opposite reason in comparison to *S. devriesei 1316*. The increase in cell inoculation from 2 330 to 9 321 may have been significant enough to allow for colonization but without evoking a substantial immune response to clear the NAS. The increase in SCC because of the immune response was just a 58-fold difference in comparison to the SCC pre-inoculation.

Secondly, trial 1 contained a mixture of NAS which suggests possible synergistic and antagonistic interactions with the other NAS. In *S. devriesei 1316's* case, since there was higher persistence in trial 1, co-aggregation could have occurred with the other NAS to help with persistence. This ultimately promotes binding to other bacteria or other surfaces inside the udder. An example of co-aggregation has been seen with *Lactobacillus* species and *S. aureus* for closer contact[104]. For *S. warneri* 2993, the interactions with the other NAS in the mixture may have been antagonistic towards its persistence. It is important to mention that work was done to understand the percentages of isolate colonies per mixture. Mixtures were made to ensure each NAS isolate had a replicable number of colony growth and were distinguishable from each other on CHROMagar. For mixture 1 with *S. chromogenes* 5978, *S. xylosus* 5435, *S. warneri* 1052, and *S. devriesei* 1316, the average percentages of colonies were 12.2%, 6.1%, 59.9%, and 21.9%, respectively. For mixture 3 with *S. warneri* 2140, *S. caprae* 4023, *S. equorum* 2121, and *S. warneri* 2993, the average percentage of colonies were 9.6%, 62.2%, 8.4%, and 19.9%. These percentages of colonies were based on an equal number of cells per inoculum unlike the mixtures in trial 1 and 2 which had varying amounts due to the CFU quantification mistake. Another difference is that these percentages were derived from *in-vitro* conditions and not *in-vivo* conditions. Therefore, this makes drawing direct comparisons difficult between the two difficult.

Thirdly, trial 1 and trial 2 contained a different set of cows that may have contained immunity to certain NAS or a different microbiome suitable to certain NAS. Thus, this different set of cows might show differences in the ability of *S. warneri* 2993 or *S. devriesei* 1316 to persist inside the udder. Other

studies have shown that different strains of *S. aureus* can influence the type of host immune response alongside the severity of the intramammary infection inside the same cow[105,106]. These different immune responses would most likely extend to different sets of cows as well. Furthermore, differences in genetics between cows can play a role in its susceptibility to NAS infections. Polymorphisms in the bovine major histocompatibility complex gene has shown to have an effect on the bacterial communities present in colostrum and milk samples[107]. Cows with a specific BoLA genotype had higher levels of potentially beneficial bacteria in their milk samples, while other genotypes had more potentially harmful bacteria[107]. Thus, these differences in microbiota may affect the NAS' ability to colonize the mammary glands.

There were several limitations in trials 1 and 2 that could be addressed in future experiments to provide clearer answers. These limitations were partly due to the trial being a pilot study and the experiment being performed for the first time at the University of Calgary. Two immediate limitations that came up were the inconsistency in cell inoculation because of improper CFU quantification. If each NAS had a consistent number of cell inoculation, then direct comparisons would be possible. The other limitation would be the number of cows in each trial. Since this was a pilot study, only 3 cows were used but by having more cows, more NAS mixtures could have been incorporated with less NAS isolate per mixture. This would reduce the risk of NAS co-interactions between each other and, increase the reliability of the experiment due to more replicates. However, this would require significantly more personnel to tend and milk the cows, alongside a larger housing barn for all the cows. The last limitation comes from objective 1 being a pilot study and was conducted before a larger study to test the feasibility and appropriateness of the study design, data collection methods, and analysis techniques. Consequently, the study had a lower sample size which means there is low statistical power and thus, no statistical significance was able to be detected. Once a larger study is performed, then statistical tests could be done to create statistically significant or non-significant results.

Ultimately, this trial provided us with a persistent NAS for modification with bacteriocin gene clusters and created a model to test the persistence of NAS in the mammary glands of lactating cows.

Furthermore, this seems to be the first mixture of NAS infusion experiment that has been done in the literature. This will be beneficial to anyone who wishes to explore NAS or other bacteria infusions into lactating cows. Several potential experiments could build on this lactating cow infusion study. Firstly, a dose-response study to determine the optimal bacterial dosage for persistence or to see if certain bacteria influence milk production and cow health[23]. This would be done by administrating different concentrations of the bacterial strains and monitoring milk yield, milk composition, SCC, and milk bacteriology. This experiment would be especially useful for *S. warneri* 2993 to understand its optimal dose alongside, seeing if a higher dose results in a similar clearance pattern as *S. devriesei* 1316. Secondly, a multi-strain infusion study could be performed but with even numbers of cells per bacterium to see if these strains have a synergistic or antagonistic effect on milk production and cow health. Thirdly, a long-term study to see how long NAS could potentially persist inside the mammary glands rather than the 2-week trial that was performed. This would be especially important to test on *S. warneri* 2993 and on the probiotic to understand the duration of protection it gives alongside monitoring its effect on milk yield, milk composition, and SCC. Furthermore, this long-term study would show if the introduction of the bacteriocin genes into *S. warneri* 2993 would affect its persistence compared to its wild type. Fourthly, a bacterial infusion timing study to understand the optimal timing for bacterial infusion and persistence could be performed. The study can investigate the effect of persistence during different stages of lactation such as post-calving, early-lactating, mid-lactation, late-lactation or dry period by performing bacterial infusions at different time points in the same cow[108]. For example, Condas et al.[80] that tracked the NAS distribution in lactating dairy cows over a 10-month period (305 days) and saw differences in species prevalence as the months progresses. This study also noted a difference in NAS species from low, intermediate, and high bulk milk somatic cell counts. These

differences in NAS microbiota over the months may be synergistic or antagonistic towards the ability of our persistence NAS to colonize. It would also be interesting to see if the amount of milk production affects the ability of NAS to colonize inside the mammary glands. This question comes from the trend we saw where the highest milk-producing cows had the highest persistence of NAS after infusion while the least producing had little to no persistence. Furthermore, a timing study can be conducted to see if there is a period where cows are more susceptible to NAS persisting before or post-milking[109]. Lastly, a comparison study to investigate the ability of the probiotic, a mixture of bacteriocin-producing NAS, or an antimicrobial-producing bacteria to protect against an *S. aureus* infection compared to no treatment could be done. This study can also extend to check the infusion's effect on milk yield, milk composition, and SCC as well[110,111].

Currently, the reason why *S. warneri* 2993 was the most persistent NAS out of the 16 tested is unknown. While we do have a comprehensive list of virulence factors that S. warneri 2993 has, it is unknown which factor is the main reason for its strong persistence in the udder. S.warneri 2993 has 8 adherence VFs, 10 exoenzymes VFs, 6 immune evasion VFs, 12 iron uptakes VFs, and 1 secretion VFs. More virulence factors do not mean a stronger persistence inside the udder as some NAS isolates in the mixture had double the VFs as *S. warneri* 2993. One possible future direction to understand which virulence factor(s) contribute to the persistence is to create single knock-out mutants of the respective virulence factor. Any unique VFs found only in *S. warneri* 2993 would be preferentially knocked out first. These mutants could then be infused as a mixture into the lactating cow model alongside the wild type to see for differences in persistence. Then, the strains that do not persist can be identified and can indicate which VFs are important. Two other unknowns are the optimal dosage of *S. warneri* 2993 and what is the complete duration *S. warneri* 2993 can persist inside the cow's mammary glands. Two experiments such as a dose-response study and a long-term study should be performed as mentioned above to answer these questions.

Trial 1 and trial 2 both contained a singular cow that had low persistence of bacteria despite getting the same dosage as the other cows. One similarity between both cows is that they were the lowest milk-producing cows out of the 3 cows in each trial. It would be interesting to have a trial containing cows that are in similar lactation cells. This is because for some bacteria such as *Entereococcus faecium,* early lactation cows are more susceptible to IMIs than late lactation cows[80,104]. This trend could be like the NAS used in this study but further comparative studies using cows at different lactation periods are needed. This leads to a possible modification for future lactating cow studies where cows of similar parity and lactation period should be used to normalize all external factors as possible.

Objective 2: Integration of a Bacteriocin Gene Cluster into the Recipient NAS Strain

In this study, we were able to genetically modify *S. warneri* 2993 with an *RFP* gene through allelic replacement. This *RFP* knock-in was into the *mtlF* locus of *S. warneri* 2993 as it would display a phenotypic change of no longer being able to ferment mannitol as a result. While this was successful, the creation of the plasmid containing the bacteriocin gene cluster and the subsequent knock-in of the bacteriocin gene cluster was not successful. This is particularly odd as the process of making the plasmid followed the same procedure as the *RFP* plasmid but with an extra step and needs further exploration. With the *RFP* plasmid, the *RFP* oligonucleotide was synthesized by a third-party company which gives your oligonucleotide a complexity score. Only scores below 10 can be made as the score is used to explain the possibility of creating the sequence based on the complexities in the sequence. While the bacteriocin gene cluster was PCR amplified successfully, we inputted the sequence into the third-party company to see if it was possible to make it synthetically. For the *LanM* section of the bacteriocin gene cluster, it had a complexity score of 51.1 and thus, was denied for creation by the program. It had one or

more repeated sequences greater than 8 bases comprising 65.3% of the entire gene where the limit was 40%. There was a TGTAG repeated sequence that occurred at 7 different locations, a CTTCATCTTT repeat at 2 locations, and an AGCATGTG repeat at 2 locations. These repeats ultimately lead to higher rates of sequencing errors, alongside hairpins and other formations of secondary structures which makes ligation of the fragment into the vector difficult[112]. Lastly, there was also an AACTTCAATTG hairpin at two different locations which could interfere with the fragment ligation into the plasmid. It is important to note that *LanM* is comprised of 2 962 bps out of the 6 684 bps from the entire bacteriocin gene cluster. With nearly half the cluster being a highly complex sequence, it sheds light on the immense difficulties of ligating this fragment into the plasmid. For future directions, if we are determined to use the bacteriocin gene cluster from *S. chromogenes* 803, the complex *LanM* region could be codon optimized for *S. warneri* to potentially lower its complexity. This may prove challenging as most codon optimizing tools are not designed for NAS but rather, *S. aureus*. Therefore, there is a possibility that after the codon optimization for *S. aureus*, the gene may not function properly in *S. warneri.* Another pressing future direction is to use an alternative bacteriocin gene cluster instead of the cluster from *S. chromogenes 803*. There were several other NAS bacteriocin clusters that inhibited *S. aureus* but also had a similar size bp cluster to the one currently used. These new clusters may be synthetically created by a third-party and ligated together using Gibson assembly for a higher rate of success compared to PCR-amplification of the cluster and then ligation. This logic comes from the success of the *RFP* fragment into the plasmid as it was synthetically created by a third-party. Another alternative could be to use different genes that could inhibit *S. aureus* from lactic acid bacteria (LAB), as they can produce antimicrobials, H_2O_2, and other antimicrobial compounds[113,114]. However, if the introduction of these antimicrobial genes into the plasmid keeps on providing difficulties, then an alternative of using bacteriocin-producing NAS could be used. This would simplify the process as genetic manipulation would not be needed but rather, to find a persistent bacteriocin-producing NAS. An

interesting direction could be to create bacteriocin-gene knockouts of the NAS to simply investigate the NAS' ability to colonize the mammary glands without any influence from the bacteriocin it produces. Then, after confirming which bacteriocin knock-out NAS is most effective at colonization, a dosage-response study should be performed to find the optimal number of cells to inoculate that do not cause a strong mammary gland immune response.

Creation of the bacteriocin-plasmid was attempted through Gibson assembly and sticky-end cloning which ultimately did not work. Both products unexpectedly resulted in plasmid self-ligation making the selection of the mutant with the insert significantly more difficult. Initially, the re-ligation in Gibson assembly was hypothesized to be because of a high G/C content overhang that the linearized plasmid contained. This was not the case as when the plasmid was digested with a different set of enzymes leaving a lower G/C content, self-ligation still occurred. These difficulties with creating the plasmid are hypothesized to come from the quality of the DNA fragment (secondary structures, A260/A280 and A260/A230 ratio) prepared and not the actual molecular techniques themselves. This is because the creation of the *RFP* plasmid did not lead to the same problems as the bacteriocin gene cluster fragment. The bacteriocin fragment was purified in several different ways including gel purification after PCR amplification, and PCR purification after PCR amplification. The result was still the same.

Objective 3: Bacteriocin Gene Expression Characterization

In this study, we were able to determine the gene expression levels of the bacteriocin gene cluster in *S. chromogenes* 803 which was our donor bacteriocin strain. There were three different areas of the cluster targeted which included *LanA1, LanA2,* and a portion overlapping both *LanP* and the methyltransferase gene. The average Cq value for those three genes were 23.39, 22.54, and 23.42. It is

hypothesized that all the genes in this cluster have shared regulatory elements such as promoter regions or enhancers that help control their expression in a coordinated manner.And thus, these results seem to confirm this hypothesis. When creating the primers to amplify the bacteriocin gene cluster, a promoter predictor software (BPROM) was used to find a promoter that is 71 bp downstream from the forward primer. It is suspected that this cluster shares that promoter for its gene expression. Furthermore, this cluster may have co-regulation with transcription factors that also coordinate their expression in a similar manner. The same primers used to test these regions were used on our persistent *S. warneri* 2993 to confirm it did not have the bacteriocin genes already present. The average Cq 39.91, 37.98, and no detection which was as expected. This is because when the bacteriocin gene cluster was PCR amplified, *S. warneri* 2993 was used as a negative control and subsequently, no product was amplified. It would have been interesting to see the gene expression levels of the probiotic once it was created to see if expressions dropped or remained the same. All of the future directions come from the successful creation of the probiotic. Firstly, the probiotic should have its bacteriocin gene expression characterized and compared to its level to the wildtype *S. warneri* 2993 alongside *S. chromogenes* 803[115]. Secondly, the probiotic's in-vitro killing ability should be assessed against *S. aureus,* other important mastitis pathogens (*E. coli, Streptococcus agalactiae, etc)*, and other NAS that are commonly found in a healthy cow's udder microbiome. Typically, bacteriocins are effective at killing bacteria of the same species or related genus thus it would be interesting to see its ability to inhibit different genera or the more antibiotic resistant gram-negative bacteria. It is important to understand its spectrum of activity so we understand which microorganisms can be affected by the probiotic. This would be done through the modified cross-streak method[83]. Thirdly, a mouse infection trial should be done confirm the probiotic's ability to prevent mastitis in-vivo. Previously in our lab, a similar trial was performed where lactating mice was infused with a dose of NAS at 0 h and then challenged with *S. aureus* at 24 h. The same procedure will be performed here but with our probiotic *S. warneri 2993* and the wild-type *S. warneri*

2993 for comparison instead. Fourthly, if the mouse trial proved to be successful indicating that the probiotic can prevent the growth of mastitis pathogens *in-vivo,* the next logical step would be to use a lactating cow model[23,94]. Fortunately, we have already created a successful lactating cow model thus, the same protocol would be used with the addition of several cows and correct CFU calculations. This would be achieved by infusing a cow early in lactation cow with an appropriate dose after finding out the optimal dosage for colonization and effect on SCC, and then after 12 h, a challenge with *S. aureus*. Fifthly, it would be interesting to investigate the mechanism of action of the bacteriocin to understand how it inhibits *S. aureus* and have the bacteriocin characterized. This can be achieved by first purifying through high-performance liquid chromatography and characterized in terms of molecular weight, charge, and stability. The mode of action can be investigated through electron microscopy to see how the bacteriocin may disrupt *S. aureus´* cell membrane or if it inhibits other cellular processes[116,117]. Sixth, while it was already mentioned that the appropriate dosing should be determined, another similar avenue to explore is different delivery methods. Currently, the plan was to infuse a standardized dose of the probiotic all at once into the cow's quarters. However, it would be intriguing to explore slow-release formulations of the probiotic which would allow for longer administrations of the probiotic. Furthermore, it would reduce the need for frequent application and minimize the risk of overly simulating that cow's immune system resulting in a surge of SCC and clearance of the probiotic[118]. Lastly, additional bacteriocin gene clusters can be added to the probiotic to create a stronger probiotic capable of inhibiting *S. aureus* and also, reducing the risk of mastitis pathogens developing bacteriocin resistance. This would be done through the same method initially used to introduce the 1st gene cluster with the difference being a different gene target and a different bacteriocin gene cluster.

This study also tested the in-vitro killing ability of *S. chromogenes* 803 using a modified cross-streak method. When this was first tested in 2020, *S. chromogenes* 803 produced a clear streak of inhibition after being challenged with *S. aureus*. However, when this inhibition was checked again in

2021, *S. chromogenes* no longer produced a clear streak of inhibition. Despite most *S. aureus* colonies being inhibited, there were several small colonies that grew in the zone of inhibition. This was particularly odd as the exact same technique was used down to the 48-h incubation time with *S. chromogenes 803.* One difference was the blood agar plates used in 2020 were expired and dehydrated making the agar thin. This thin agar may have increased the local concentration of bacteriocins that form and diffuse into the agar. Thus, giving it a higher concentration of bacteriocins to inhibit *S. aureus* compared to the fresh plates used in 2021. However, we made fresh blood agar plates with a thin layer of agar to simulate the conditions in 2020, and still did not achieve the same level of *S. chromogenes 803* bacteriocin inhibition. Our next hypothesis was that the strain of *S. aureus* used in 2020 was different from the strain used in 2021. Thus, we tested all the *S. aureus* strains in our collection, but we could not achieve the same level of inhibition as we initially did in 2020.

Conclusions

S. warneri 2993 was the most persistent NAS with respect to inflammation out of the 16 NAS strains tested. *S. warneri 2993* grew 28 out of the 42 times from CHROMagar milk culturing and despite having a spike in SCC from day 2, the SCC dropped below the subclinical mastitis threshold of 200,000 cells/mL from day 5 until the end of the trial. Furthermore, this was the first lactating cow infection trial done at the University of Calgary showing an intramammary NAS infusion can result in colonization of the cow's mammary glands. The creation of this trial will make future infection trials more streamlined and efficient.

Despite being able to genetically modify *S. warneri* 2993 with an *RFP* gene to gain expertise, we were not able to perform the bacteriocin gene cluster knock-in. This is because the creation of the bacteriocin pKOR1 plasmid proved difficult due to the self-ligation of the plasmid and failure of inserting

the bacteriocin gene cluster. Several different ligation attempts such as Gibson assembly, blunt-end ligation, and sticky-end ligation were performed without success. The trouble is suspected to stem from the *LanM* section of the bacteriocin gene cluster as it is highly complex with several repeated sequences comprising 65.3% of the gene. Ultimately, this led to the formation of secondary structures making ligation into the pKOR1 plasmid difficult. Future directions would include a re-attempt at doing a bacteriocin gene knock-in but with a different bacteriocin gene cluster.

We examined the gene expression levels of wildtype *S. warneri 2993, S. chromogenes 803,* and the respective controls needed in RT-qPCR. Specifically, we targeted *LanA1, LanA2*, and a section in between *LanP* and the methyltransferase gene. Cq values for *S. chromogenes* 803 indicated that the cluster is controlled by a single promoter. Future directions would include gene expression studies and killing assays of the probiotic. The probiotic will then need to be tested in an *in-vivo* intramammary infection experiment in mice and then in cows following the protocols we have designed.

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