Inhibitory activity of commensals isolated from the bovine teat skin microbiota against the major mastitis pathogen *Staphylococcus aureus*

Theresa J. Astmann
John W. Barlow

Follow this and additional works at: https://scholarworks.uvm.edu/hcoltheses

**Recommended Citation**

Astmann, Theresa J. and Barlow, John W., "Inhibitory activity of commensals isolated from the bovine teat skin microbiota against the major mastitis pathogen *Staphylococcus aureus*" (2019). *UVM Honors College Senior Theses*. 278.
https://scholarworks.uvm.edu/hcoltheses/278

This Honors College Thesis is brought to you for free and open access by the Undergraduate Theses at ScholarWorks @ UVM. It has been accepted for inclusion in UVM Honors College Senior Theses by an authorized administrator of ScholarWorks @ UVM. For more information, please contact donna.omalley@uvm.edu.
Theresa Astmann 955088287 Animal science

Thesis reviewed by the following faculty (3 required):

Faculty name: Ruth Blauwinkel  Department: ASCI
Faculty signature: 
Date: 4/5/2019

Faculty name: Matt Wargo  Department: MMG
Faculty signature: 
Date: 5 Apr 2019

Faculty name: John Barlow  Department: ASCI
Faculty signature: 
Date: 8/4/2019

Presented in a department seminar or presentation to at least three faculty members on (date):
4/4/19

Endorsed by the department chair or equivalent:

I, David Townson (name of department chair), have the support of the faculty of my department (unit or equivalent as applicable) to confirm that (student’s name) Theresa Astmann has completed a thesis and presentation of very high quality, thereby meeting the standards set by (name of department) Animal and Veterinary Sciences necessary to be recognized for Distinguished Undergraduate Research.

Chair's signature: 
Date: 4-9-19
Inhibitory activity of commensals isolated from the bovine teat skin microbiota against the major mastitis pathogen Staphylococcus aureus

Theresa J. Astmann* and John W. Barlow

Theresa.Astmann@uvm.edu

John.Barlow@uvm.edu

* Corresponding author.
For further correspondence: Theresa.Astmann@uvm.edu

a 221 Terrill, Department of Animal and Veterinary Sciences, University of Vermont, Burlington, VT 05405
Abstract

Many studies suggest that certain potentially beneficial commensal bacteria residing in the teat skin environment may protect the bovine mammary gland from mastitis by actively inhibiting colonization by major mastitis pathogens. To investigate the contribution of these commensals to differences in susceptibility to mastitis observed between healthy cows and chronically infected cows, teat skin swab samples were collected from 8 mature, lactating Holstein cows – 4 animals having demonstrated consistently low somatic cell counts and 4 animals having demonstrated consistently high somatic cell counts – and screened for the presence of organisms demonstrating antagonism against the major mastitis pathogen *Staphylococcus aureus*. Presumptive antagonists identified were also individually tested against a second *S. aureus* strain to confirm inhibition. 130 isolates total were characterized as *S. aureus* antagonists over the course of this study, and 36 representative isolates were selected for 16S rRNA sequencing to establish species identity. While the original hypothesis was not supported by the data, potential flaws in study design that may have influenced the results were identified and used to pinpoint targets to address through further investigation. Ultimately, this study did support the idea that the bovine teat skin microbiota represents a rich source of *Bacillus* and *Paenibacillus* commensals demonstrating effective *S. aureus* antagonism which, via the purification of antimicrobial substances and/or the development of novel probiotics, could potentially be applied to alternative strategies of mastitis control.

Key words: mastitis, teat skin, somatic cell count (SCC), growth antagonism, *Staphylococcus aureus*
1. Introduction

Mastitis is the most common and most costly production disease of dairy cattle, imposing a considerable economic impact on dairy farmers in Vermont as well as globally and serving as the principal reason for antibiotic use on farms (Carson et al., 2017; Pol and Ruegg, 2007).

Given the decrease in milk quality that results from infection in one or more quarters as well as the high risk of infection spreading to other animals, there are major incentives for non-organic dairy farms to pursue antibiotic treatment of clinical cases of mastitis. Antibiotic use associated with mastitis control is attributed to both therapeutic as well as prophylactic indications. On dairy farms in the United States, dry cow therapy represents an extremely significant percentage of antibiotic use, as over 90% of dairy farms in the U.S. administer intramammary antibiotics at dry off and at least 80% of these farms implement blanket dry cow treatment for all of their animals (Oliver et al., 2011). Such extensive antibiotic use places heavy selection pressure on mastitis pathogens and contributes to the emergence of antimicrobial-resistant strains, particularly against classes of drugs that are most frequently used for mastitis prevention and/or treatment, such as cephalosporins and other β-lactams (Oliver et al., 2011).

Considering the implications for public health posed by growing antibiotic resistance, there is a significant demand for research focused on mastitis epidemiology to aid in the development of alternative strategies of mastitis control. The existing mastitis research literature contains a long history of attempts to characterize the species composition of the bovine mammary gland microbiota and its relationship to the incidence of disease, and recent studies have identified a number of commensal species colonizing the teat skin that have either never or very rarely been associated with cases of mastitis (Falentin et al., 2016). Furthermore, many studies suggest that certain potentially beneficial commensal bacteria may actually protect the mammary gland from infection by actively inhibiting colonization by major mastitis pathogens.
such as *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae*, and the coliforms (e.g. *Escherichia coli* and *Klebsiella* spp.) (Al-Qumber and Tagg, 2006; Carson *et al.*, 2017; De Vliegher *et al.*, 2004). Further understanding of the organisms colonizing the teat skin and the potential role of competitive exclusion or growth antagonism of bacterial species or communities of the teat skin habitat may provide new insights or tools for mastitis epidemiology and control (Klaas and Zadoks, 2017).

The production of antimicrobial substances, including antibiotics, bacteriocins, and antimetabolites, has been widely observed among various species and proposed as a fundamental mechanism by which certain teat skin commensals confer protection against infection by major mastitis pathogens (Woodward *et al.*, 1987); these substances may therefore represent a useful avenue to explore in the search for novel methods of mastitis control. The potential value of this nature of research is exemplified by a study conducted by Woodward *et al.* (1987), in which 25% of isolates obtained from teat skin swab samples collected from dairy heifers – representing multiple different genera, including *Bacillus, Aerococcus*, and *Corynebacterium* – inhibited both Gram-positive and Gram-negative mastitis pathogens *in vitro*. More recently, De Vliegher *et al.* (2004) described multiple *Staphylococcus chromogenes* isolates of teat skin origin which were found to secrete a bacteriocin capable of diffusing through agar gel to inhibit the growth of *S. aureus in vitro*, and Barboza-Corona *et al.* (2009) found that multiple strains of *S. aureus* were susceptible to five different bacteriocins produced by *Bacillus thuringiensis*. The results from these studies suggest that species of a variety of different genera isolated from the udder microbiota may serve as a viable source of antimicrobial compounds with the potential to be applied to mastitis prevention.
One factor that may help identify individual animals more likely to possess beneficial, antimicrobial-producing commensals colonizing the mammary gland is somatic cell count (SCC), defined as the concentration of somatic cells (predominantly leukocytes) per milliliter of milk. Somatic cell count is widely used in the dairy industry as an indicator of milk quality, but also has an important role in assessing individual animal health; typically, an SCC lower than 100,000 cells/mL for an individual cow would indicate a healthy animal whereas an SCC greater than 200,000 cells/mL would suggest a case of intramammary infection (Ruegg and Pantoja, 2013). In a study performed by Al-Qumber and Tagg (2006), milk and teat skin swab samples were taken from healthy lactating cows selected based upon the observation of consistently low somatic cell counts; from those samples, bacilli with inhibitory activity against mastitis-associated bacteria were successfully identified, therefore suggesting a potential correlation between somatic cell count and the presence of mastitis pathogen antagonists.

The aim of this present study was to sample the teat skin microbiota of adult lactating Holstein cows from the University of Vermont CREAM herd and screen these samples for isolates demonstrating antagonism against multiple strains of *Staphylococcus aureus* originating from other dairy farms in Vermont. The general hypothesis inspiring this work was that healthy, mastitis-free cows possess teat skin microbiota with a species distribution that protects against intramammary infection by major mastitis pathogens such as *S. aureus*, and that this protective effect is potentially due to the presence of bacterial strains which secrete antimicrobial compounds that actively inhibit *S. aureus* growth and colonization of the mammary gland. It was therefore hypothesized that healthy cows from the UVM farm with a history of consistently low milk somatic cell counts would possess a teat skin microbiota with a greater density of *S. aureus* antagonists than chronically infected cows from the same farm, established as such based on a
history of consistently high milk somatic cell counts. Upon identifying a set of antagonists isolated from these samples, the secondary goal of this study was to use 16S genotyping methods to analyze species composition and diversity among these isolates, comparing similarities and differences between healthy and chronically infected cows as well as comparing types of species identified to those characterized as *S. aureus* antagonists in previous, similar studies.

2. Materials and Methods

2.1. Study Design

During three different sampling events over a 5 month period lasting from September 2018 to March 2019, teat skin swab samples were collected from 8 mature lactating Holstein cows belonging to the CREAM herd of the University of Vermont dairy farm. Cows were selected based on assessment of the most recent five months of their milk somatic cell count history, as reported by monthly milk quality testing conducted by the Dairy Herd Improvement Association (DHIA). 4 mid-lactation cows (ID numbers 3799, 3863, 3814, and 3837) were chosen for enrollment in Group 1 of the study based on the demonstration of consistently low somatic cell counts (< 60,000 cells per mL) for at least 5 consecutive months; conversely, 4 mid-lactation cows (ID numbers 3833, 3792, 3864, and 3894) were enrolled in Group 2 of the study based on evidence of chronic mastitis represented by consistently elevated milk somatic cell counts (>280,000 cells per mL) for at least 5 consecutive months. The single exception within this group was cow 3894, whose somatic cell count had been elevated for only 4 consecutive months at the time of sampling; this exception was made due to the absence of a fourth animal within the same herd that fit all of the specifications indicated for Group 2. The number of
lactations completed by each cow was also taken into account when selecting animals for the study. With the exception of cow 3894 (a first-calf heifer), animals ranged from second-lactation dams to fourth-lactation dams, and individuals in each group were matched on the basis of this parameter.

2.2 Sample Collection

The procedure used to collect all samples was approved by the University of Vermont Institutional Animal Care and Use Committee prior to the beginning of the study (IACUC protocol #19-014). Teat skin swab samples were collected while the animals were standing in the milking parlor for their normal afternoon milking. Samples were taken from all four teats, at three different locations on or inside the teat: the external teat barrel (TB), the external teat end (TE), and within the distal streak canal (SC). Before collecting a sample, any obvious fecal debris or bedding material was removed from the teat surface using a clean, non-sterile towel. Samples were then collected using sterile swabs (100 mm normal flocked swabs were used for TB and TE samples while 80 mm mini-tip flocked swabs were used to access the internal streak canal) dipped in sterile water. Swabs were kept on ice in tubes of sterile water and transported back to the lab immediately following the completion of sampling. Samples were vortexed for 5 minutes prior to sterile removal of the swab and then stored in 1 mL aliquots at -20 °C.

2.3 Antagonism Assays

To initially screen samples for the presence of S. aureus antagonists, samples were inoculated in serial ten-fold dilutions out to $10^{-3}$ on Tryptic Soy Agar with 5% sheep blood (Northeast Laboratory) and tested for simultaneous antagonism against SA AC8-MS3, a S.
aureus strain isolated from the bulk tank of an organic Vermont dairy farm. Briefly, on day 1 of this trial, plates were inoculated with 100 μL of sample and incubated for 24 hours at 37°C. After 24 hours, the number of colony-forming units (CFUs) on each plate was counted, and the agar gel was flipped onto the lid of the plate by circularly loosening and freeing the gel with a sterilized spatula. On the newly exposed side of the agar, 100 μL of a 1:1000 dilution of a 0.5 McFarland solution of SA AC8-MS3 was inoculated and spread across the plate using a sterile L-shaped cell spreader. Following another 24 hours of incubation, plates were interpreted by determining the number of zones of inhibition, as indicated by clear zones of no S. aureus growth. When possible (i.e. provided that the density of colonies on the original side of the agar was light enough that separate colonies could be differentiated), a sterile scalpel was used to cut into the agar around the presumptive antagonist so that this colony could be picked up with a sterile inoculating loop and streaked on a new blood agar plate.

Following establishment of pure cultures, each isolate was then tested against an additional S. aureus isolate to confirm inhibition. SA CP20, an isolate derived from an infected quarter of a cow on a Vermont dairy farm which was found to be strongly inhibitory against numerous strains of S. chromogenes as well as other strains of S. aureus from the same farm (Bachmann et al., unpublished), was chosen as the indicator strain for this assay as it was expected to be a more robust S. aureus strain as compared to others. Isolates were individually tested using the functional simultaneous antagonism assay previously described by De Vliegher et al. (2004). Briefly, this method involves inoculating a 0.5 McFarland solution of the isolate being tested as a growth antagonist as a single streak across the center of a blood agar plate, incubating at 37°C for 24 hours, and again flipping the agar gel using a sterilized spatula. On the opposite side of the gel, a 1:1000 dilution of a 0.5 McFarland solution of SA CP20 was
inoculated as a lawn across the surface. Following an additional 24 hours of incubation, each plate was examined for evidence of inhibition by looking for a zone of no *S. aureus* growth overlaying the original center streak of the presumptive inhibitor strain. When present, the width of this zone of inhibition was measured in centimeters. Control plates were also implemented in this assay by testing each isolate against itself, using a 1:1000 dilution of the same 0.5 McFarland solution to inoculate the flipped side. Additionally, the same diluted solution of SA CP20 used for each of the test plates was inoculated on a fresh blood agar plate to serve as a negative control and to demonstrate the growth pattern expected in the absence of an inhibitor.

### 2.4 16S rRNA Sequencing and Species Identification

36 representative isolates from the overall set of inhibitors identified were chosen for 16S rRNA gene sequencing and species identification. Isolates were selected based on the apparent strength of inhibition demonstrated as well as through comparison of macroscopic morphology to other inhibitors identified, such that those chosen to be sequenced out of the greater set of all inhibitors would theoretically represent the overall diversity of species present. DNA extraction was performed on these selected isolates grown for 12-24 hrs at 37 °C in Trypticase Soy Broth (BD™) using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), and extracted DNA was stored at -20 °C. Forward and reverse 16S rDNA primers (Table 1) first described by Wilson *et al.* (1990) and subsequently utilized by Al-Qumber and Tagg (2006) in the identification of *Bacillus* spp. isolated from the bovine teat skin microbiota were used to perform polymerase chain reaction in a Bio-Rad® C1000 thermal cycler. PCR reactions were carried out using 5 μL of 10X PCR buffer, 3 μL of 50 mM MgCl₂, 1 μL of each primer, 1 μL of 10 mM deoxynucleotide mix, 0.5 μL of Platinum *Taq* polymerase, 2 μL of bacterial DNA, and
36.5 μL of sterile molecular grade water to achieve a volume of 50 μL per reaction. After initial
denaturation at 95 °C for 3 minutes, subsequent denaturation reactions were performed at 95 °C,
annealing reactions at 41 °C, and elongation reactions at 72 °C for 30 seconds each; these
conditions were repeated for a total of 30 cycles, after which the final elongation step was carried
out at 72 °C for 10 minutes. Gel electrophoresis was used to verify the success of the 16S
primers and of each round of PCR amplification by confirming the visualization of bands at
approximately 790 base pairs for each isolate (Figure 8), as described by Wilson et al. (1990).

Each sample of PCR product was treated with ExoSAP-IT™ (ThermoFisher Scientific)
to clean up excess primer and nucleotides and subsequently submitted to the Vermont Integrative
Genomics Center of the UVM Medical Center for 16S rRNA sequencing. Forward and reverse
sequences were trimmed and aligned using Geneious® software in order to determine an overall
consensus sequence to then be entered into the search engine of the NCBI BLAST database and
compared to other known DNA sequences.

3. Results

3.1 Screening Assays

In total, 96 skin swab samples from three different locations (teat barrel, teat end, and
streak canal) on all four quarters of 8 cows were tested for the presence of S. aureus antagonists.
All cows from Group 1 (low somatic cell count group) were sampled during one session at the
beginning of the study and these samples remained frozen at -20 °C until ready for screening; in
contrast, sample collection for Group 2 (high somatic cell count group) was divided into two
sampling sessions focused on two cows each. This difference was due to the fact that at the time
of the first sampling session for Group 1, four cows were available that fit the specifications for
this group, whereas at the time of the first sampling session for Group 2, only two cows had
demonstrated high somatic cell counts for five consecutive months. Therefore, subsequent
sample collection was delayed until two more animals qualified for the study.

Samples were diluted in series as needed in order to obtain accurate CFU counts and
permit differentiation between potential inhibitory colonies. Generally, samples of streak canal
(SC) origin had much fewer organisms present than samples of teat end (TE) or teat barrel (TB)
origin; therefore, SC samples were usually tested at a maximum of one ten-fold dilution while
TE and TB samples were diluted out to $10^{-2}$ or $10^{-3}$. If multiple inhibitors were clustered together
on the original side of the agar gel, causing zones of inhibition to overlap, the number of zones
on the plate was estimated as carefully as possible. If the density of inhibitors on the original side
of the gel was high enough that the zones of inhibition could not be differentiated from one
another at all, the plate was recorded as having too many zones to count. Since many of the
undiluted sample plates had very high CFU counts and completely overlapping zones of
inhibition, only potentially inhibitory colonies that were reasonably well isolated were picked up
with a sterile loop and cultured for further testing. Therefore, not all colonies that demonstrated
inhibition were chosen for the individual antagonism assays. An example of a test plate
demonstrating the presence of a well-isolated *S. aureus* antagonist can be seen in Figure 1.

To compare the frequency of inhibitors between the low somatic cell count group and the
high somatic cell count group, the ratio of the number of zones of inhibition observed to the total
CFU count in the sample (both calculated as values per mL based on the final dilution factor of
the best countable plate) was analyzed (Figure 3). For modeling purposes, this data was
transformed to a log scale in order to normalize the distributions. Data points that were
significant outliers or considered untrustworthy were removed from the data set prior to analysis.

For example, a plate in which only 1 colony grew and that colony demonstrated a zone of inhibition resulted in a ratio of 1.0; however, this value was not trusted because it is extremely unlikely that 100% of CFUs from that sample were antagonists. The majority of these unreliable data points came from samples of SC origin, as many of these plates had very low overall CFU counts (i.e. less than 10). Data points from plates where no colonies grew at all were also removed because these plates did not give any information about the frequency of inhibitors in the sample. Samples that were kept in the freezer for longer before testing (for example, samples from cow 3799, which were worked with last out of the set of samples from Group 1 and had been kept frozen for about three months) tended to result in poorer overall growth on all plates.

Greater variation in the ratio of zones of inhibition to total CFU count was observed among Group 1 as compared to Group 2 (Figure 3). The average zones-total CFU count ratio was 0.4867 ± 0.0641 for Group 1 and 0.5832 ± 0.0388 for Group 2. The results of an independent t-test performed to compare these means indicated that the difference between them was not statistically significant (p = 0.1857, df = 73). The average zones-total CFU count ratio was also compared among different sampling locations (Figure 4). Of the three sample locations, samples isolated from the streak canal had the lowest average zones-total CFU count ratio (M = 0.2756 ± 0.0675) while samples isolated from the teat barrel had the highest average ratio (M = 0.6649 ± 0.0508). A one-way ANOVA was performed to compare these means and a statistically significant difference was observed between means (p < 0.0001, df = 2).

The log of zones of inhibition per mL was also plotted as a function of the log of CFU count to determine if a linear relationship existed between these two variables (Figure 5). Regression analysis was performed and a positive linear relationship was supported (m = 0.875,
$R^2 = 0.424$), suggesting that the number of zones of inhibition increases as the total CFU count of a sample increases.

3.2 Individual Antagonism Assays

130 isolates were generally characterized as *S. aureus* antagonists over the course of this study. 57 of these isolates originated from samples obtained from Group 1 and 73 isolates originated from samples obtained from Group 2. Figure 2 shows an example of an isolate demonstrating successful SA CP20 inhibition on an individual test plate, as compared to two types of control plates. Each antagonist was classified into one of four categories based on the width of the zone of inhibition measured on the SA CP20 test plate and/or the overall pattern of inhibition observed (Table 2).

Control plates were performed for each antagonist tested. On almost all control plates, a narrow zone of no growth directly overlaying the initial streak (and often the zone of hemolysis) was observed (Figure 2). In these cases, growth typically stopped very abruptly at the border of the initial streak or zone of hemolysis, with potentially two or three colonies invading into the outer edges of the zone. In contrast to true SA CP20 inhibition, this pattern of growth was suspected to be the result of minimal nutrient availability at that zone of the gel due to thick growth at the level of the initial streak. Therefore, SA CP20 test plates were compared to control plates in order to verify inhibition, and plates with a narrow zone of inhibition in which SA CP20 growth only stopped adjacent to the zone of hemolysis were suspected of demonstrating weak or non-inhibitors, with regards to their activity against SA CP20 (Table 2).
3.3 Species Identification

Based on microscopic and macroscopic morphology as well as preliminary Gram staining, antagonists were presumptively established as members of the Bacillus genus. This was also supported by gel electrophoresis which confirmed the success of the primers PC3mod and P0mod in PCR amplification of the 16S gene (Figure 8), which had been used previously to classify unknown isolates as Bacillus spp. (Al-Qumber and Tagg, 2006). 36 representative isolates demonstrating a clear zone of inhibition were selected for 16S sequencing, and all 36 isolates were established as members of either the Bacillus or Paenibacillus genus. Due to major similarities within the 16S gene observed between species of these genera, the comparison results for sequences entered into the BLAST search engine typically came up with multiple species demonstrating 99-100% matching identity for each isolate. Therefore, isolates could not be completely narrowed down to the individual species level and were instead classified as belonging to a particular species cluster consisting of two or more species between which 16S sequencing was unable to discriminate.

The four main species clusters identified, in order of decreasing frequency of observation, were established as follows: (1) the Bacillus pumilus* cluster (*indistinguishable from B. zhangzhouensis, B. safensis, B. australimaris, B. aerophilus, B. altitudinis, or B. stratosphericus), (2) the Bacillus licheniformis** cluster (**)indistinguishable from B. haynesii, B. paralicheniformis, or B. glycinifermentans), (3) the Paenibacillus barcinonensis*** cluster (***indistinguishable from P. amylyolyticus, P. oceanisediminis, or P. silvae), and (4) the Paenibacillus cookii**** cluster (****indistinguishable from Acetobacter pasteurianus or P. relictisesami). A higher frequency of inhibitors with zones of inhibition greater than 3 cm wide was observed among B. pumilus* and B. licheniformis** isolates as compared to the two
Paenibacillus species clusters, with no Paenibacillus isolates demonstrating zones of inhibition falling into the uppermost range for zone width (i.e. greater than 5 cm) (Figure 6). The proportions of inhibitors identified as B. pumilus* with zone widths in the middle range (3.1-5 cm) and the uppermost range (greater than 5 cm) were markedly higher than the proportions of all other species clusters in each respective category (Figure 6). The frequency of isolates identified as B. pumilus* among sequenced isolates from the low somatic cell count group was also higher than the frequency of B. pumilus* isolates among sequenced isolates from the high somatic cell count group; the opposite trend was true for B. licheniformis** and P. barcinonensis*** (Figure 7). No isolates identified as P. cookii**** were isolated from the high somatic cell count group.

4. Discussion

In this study, no significant difference was reported in the ratio of zones of inhibition to total CFU count between Groups 1 and 2. Therefore, the hypothesis that a higher frequency of S. aureus antagonists would be observed among the low somatic cell count group as compared to the high somatic cell count group was not supported. However, the apparent presence of a positive, linear relationship between the total CFU count and the number of zones of inhibition (Figure 5) does suggest that total CFU count may be a predictor of the density of S. aureus antagonists present within the microbial community of the teat environment – in essence, that “dirtier” teats result in a higher frequency of antagonists because this frequency is simply a function of the number of microorganisms present and is not necessarily related to the infection status of the mammary gland. This hypothesis is also supported by the fact that a higher mean zones-total CFU count ratio was observed among samples from the teat barrel as compared to
samples from the teat end or streak canal (Figure 4), as TB samples typically had the highest initial CFU counts.

The theory that antagonist frequency might be a function of CFU density is logical because all of the antagonists identified were established as members of either the *Bacillus* or *Paenibacillus* genus, both of which are largely comprised of soil bacteria that would be ubiquitous in a farm environment and therefore naturally present on the surface of the teat skin.

Previous studies have already indicated that *Bacillus* spp. make up a significant percentage of the normal bovine teat skin microbiota, and that many of these species demonstrate inhibitory activity against mastitis pathogens (Al-Qumber and Tagg, 2006; Woodward *et al*., 1987, 1988).

Provided that the proportion of the microbial community consisting of *Bacillus* spp. is relatively constant, a teat surface colonized by a higher number of microorganisms would inevitably have a higher number of *Bacillus* organisms present and thus, in theory, a higher number of antagonists present. This finding does not, however, explain the difference in susceptibility to intramammary infection observed between the high and low somatic cell count groups, which may be more tied to individual cow factors than teat skin microbiota factors, or which could potentially be elucidated by further investigation into this specific area of research.

It is important to note that the original hypothesis does not factor the respective inhibitory strength of these antagonists into the equation, which could potentially represent an important piece of the puzzle. *Bacillus pumilus* was identified as the species cluster with the highest frequency of inhibitors producing zone widths in both the middle (3.1-5 cm) and upper ranges (>5 cm) of the spectrum (Figure 6), and the proportion of sequenced Group 1 isolates represented by members of this species cluster was higher in comparison to the proportion of sequenced Group 2 isolates (Figure 7). The observation of greater zone widths does not necessarily
establish isolates as “stronger” inhibitors, as there may be other confounding variables at play at the molecular level (i.e. rates of diffusion through the agar) which have not yet been fully assessed; however, it is certainly possible that those isolates produced antimicrobial compounds which were structurally different from those which typically fell into the lower ranges for zone width and potentially more effective in terms of the extent of their activity. If this were true, it would be conceivable to imagine based off of the results from this study that there might be a difference in the frequency of isolates and species producing more effective antimicrobial substances between the two study groups, and that variation in this parameter as opposed to the overall frequency of inhibitors present might play more of a role in determining individual cow susceptibility. Sequencing of the remainder of the antagonists identified in this study would provide more data from which to draw conclusions regarding the relative frequencies of individual species; given that 16S sequencing was unable to differentiate between numerous species within the two main genera identified, targeting a different gene – such as the rpoB housekeeping gene, which has demonstrated success in enabling differentiation between more closely related Bacillus and Paenibacillus species (Durak et al., 2006) – may be useful in gathering more precise species data. Additionally, further characterization of the antimicrobial substances described in this study would be a helpful first step toward better understanding their respective levels of activity and modes of action, as well as how these factors may be related to the relative “strength” of each individual inhibitor.

There are several factors related to study design that may have influenced the results of this study and that could also serve as targets for continuation of this work. First, it is possible that the different lengths of freezer storage time between low SCC samples and high SCC samples may have skewed the data, as samples from cow 3799 (which, out of all samples, were
stored in the freezer for the longest before testing) had very minimal growth all around and likely
did not reflect the true microbial community associated with the teat skin of that animal. Since
samples were initially frozen in sterile water as opposed to a glycerol growth medium designed
to support long-term storage, it may be possible that CFU count decreased with the length of
storage time, and this could have easily affected the data for the low SCC group. Since all high
SCC samples were worked with closer to the original sampling date, this could have influenced
the antagonist frequency results to be more accurate for Group 2 and potentially less accurate for
Group 1 (at least for samples analyzed last, which still made up a significant percentage of the
overall data set for Group 1). This would account for the more extensive variation observed in
the data for Group 1 as compared to Group 2 (Figure 3). Therefore, for future continuation of
this work, it would most likely be prudent to collect fewer samples at a time and test them sooner
to avoid potential deterioration of the samples over time.

An additional factor to consider when analyzing the results of the data in terms of the
original hypothesis is the fact that neither of the two S. aureus isolates used as indicator strains
originated from the UVM CREAM herd. Therefore, the results from this study do not necessarily
reflect the specific relationship between antagonists isolated from the teat skin microbiota and
the pathogen strains that they presently co-exist with, some of which may have developed
adaptations to make them more resistant to antimicrobial substances produced by antagonists
residing in the same niche. It may be possible that some of the isolates that actively inhibited
both S. aureus strains in this study would fail to inhibit S. aureus strains originating from the
same farm, which have potentially accumulated mutations providing them with growth
advantages specific to their own environment and which may include resistance to certain
antimicrobials. As a follow-up study, it would be worthwhile to test all antagonists identified in
this study against two or more *S. aureus* strains isolated from clinical cases of mastitis at the UVM farm, and then compare those results between low and high SCC cows and between different species. If resistance to these antagonists was more frequent among the high SCC group compared to the low SCC group, this could potentially support the original hypothesis – in other words, even though the present study suggests that both groups have a similar density of antagonists, it may be true that cows more resistant to mastitis have a teat skin microbiota favoring a higher density of antagonists that are more competitive in their specific environment, as a result of the production of antimicrobial substances with a stronger effect against indigenous mastitis pathogens.

The simultaneous antagonism assay described by De Vliegher et al. (2004) was chosen to test individual isolates for *S. aureus* antagonism because this method specifically allows for the identification of isolates which exert their inhibitory effect through the production of diffusible antimicrobial compounds; patterns of inhibition more likely to be caused by other factors, such as nutrient competition, can easily be distinguished through the aid of control plates (Figure 2).

The production of antimicrobial compounds by bovine teat skin commensals with activity against mastitis pathogens has been observed among coagulase-negative staphylococci, numerous *Bacillus* and *Paenibacillus* spp., and other species (Carson et al., 2017; Nascimentoa et al., 2005; Ding et al., 2011). Bacitracin, an antibiotic with widespread clinical use that is known to be effective against *S. aureus*, is one example of a well-characterized antimicrobial peptide produced by *B. licheniformis* (Sumi et al., 2014) and one which may have been produced by certain isolates classified as members of the *B. licheniformis*** cluster. Follow-up analysis of the antagonists identified in the present study should include purification and characterization of the antimicrobial compounds being produced, along with comparison to conventional antibiotics.
such as bacitracin. Subsequent in vitro testing could be employed to measure the susceptibility of a panel of different *S. aureus* strains to these compounds, quantify their minimum inhibitory concentrations (MICs), and assess the potential development of resistance. Such work could ultimately inform future in vivo studies designed to assess the efficacy of antibiotics derived from the normal microbiota of the teat skin in prophylactic and/or therapeutic management of mastitis.

The present research supports the idea proposed by Al-Qumber and Tagg (2006) suggesting that *S. aureus* antagonists of the *Bacillus* genus may merit further consideration in terms of their potential application as a probiotic designed to support mammary gland health (and also identifies antagonists of the *Paenibacillus* genus which could be applied for this use as well). The idea of intentionally colonizing the mammary gland with species known to be inhibitors of mastitis pathogens to confer protection against these pathogens is not novel (Matthews et al., 1990); however, the main downside of this strategy is the risk of the new species introduced causing more harm than benefit by triggering an elevation in somatic cell count. The advantage of using antagonists such as those identified in this study (particularly those isolated from Group 1 cows) would be that these species make up part of the normal microbiota of the teat surface and streak canal and therefore would not be expected to negatively impact the mammary gland as a result of their colonization. In a recent appraisal of the potential of probiotics as a strategy for mastitis control, it was concluded that although requiring extensive research prior to implementation, a probiotic delivered at the level of the teat apex (as opposed to intramammary or oral delivery) could hypothetically offer a real possibility of preventing mastitis (Rainard and Foucras, 2018). Numerous *Bacillus* spp. are already widely used as probiotics to supplement the diet in humans and animals, including *B. coagulans, B. clausii, B.*
cereus, B. subtilis, and B. licheniformis (Mingmongkolchai and Panbangred, 2018). The general safety of these species in addition to their demonstrated capacity to shift the balance within the microbiome to favor overall eubiosis has established them as effective probiotics for gastrointestinal health, indicating that certain Bacillus species could reasonably have the same effect on mammary gland health.

Good hygiene practices (i.e. bedding management, milking system sanitation, overall farm cleanliness etc.) serve as the first and most important step in controlling mastitis, but due to the ubiquity of pathogens in the environment, it can be challenging to keep animals infection-free even under the best of management systems. Antimicrobial drugs are currently the best tool available to farmers and veterinarians in treating cases of mastitis once they arise, and are also used heavily in a prophylactic sense on most farms in the U.S. to help protect the mammary gland for future lactations once cows enter the dry off period. Such a high dependence on these drugs for maintaining herd health, however, poses serious risks for livestock and humans alike as antibiotic resistance becomes a rapidly growing public health concern. Research focused on addressing knowledge gaps in mastitis epidemiology as well as investigating novel mastitis treatment strategies is of critical importance in supporting the goal of reducing the use of antibiotics in agricultural practices. Reflecting the increasing level of significance that is currently being accorded to the microbiome by emerging research in the field, the findings in this present study ultimately contribute to a growing understanding of the importance of the relationship between eubiosis within the udder and teat skin microbiota and overall animal health.
5. Conclusion

Although the original hypothesis that a significant difference would be observed between the frequency of *S. aureus* antagonists isolated from healthy, low somatic cell count cows and chronically infected, high somatic cell count cows was not supported, the present study was successful in identifying numerous isolates from both groups with the capacity to inhibit the growth of two different strains of *S. aureus*. Furthermore, multiple factors have been identified that may have influenced the data and are worth further investigation before ruling out the hypothesis. Ultimately, the findings in this study support those reported in other similar studies which suggest that the teat skin microbiota is a rich source of commensals, particularly those of the *Bacillus* and *Paenibacillus* genera, with the capacity to secrete antimicrobial compounds effective in inhibiting the growth of *S. aureus*. Be it through purification and characterization of these compounds or through assessment of the suitability of these commensals for use as a probiotic, the *S. aureus* antagonists identified in this study offer a wealth of further research avenues to explore and suggest that *Bacillus* and *Paenibacillus* species with a demonstrated range of activity against mastitis pathogens may represent a valuable resource in the development of alternative methods of mastitis control.

Acknowledgements

The author would like to thank Dr. John Barlow, Ashma Chakrawarti, Sam Keum, and Ariela Burk for their guidance and support throughout the research process; Dr. Ruth Blauwiekel and Dr. Matt Wargo, for reviewing this manuscript; and the Animal Science department as well as the Institutional Animal Care and Use Committee at the University of Vermont, for providing the opportunity to make this study a reality.
**Funding**

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Conflict of Interest Statement**

The author declares no conflicting interests in regards to the publication of this manuscript.

**References**


Table 1: PCR primers used for 16S sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3mod (forward)</td>
<td>5’ GGA CTA HAG GGT ATC TAA T 3’</td>
</tr>
<tr>
<td>P0mod (reverse)</td>
<td>5’ AGA GTT TGA TCM TGG 3’</td>
</tr>
</tbody>
</table>

Table 2: Distribution and classification of *S. aureus* antagonists identified from all cows enrolled in the study.

<table>
<thead>
<tr>
<th>Cow</th>
<th>Zone of Inhibition Width</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weak a</td>
</tr>
<tr>
<td>3863</td>
<td></td>
</tr>
<tr>
<td>3814</td>
<td></td>
</tr>
<tr>
<td>3837</td>
<td></td>
</tr>
<tr>
<td>3799</td>
<td></td>
</tr>
<tr>
<td>3792</td>
<td></td>
</tr>
<tr>
<td>3833</td>
<td></td>
</tr>
<tr>
<td>3864</td>
<td></td>
</tr>
<tr>
<td>3894</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
</tr>
</tbody>
</table>

a Isolates for which the test plates demonstrated SA CP20 growth very close up to the zone of hemolysis/initial streak were perceived as weak or questionable inhibitors.

b Includes isolates for which SA CP20 growth was completely absent.
**Figure 1** Test plate demonstrating a clear zone of SA AC8-MS3 inhibition around the colony at the top of the plate.

**Figure 2** Right: example of test plate demonstrating SA CP20 inhibition by an isolate characterized as a very strong inhibitor; Bottom left: example of control plate in which the same isolate is inoculated on both sides of the gel (note lack of growth along central streak); Bottom right: example of negative control plate demonstrating dense growth of diluted SA CP20 in the absence of an inhibitor streak.
Figure 3  Box-and-whisker plot representing the lower and upper extremes, lower and upper quartiles, and medians of the zone-total CFU count ratios for both groups of the study, with outliers and untrustworthy data points removed (n = 75). More extensive variation in the data is evident in the distribution for Group 1, representing the low somatic cell count cows, as compared to Group 2. The difference in mean ratio values between Groups 1 and 2 (M₁ = 0.4867 ± 0.0641, M₂ = 0.5832 ± 0.0388) was not determined to be statistically significant (p = 0.1857, df = 73).
Figure 4 Box-and-whisker plot comparing the distribution of the ratio of zones of inhibition to total CFU count among the three sampling locations on the teat: the streak canal (SC), the teat barrel (TB), and the teat end (TE). Outliers and untrustworthy data points have been removed from the model (n = 75). The results of one-way ANOVA indicated that the difference in mean ratio values between sampling locations ($M_{SC} = 0.2756 \pm 0.0675$, $M_{TB} = 0.6649 \pm 0.0508$, $M_{TE} = 0.5651 \pm 0.0526$) was statistically significant ($p < 0.0001$, df = 2), with the teat barrel having the highest mean zone-total CFU count ratio and the streak canal representing the lowest.
Figure 5  Linear regression model depicting a positive linear trend \((m = 0.875)\) in the relationship between the total CFU count per mL and the zones of inhibition per mL, with outliers and untrustworthy data points removed \((n = 75)\). The \(R^2\) value is 0.424, indicating that the model accounts for 42.4% of the variation in the data.
Figure 6  Bar graph comparing the observed frequencies of each species cluster with respect to width of zone of inhibition. Frequencies were calculated by determining the number of isolates of a given species cluster classified into a specific range based on width of zone of inhibition and dividing by the total number of isolates demonstrating a zone of inhibition falling within that range (n_{total} = 36).
**Figure 7** Bar graph comparing the distribution frequencies of each species cluster between Groups 1 and 2. Frequencies were calculated by determining the number of isolates identified as members of a given species cluster and dividing by the total number of isolates sequenced from that group (n_{Group 1} = 24, n_{Group 2} = 12; n_{total} = 36).
**Figure 8** Gel demonstrating successful PCR amplification of the 16S gene on 14 isolates using the primers PC3mod and P0mod, as indicated by the presence of clear, dark bands of approximately 790 base pairs. Lane 1 represents the DNA ladder, and lanes 2 and 10 represent negative controls.
Reflection

Although the DUR process was one of the most challenging undertakings of my academic career, it is one that I am extremely glad I chose to pursue. I am grateful to have had the opportunity to design a research project tailored to my scientific interests and see it through to the end, through all the highs and lows that I encountered along the way. I believe that a rather unique aspect of Dr. Barlow’s laboratory as compared to others is the level of independence that is accorded to his students; while it may have been a bit unnerving at first, I quickly learned the necessity of taking initiative and now understand how beneficial this has been for my own personal and academic growth. I have come to realize that I am the kind of student who really values and enjoys individual work because it allows me to set my own standards of rigor and challenge myself to reach or even exceed them.

Throughout this process I have had to learn how to perform numerous different procedures rather quickly and then be able to perform them on my own right away, as a result of which I have gained a strong sense of confidence when it comes to a variety of methods and techniques commonly utilized in the fields of microbiology and molecular biology. I can truthfully say that I have devoted hundreds of hours to the lab over the past year, and while I did receive help on some occasions from fellow students, for the most part I completed all of the wet lab work on my own. I definitely benefitted from Dr. Barlow’s support when I had questions or ran into issues, but I also become increasingly more independent over the course of this project in terms of troubleshooting problems on my own. I would first seek out answers in the research literature when I had questions, and often found myself getting inspired with new hypotheses to consider or ideas to explore.
The most important thing I will take away from this experience is the realization of how much I enjoy independent research. Despite the long hours spent in the lab, the setbacks I encountered, and the stress of the past few months, throughout the entire process I genuinely felt passionate about the work I was doing. Ultimately, the time crunch that I ran into toward the end – the greatest challenge I had to overcome during this process – was due to my own obstinacy in wanting to collect more data that I was personally interested in but which was theoretically superfluous in terms of the amount I needed for an undergraduate-level thesis. I pushed myself harder than I ever have before to finish all of my data collection on time, but ultimately I feel that the work I put into this project paid off and I will submit this document feeling proud of what I have accomplished. I also plan to continue working on this research over the coming months and hope to have a manuscript worthy of publication by the end of the summer.

For most of my college career, I have envisioned veterinary school and veterinary practice as my determined path; now, I have begun to consider the other options that are available to me, particularly those associated with research. My new plan is to attend graduate school for a Master’s thesis in animal science or microbiology, after which I may decide to pursue a dual DVM/PhD degree. While I am still interested in going into practice, I can also see myself staying in academia and focusing my career on conducting research within some sector of veterinary medicine. Regardless of what I do decide on, completing the DUR has been a truly invaluable part of my college experience and has given me a novel appreciation for the scientific process and the field of veterinary microbiology as a whole. I am excited to see where this leads me next!