EVALUATING SAMPLING AND DNA EXTRACTION TECHNIQUES FOR CULTURE-INDEPENDENT ANALYSIS OF BOVINE MAMMARY GLAND NORMAL FLORA

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INTERPRETIVE SUMMARY

Methods for determining the varieties of bacterial species that normally reside on cow teats were reviewed. Milk and skin swab samples were collected and processed following various techniques described in previous research studies, and the ability of these techniques to yield bacterial DNA was assessed. The two methods for extracting bacterial DNA directly from cow's milk did not achieve large yields, and none of the evaluated skin swabbing methods exhibited a statistically significant advantage over the other methods in recovering bacterial DNA. The use of bacterial DNA to distinguish bacterial species in cow teat environments was discussed.

ABSTRACT

Udder health in dairy cattle is not solely influenced by bacteria present within the teat's internal structures but also by bacteria growing at external epidermal surfaces on the mammary gland. Culture-independent methods for surveying bacterial diversity of epidermal surfaces have created more accurate profiles of bovine teat microbiota that contribute to an enhanced understanding of mastitis etiology and prevention. This study evaluated techniques for bacterial DNA extraction from milk and skin swab sampling to determine their applicability for use in future culture-independent investigations of this microbiome. Two commercial column-based kits (the Norgen Milk Bacterial DNA Isolation Kit and the Qiagen QIAamp DNA Mini Kit) were used to extract DNA from gradients of milk dilutions spiked with *S. aureus* (0 cfu/mL to ~10^5 cfu/mL) so that a threshold of reliable PCR detection of extracted DNA could be established for each kit. Additionally, three individual comparisons were made for skin swab transportation media (ddH_2O, "Swab Buffer" and "Milk Buffer"), swab moisture status at the time of sampling (Wet vs. Dry) and swab agitation methods following sample collection (Vortexing vs. Stomaching) by analyzing both aerobically-cultured bacterial yields and extracted bacterial DNA concentrations from skin swabs of Holstein-Friesian and Jersey dairy cows. The two commercial milk bacterial DNA extraction kits were generally ineffectual in recovering amplifiable bacterial DNA extracts from low concentrations of bacteria and as such should be avoided for culture-independent analysis of bacterial communities in non-mastitic milk unless their protocols are amended with improved milk fraction treatment and more rigorous cellular lysis conditions. Moreover, no clearly superior skin swab sampling and processing methods were identified from any of the three comparisons, so future investigations covering larger herds or employing altered study designs are necessary to obtain more definitive results.

Key Words: Mastitis, Mammary Gland, Normal Flora, DNA Extraction

INTRODUCTION

The importance of mastitis control to the bovine dairy industry cannot be overstated. In 2007, intramammary infections (IMI's) represented the single most prevalent disease amongst U.S. dairy cattle, afflicting a proportion of nearly 1 in every 5 animals (USDA, 2009). Moreover, approximately 90 percent of the mastitic cattle from this year were administered antibiotics (USDA, 2009), a fact that qualifies mastitis treatment as a major cost to American dairy farmers. However, producers also incur financial detriments from reduced cow productivity and loss of sale premiums due to diminished milk quality (Seeger et al., 2003), as well as from preventative measures like sanitizing iodine teat dips (Foret et al., 2005). For these reasons, mastitis therapies and prevention strategies have been and continue to be the subject of intensive scientific research so that superior products and practices may be developed.

Mastitis-causing pathogens generally access mammary gland tissue via passage through the teat (or streak) canal and lumen (Paulrud, 2005). As is the case with superficial skin, the epidermal surfaces of these structures are composed of continuous sheets of keratinocytes that serves as a primary physical barrier against such pathogens (Paulrud, 2005). It would therefore be tempting to hypothesize that the burden of protection against teat infection lies squarely upon these deeper tissues, but an existing body of literature suggests that external teat conditions also contribute to the mammary gland's overall health. For instance, Neijenhuis et al. (2001) demonstrated that teat end callosity accrued after prolonged machine milking exhibited a direct relationship with prevalence of clinical mastitis and additionally highlighted prior evidence that the presence of lesions (erosions or scabs) on teat end skin is positively associated with

subclinical mastitis. Such modifications of the teat skin's epidermal surface may create different microenvironments with more favorable conditions (e.g. altered pH, moisture, temperature, etc.) for pathogen persistence and proliferation (Weese, 2013).

Changes in epidermal microenvironments affect not only transient pathogens but also the resident or "normal" flora that generally occupy them (Grice & Segre, 2011). Some normal flora species establish mutualistic relationships with their hosts by producing substances the host's cells cannot manufacture or by inhibiting the growth of harmful organisms, so when a change in an epidermal microenvironment is significant enough to disturb the composition of local normal flora, disease states may result (Grice & Segre, 2011). It is important to consider, however, that microflora communities are not homogenously distributed across the epidermis. The bacterial components of normal flora communities have been confirmed to vary widely across the spatial terrain of the skin – in fact, a community at a given site on one individual is likely to be more similar to a community at the same site on a different individual than to a community at a different site on the same individual (Weese, 2013). Separate skin environments should consequently be assessed individually when disease states do arise, for different pathogens may be responsible across various sites despite shared signs or symptoms of infection.

Several studies have sought to characterize the bacterial normal flora of bovine teat skin (Woodward et al., 1987; De Vliegher et al. 2003; Braem et al., 2012; Braem et al., 2013). Consistent with findings of human skin (Weese, 2013), these studies unanimously identified coagulase-negative staphylococci (**CNS**) as highly prevalent bovine teat skin colonizers. Although many CNS species may opportunistically infect the mammary gland and instigate subclinical and clinical mastitis (Braem et al., 2013), cattle with CNS intramammary infections (IMIs) early in lactation have been able to out-produce uninfected herd-mates across the span of the lactation (Piepers et al., 2011). Moreover, De Vliegher et al. (2003) concluded that teat end colonization by *Staphylococcus chromogenes* could be protective against IMI in early lactation, and *in vitro* assays by Woodward et al. (1987) demonstrated growth inhibition of major mastitis pathogens (namely, *S. aureus, S.* agalactiae, S. *dysgalactiae, S. uberis* and *E. coli*) by 25% of normal flora species (including CNS species). Similar mutualistic relationships have been noted in the human skin microbiome, such as the recent observation that common skin resident *Propionibacterium acnes* can inhibit the growth of methicillin-resistant *S. aureus* (MRSA) by secreting fermentative byproducts into infected wounds (Shu et al., 2013). These examples illustrate that an understanding of bovine teat normal flora is essential if knowledge of mastitis etiology and disease prevention is to progress.

Traditional, culture-based methods of skin microbiology do not adequately characterize normal flora communities because they cannot cultivate the growth of anaerobic, microaerophilic or fastidious aerobic species otherwise present on or within the teat epithelium (Weese, 2013). Modern culture-independent methods employ molecular genetics to detect these "absent" species and thus give a more accurate portrayal of the skin microbiome (Braem et al., 2013). In essence, the success of culture-independent studies does not hinge upon the isolation of the organism itself but rather the harvest of its DNA. The unique genetic markers (i.e. single-nucleotide polymorphisms, or SNPs) of the DNA are distinguished by genetic analysis so that the presence of a given organism can be confirmed or refuted. In this way, an additional benefit of culture-independent analysis is that collected cells do not have to be harvested alive to be detected. However, sample collection and processing are still extremely important for culture-independent methods to function properly, for some species may only be represented by a sparse distribution

of individuals across an epithelial surface. Cells must be collected in sufficient quantities to yield workable concentrations of DNA for downstream processing.

Methodological comparisons are therefore important in determining which techniques are most effective in recovering yields of bacterial DNA that are both high (or at least sufficient) in concentration and representative of the community sampled. Therefore, the aim of the present investigation was to evaluate methods of sampling and DNA extraction as they pertain to the various regions of the bovine teat epidermis so that more accurate profiles of this microbiome may be realized. In particular, milk collection (representing bacteria from the teat cistern epidermis suspended in mammary gland secretions) and skin swabbing (representing bacteria from the external teat and streak canal epidermis) were identified as the primary means of capturing the microflora of all possible teat environments. In total, two commercial milk bacterial DNA extraction kits were examined individually, and three separate experiments testing skin swab collection and processing methods were investigated. This second component of the study is of particular interest because, to the author's knowledge, there have not yet been explicit methodological evaluations for bovine teat skin sampling in the existing literature. End-point PCR was conducted when possible to demonstrate the ability of extraction and sampling methods to yield amplifiable bacterial DNA.

MATERIALS AND METHODS

Experiment A: Norgen Milk Bacterial DNA Extraction

Milk Collection. Two Holstein-Friesian cows (3593 Mandrake and 3686 Beyonce) from the UVM CREAM herd (Miller Research Complex, Burlington, VT) were selected as likely candidates for sterile milk production due to their low somatic cell count (**SCC**) as measured by monthly Dairy Herd Improvement Association (**DHIA**) testing. The cows were housed in a tie-stall facility accommodating a total of 34 lactating cattle and were milked twice daily (approximately every 12 hours). In April of 2012, between 500 mL and 1000 mL of milk were collected aseptically from each of the cows' quarters by hand-milking following teat sterilization with FS-103X iodine pre-dip (IBA Incorporated, Millbury, MA) and 70% ethanol gauze pads. Aliquots of approximately 50 mL were taken from each quarter's volume and measured using a DeLaval Cell Counter DCC (DeLaval International AB, Tumba, Sweden) to obtain precise SCC values. Additionally, 100 µL from each aliquot were streaked for confluency in triplicate on blood agar plates (**BAPs**) of tryptic soy agar with 5% sheep's blood (Northeast Laboratories, Waterville, ME), which were incubated aerobically overnight at 37°C in order to obtain rough estimates of bacterial concentration.

S. aureus Preparation and Inoculation. 5 mL of tryptic soy broth were inoculated with a single colony from a stock plate of *S. aureus* American Type Culture Collection (**ATCC**) strain 25923 (ATCC, Manassas, VA) and incubated aerobically overnight at 37°C. Suspended cells were then sedimented by centrifugation at 3,600 × *g* for 15 minutes at 4°C and washed twice with sterile double-distilled water (ddH_2O) using the same centrifuge conditions. The washed cells were re-suspended in 5 mL of sterile ddH_2O and diluted serially in 1:10 increments until a concentration of 10^{-7} was attained. The final three dilutions in the series (10^{-5}, 10^{-6} and 10^{-7}) were streaked for confluency in triplicate on BAPs, which were then incubated aerobically overnight at 37°C in order to obtain a known colony forming unit (**cfu**) count of the bacterial suspension. This calculated cfu count was then employed to discern the volume of bacterial suspension representing a 10^6 cfu inoculum.

Creation of Spiked Milk Gradient. Based on the bacterial suspension cfu concentration calculations, 20 µL of bacterial suspension were inoculated into 980 µL of milk from 3686 Beyonce's right front quarter (selected based on its low SCC and lack of bacterial growth in culture) to give a 10^6 cfu/mL concentration. This spiked milk sample was then diluted serially in 1:10 increments into sterile milk from the same aliquot until a concentration of 10^0 cfu/mL was attained. A range of seven sequential spiked milk dilutions (10^0-10^5 with an added sterile milk negative control) were sent to Lancaster (PA) DHIA for two purposes: 1) to correlate cfu counts obtained by streaking the dilutions for confluency in triplicate on BAPs and aerobically incubating them overnight at 37°C with Lancaster DHIA's Pathoproof real-time PCR cycle threshold ($\mathbf{C_t}$) values obtained for *S. aureus*; and 2) to confirm the extractability of the *S. aureus* DNA via Lancaster DHIA's Pathoproof real-time PCR.

Bacterial DNA Milk Extraction. Bacterial DNA from the seven spiked milk dilutions and an additional positive control aliquot of pure *S. aureus* ATCC strain 25923 bacterial suspension was extracted using a Norgen Milk Bacterial DNA Isolation Kit (Norgen Biotek Corp., Ontario, Canada) according to the manufacturer's instructions. Milk samples in microfuge tubes were centrifuged at 14,000 × *g* for 2 minutes to obtain pellets, which were then isolated by removing the supernatant and cream. The pellets were subjected to incubation at 37°C for 45 minutes in 100 µL of Digestion Buffer containing lysozyme and lysostaphin (1.0×10^{-4} mg/mL). 300 µL of Lysis Solution and 10 µL of proteinase K (reconstituted in microbiology-grade H_2O) were added to the mixture, which was subsequently incubated at 55°C for 45 minutes. 40 uL of Binding Solution and 180 µL of 100% ethanol were added after this incubation, and the mixture was centrifuged at 14,000 × *g* for 10 seconds. The resultant clear aqueous phase of the mixture was transferred to a silica-based spin column and centrifuged at 14,000 × *g* for 3 minutes. Two wash buffers (Wash Solutions 1 and 2) were employed in successive column washing steps with centrifugation at 14,000 × *g* for 2 minutes, after which the column was dried by centrifugation at 14,000 × *g* for 3 minutes. DNA was finally eluted into 200 µL of Elution Buffer by two successive centrifugations at 2,600×*g* for 2 minutes and 14,000 × *g* for 2 minutes. DNA concentrations were measured using a Thermo Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and remaining DNA was stored at -20°C until further processing.

S. aureus Multiplex PCR Amplification of Extracted DNA. Primers to amplify the *S. aureus*-specific thermonuclease (*nuc* – Forward: 5'-GCGATTGATGGTGATACGGTT-3'; Reverse: 5'-AGCCAAGCCTTGACGAACTAAAGC-3'), β-lactamase (*blaZ* – Forward: 5'-AAGAGATTTGCCTATGCTTC-3'; Reverse: 5'-GCTTGACCACTTTTATCAGC-3') and methicillin resistance (*mecA* – Forward: 5'-AACAGGTGAATTATTAGCACTTGTAAG-3'; Reverse: 5'-ATTGCTGTTAATATTTTTTGAGTTGAA-3') genes were employed to identify the presence of *S. aureus* DNA in the eluted samples from the Norgen extraction based upon their application in multiple investigations of *S. aureus* transmission dynamics (Vesterholm-Nielsen et al., 1999; Martineau et al., 2000; Barlow et al., 2013). Additional reagents in the PCR master mix included PCR-certified H_2O (Teknova, Hollister, CA), 10X ThermoPol Reaction Buffer (New England BioLabs, Ipswich, MA), 50mM $MgCl_2$ (Invitrogen), deoxynucleotide solution mix (New England BioLabs) and Taq DNA Polymerase (New England BioLabs). The Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) conditions for the reaction included an initial denature step at 95°C for 15 minutes, 35 cycles of denaturation, annealing and elongation at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, respectively, and a final extension step at 72°C for 10 minutes. The PCR amplicons were loaded

into a 1.5% agarose gel and electrophoresed at 100 V for one hour to visualize the presence of the *S. aureus*-specific genes in each sample.

The *S. aureus* preparation, milk spiking, DNA extraction and PCR phases of this experiment were replicated one time.

Experiment B: Qiagen Milk Bacterial DNA Extraction

The same protocol for the Norgen kit detailed above was conducted for an assessment of the Qiagen QIAamp DNA Mini Kit (Qiagen GmBH, Hilden, Germany) in October of 2012. The following subtleties distinguished this new experiment from the prior procedure:

- Following a new round of milk collection and assessment, milk from 3593 Mandrake's left hind quarter was selected for use in creating spiked dilutions.
- *S. aureus* ATCC strain 33591 was employed instead of the former strain 25923 based on laboratory inventory at the time of the new experiment.
- The relatively higher calculated concentration of the new *S. aureus* suspension required that only 2 µL be added to 1998 µL of sterile milk to achieve a 10^6 cfu/mL spiked concentration.
- The Qiagen kit directions' initial digestion step was performed on whole, non-centrifuged milk, and the buffer employed (Buffer ATL) contained neither lysozyme nor lysostaphin. 200 µL of whole milk, 180 µL of Buffer ATL and 20 µL of proteinase K were combined and incubated at 70°C for 30 minutes. Addition of 200 µL of Buffer AL was followed by a subsequent incubation at 70°C for 10 minutes. 200 µL of 100% ethanol were added to the mixture, which was then transferred to a silica-based spin column and centrifuged at 15,000 × *g* for one minute. 500 µL of two wash buffers (Buffers AW1 and AW2) were employed in successive column washing steps with centrifugation at 15,000 × *g* for 1 minute, after which the column was dried by centrifugation at 15,000 × *g* for 3 minutes. DNA was finally eluted into 200 µL of Buffer AE by allowing one minute of incubation at room temperature in the column and subsequently centrifuging at 6,000 × *g* for one minute.

Experiment C: Skin Swab Transport Media Comparison

***Preparation of Transport Media*.** Three different transport media were prepared based upon notable examples from previous literature (Verdier-Metz et al., 2012; Braem et al., 2013). The first, denoted H_2O, was simply composed of sterile ddH_2O. The second, denoted Swab Buffer, consisted of a sterile ddH_2O base with 1 g/L of Tween 80 and 9 g/L of NaCl. The third, denoted Milk Buffer, consisted of a sterile ddH_2O base with 1 g/L of Tween 80, 9 g/L of NaCl and 0.5% milk powder. Aliquots of 5 mL from each transport medium stock were pipetted into 5 mL round bottom tubes prior to sample collection.

Skin Swab Collection. Two cows (3593 Mandrake and 3540 Poppy) that exhibited an SCC score less than 2.0 (as measured by monthly DHIA testing) and were within 30 days in milk (DIM) of each other were selected from the UVM CREAM herd for teat skin sampling in February of 2013. Without prior teat cleaning (i.e. without pre-dipping or removal of environmental debris), sterile FLOQSwabs (Copan, Brescia, Italy) were individually rubbed upon one of three regions of the right hind teat of each cow: teat barrel skin (**TBS**), teat orifice skin (**TOS**) and streak canal epithelium (**SCE**). The same quarter was sampled from each cow to account for the possibility of intra-animal variation of skin flora on different quarters. FLOQSwab model 502CS01 was employed for the TBS and TOS regions due to its relatively larger swab tip and sturdier construction, while model 501CS01 was employed for the SCE

region due to its small, thin swab tip, which allowed for smoother insertion into the streak canal. A plastic stencil with an open aperture of 1.5 cm^2 was ethanol-sterilized and used to standardize the size and location of the surface area sampled from the TBS region. The TBS region was always sampled first, followed by the TOS region and finally the SCE region; before swabbing the SCE, 70% ethanol cause pads were used to sanitize the exterior teat skin in case the swab came in contact with this surface before insertion into the streak canal. Three swabs were used at each sample site. Swab tips were then snapped off into the transport media-containing 5 mL round bottom tubes; samples were arranged so that one swab from each sample site was transported in one of the three media, thus comprising a total sample population of 18 (3 sites × 3 types of media × 2 cows). Positive controls for each transport media treatment were created by swabbing exceptionally dirty surfaces in the barn (e.g. manure-encrusted or hairy regions on cattle), and negative controls were created by placing swabs in transport media immediately following removal from sterile packaging.

Swab Processing and Aerobic Culture. All samples were processed immediately upon return to the laboratory. Tubes containing swabs tips were vortexed at 2,500 rpm for 5 minutes using a Fisher Scientific Multi-Tube Vortexer (Thermo Fisher Scientific) to remove bacteria from the surface of the swabs. The resulting bacterial suspensions were poured into clean conical tubes and were subsequently streaked for confluency in duplicate on BAPs, which were aerobically incubated at 37°C for 48 hours. Tenfold and hundredfold dilutions of the TBS samples were also streaked for confluency in duplicate on the same media and incubated under the same conditions, as were tenfold dilutions of the TOS samples. Incubated plates were examined visually to differentiate all colony morphologies present, and isolates of the most prevalent morphologies were streaked onto fresh BAPs and incubated at 37°C for 48 hours. Identification of the isolated colonies was conducted using Gram stains, catalase tests and, when applicable, coagulase tests.

Experiment D: Swab Moisture Comparison

Skin Swab Collection. A mixed group of six Jersey and Holstein-Friesian cows (3748 Lima, 3758 Mable, 3645 Gillian, 3734 Sangria, 3496 Siobhan and 3747 Faline) that exhibited an SCC score less than 2.0 (as measured by monthly DHIA testing) and formed pairs within 30 days in milk (DIM) of each other were selected from the UVM CREAM herd for teat skin sampling in March of 2013. The sampling procedure from Experiment C was executed with the following exceptions:

- The plastic stencil employed for TBS sampling was not used, as it was perceived to restrict swabbing area to a degree that negatively affected bacterial yield.
- Milk Buffer was the only transport medium used based upon relatively favorable bacterial yields in Experiment C (see Results).
- Only two swabs were used at each sample site on each cow. One swab was applied directly to the teat after removal from sterile packaging as previously described (a "Dry" swab treatment), while another swab was first dipped in Milk Buffer before contact with the teat skin (a "Wet" swab treatment). Therefore, the total sample population population was 36 (3 sites × 2 swab treatments × 6 cows).

Swab Processing and Aerobic Culture. All samples were processed immediately upon return to the laboratory. Again, tubes containing swab tips were vortexed at 2,500 rpm for 10 minutes using a Fisher Scientific Multi-Tube Vortexer. Flame-sterilized forceps were then used to press swab tips against the side of the tubes in order to squeeze out as much bacterial

suspension as possible. The swab tips were removed using the forceps, and the remaining bacterial suspensions were all streak for confluency at neat and 1:10 concentrations on BAPS, which were aerobically incubated at 37°C for 48 hours.

DNA Extraction. The remaining bacterial suspensions were transferred to 15 mL conical tubes and frozen at -20°C overnight. The suspensions were then thawed and centrifuged at 5,251 × *g* for 30 minutes at 4°C. The supernatants were poured off to isolate the bacterial pellets, which were placed in storage at -20°C until they were ready for reception at the Vermont Cancer Center DNA Analysis Facility. Here, DNA from three randomly-selected cows was extracted utilizing a DNeasy Blood & Tissue Kit (Qiagen GmBH) and a modified manufacturer's protocol. The thawed pellets were re-suspended in 1.5 mL of PBS, and the new suspensions were transferred to 2 mL microfuge tubes and centrifuged at 5,000 × g for 10 minutes. After the supernatants had been discarded, the pellets were re-suspended in 80 µL of PBS and 5 µL of lysozyme (10mg/mL). Tubes were incubated at 37°C for 60 minutes. 100uL of Buffer ATL and 20uL of proteinase K were added to the tubes and they were incubated at 56°C for 30 minutes. Next, 200 µL of Buffer AL was added and the tubes were incubated at 70°C for 10 minutes. The cells were then homogenized by adding AlO_3 abrasives to the suspensions and subjecting them to physical disruption in a FastPrep-24 (Zymo Research Corporation, Irvine, CA) set at a speed of 6.5 for 15 seconds. The homogenized suspensions were transferred to new microfuge tubes and centrifuged at 5,000 × *g* for 10 minutes. 200 µL of 100% ethanol was added to each tube, and the resulting mixtures were transferred to silica-based spin columns and centrifuged at ≥ 6,000 × *g* for 1 minute. 500 µL of two wash buffers (Buffers AW1 and AW2) were employed in successive column washing steps with centrifugation at ≥ 6,000 × *g* for 1 minute, after which the column was dried by centrifugation at 15,000×*g* for 3 minutes. DNA was finally eluted into 200 µL of Buffer AE by allowing one minute of incubation at room temperature in the column and subsequently centrifuging at 6,000 × *g* for one minute. DNA concentrations were measured using a Qubit 2.0 Fluorometer and its associated reagents (Invitrogen Technologies Corporation) according to manufacturer guidelines.

Ubiquitous Bacterial Primer PCR Amplification of Extracted DNA. Bacterial DNA extracts were amplified using ubiquitous bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991) for amplification of the bacterial 16S rRNA gene, a widely used amplicon in culture-independent studies of bacterial diversity in many types of environments (Delbès et al., 2007; Gao, Z. et al., 2007; Fierer et al., 2008; Liles et al., 2010; Schatz et al., 2010; Braem et al., 2012; Braem et al., 2013). Additional reagents in the PCR master mix included PCR-certified H_2O, 10X ThermoPol Reaction Buffer, deoxynucleotide solution mix and Taq DNA Polymerase. The Bio-Rad C1000 Thermal Cycler conditions for the reaction included an initial denature step at 95°C for 10 minutes, 35 cycles of denaturation, annealing and elongation at 95°C for 30 seconds, 53°C for 30 seconds and 72°C for minutes, respectively, and a final extension step at 72°C for 10 minutes. The PCR amplicons were loaded into a 1.5% agarose gel and electrophoresed at 110 V for 45 minutes to visualize the presence of bacterial 16S rRNA genes in each sample.

Experiment E: Swab Agitation Comparison

Skin Swab Collection. Three Jersey cows (3496 Siobhan, 3737 Almond and 3747 Faline) with SCC score less than 2.0 (as measured by monthly DHIA testing) were selected from the UVM CREAM herd for teat skin sampling in April of 2013. The sampling procedure from Experiment D was executed with the following exceptions:

- 15 mL conical tubes were employed for initial sample collection (rather than 5 mL round bottom tubes).
- All swabs were dipped in Milk Buffer before contact with the teat skin (as in the "Wet" treatment group from Experiment D) based upon relatively favorable DNA yields (see Results).
- An effort was made to rub swabs more vigorously against the teat skin in order to maximize bacterial yields.
- An additional sampling site on the bacteria-laden perineal region of each cow was added in order to create a set of positive controls. Negative controls were still created by placing swabs in transport media immediately following removal from sterile packaging.
- Two swabs were used at each sampling site, resulting in a total sample population of 24 (4 sites × 2 swabs × 3 cows).

Swab Processing and Aerobic Culture. All samples were stored at 4°C overnight and were processed within 30 hours of collection. Of the pairs of swabs taken at each sample site, one swab was agitated by vortexing as described in Experiment D; the other was processed using stomacher agitation. Swabs in this second treatment group were removed from their tubes using flame-sterilized forceps and dropped into sterile Whirl-Paks (Nasco, Fort Atkinson, WI) to orient the swab tip at the bottom; the transport media remaining in the tube was poured in afterwards with an additional 5 mL of Swab Buffer to ensure coverage of the swab tip. The Whirl-Paks were tied off and inserted into a Stomacher 400 Circulator (Seward, West Sussex, UK), which was then run at 260 rpm for 5 minutes. The resulting bacterial suspensions were pipetted from the Whirl-Paks to their original conical tubes, and all suspensions from both treatment groups were streaked for confluency at neat and 1:10 concentrations on BAPS, which were aerobically incubated at 37°C for 48 hours.

DNA Extraction. Due to time constraints, a selected assortment of samples were processed for DNA extraction according to the procedure described in Experiment D.

Ubiquitous Bacterial Primer PCR Amplification of Extracted DNA. The 16S rRNA from all extracted DNA was amplified following the protocol described in Experiment D.

Data Analysis

All data were analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA). Data of culture-determined bacterial concentration and real-time PCR C_t values from Experiments A and B were fitted to exponential linear regressions. Concentrations of extracted DNA across treatment groups from experiments D and E were also fitted to exponential linear regressions against extracted DNA concentrations but were additionally analyzed using two-tailed t-Tests in order to determine the significance of differences observed between sampling and swab processing methods, respectively. A paired t-Test was preferred for Experiment D because samples from the same cow and epidermal site could be paired between the two treatment groups of each experiment. A t-Test assuming unequal variance was preferred for Experiment E because the samples from which DNA was extracted were not paired and furthermore exhibited unequal variances. Two-tailed t-Tests were preferred in both situations because none of the treatments from either experiment were initially hypothesized to be more or less effective in improving swab bacterial DNA yield.

RESULTS

Experiment A: Norgen Milk Bacterial DNA Extraction

The *S. aureus* growth gradient established by spiked milk dilutions did not correspond exactly with the intended 0-10^5 range as calculated by the *S. aureus* suspension inoculum, but an acceptably continuous gradient was nevertheless created. BAP plate counts indicated steadily rising stepwise concentrations in both renditions of the experiment (Table 1). The gradient was intended to establish an estimated threshold of detection for milk bacterial concentration using the Norgen extraction kit (as confirmed by end-point *S. aureus* multiplex PCR).

Intended Dilution Scheme	0 cfu/mL	10^o cfu/mL	10^1 cfu/mL	10^2 cfu/mL	10^3 cfu/mL	10^4 cfu/mL	10^5 cfu/mL
Rendition 1 True Gradient	0 cfu/mL	17.5 cfu/mL	31 cfu/mL	278 cfu/mL	2,567 cfu/mL	26,667 cfu/mL	276,667 cfu/mL
Rendition 2 True Gradient	4 cfu/mL	39 cfu/mL	104 cfu/mL	303 cfu/mL	4,433 cfu/mL	53,333 cfu/mL	490,000 cfu/mL

Table 1: *S. aureus*-spiked milk dilution gradients achieved in each rendition of Experiment A

Lancaster DHIA's real-time PCR services were solicited in order to corroborate the bacterial concentrations of the spiked milk dilutions calculated from aerobic culture and to confirm the extractability of the *S. aureus* DNA from the milk. Lancaster DHIA organizes the prevalence of bacterial species within milk in a semi-quantitative manner based upon C_t values. Species prevalence in a given sample is noted as Suspect (C_t is between approximately 36 and 40), Low (C_t is between approximately 30 and 36 cycles), Moderate (C_t is between approximately 25 and 30 cycles) or High (C_t is lower than approximately 25 cycles). These intervals were inferred by the author by comparing Lancaster DHIA categorization with precise C_t value (Figure 1). Dilutions negative for *S. aureus* detection by real-time PCR naturally did not have corresponding C_t values reported by Lancaster DHIA, but for the purposes of graphical representation, they were given a C_t value of 40 (the largest number of cycles executed in this real-time PCR). Moreover, because the logarithmic y-axis prevented the depiction of data points with y values of 0 (i.e. with *S. aureus* concentrations of 0 cfu/mL), one data point was altered to have an *S. aureus* concentration of 1cfu/mL so that it could be shown on the graph. As would be expected, the bacterial concentrations calculated from plate counts in aerobic culture work exhibited an inverse relationship with real-time PCR C_t values; an R^2 of 0.9523 was calculated from an exponential regression of these two variables when data both renditions of the experiment were pooled (Figure 1).

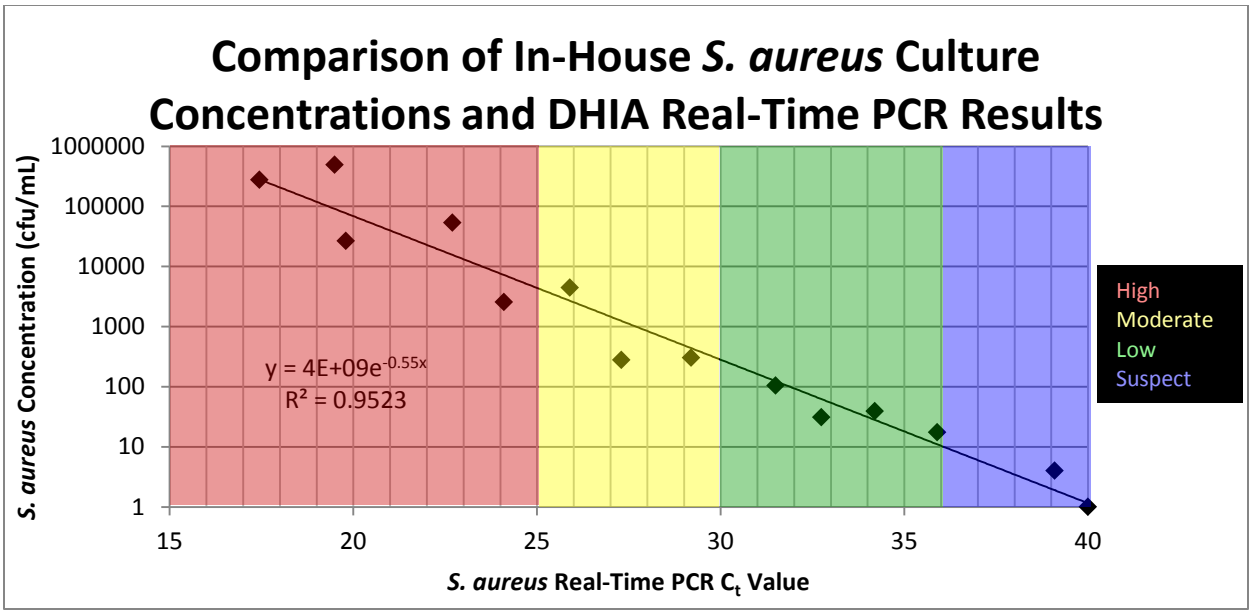


Figure 1: The BAP plate counts of *S. aureus* concentrations in the spiked milk dilutions showed a strong inverse relationship with Lancaster DHIA real-time PCR C_t values ($R^2 = 0.9523$). Prevalence categories were inferred by comparing Lancaster DHIA categorization with precise C_t values from the spiked milk dilutions. Dilutions negative for S. aureus detection by real-time PCR naturally did not have corresponding C_t values reported by Lancaster DHIA and were consequently given a C_t value of 40 so that they could be included in the graphical representation. Also, the logarithmic y-axis prevented the depiction of data points with y values of 0, so one data points representing *S. aureus*-negative dilutions was altered to have a bacterial concentration of 1 cfu/mL in order to be included on the graph.

The *S. aureus* concentrations of the spiked milk dilutions were compared to the corresponding amount of DNA extracted using the Norgen kit (Figure 2). A poor relationship was exhibited between these two variables – the R^2 value obtained from an exponential regression of the data was 0.0133. Three dilutions containing 4 cfu/mL, 104 cfu/mL and 4,433 cfu/mL of *S. aureus* produced visible bands of base-pair length characteristic of the *nuc* gene (~300 bp) encoding the *S. aureus* thermonuclease enzyme (the only one of the three multiplex genes possessed by *S. aureus* ATCC strain 25923), but bands at this position were not consistently noted for bacterial concentrations below 53,333 cfu/mL (Figure 3). It was therefore assumed that the *nuc* gene was successfully amplified in these band-positive samples.

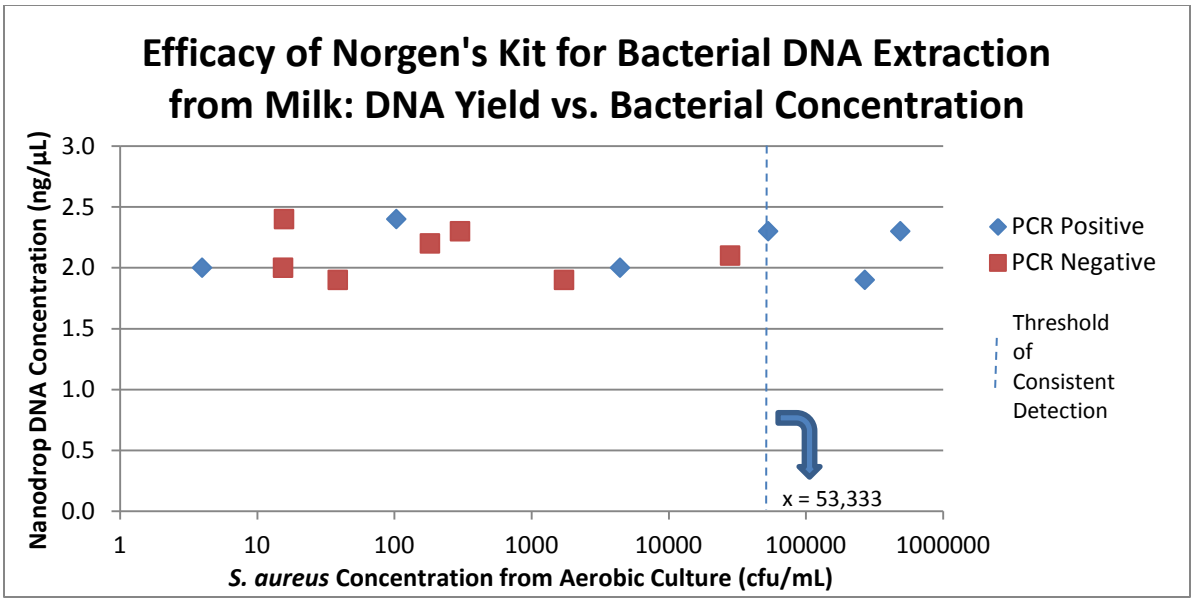
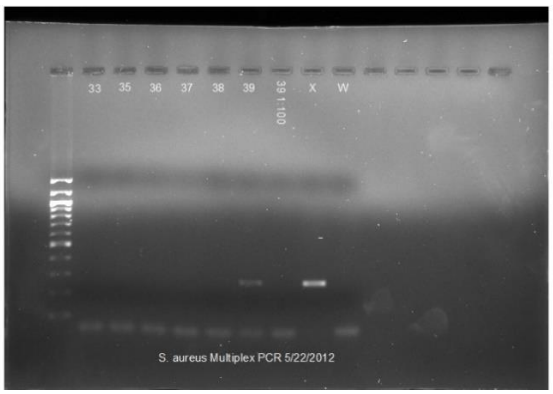
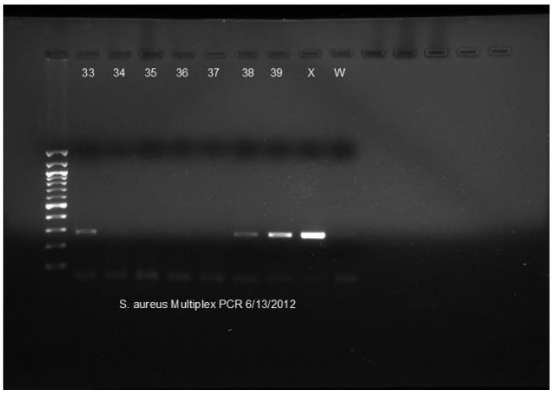


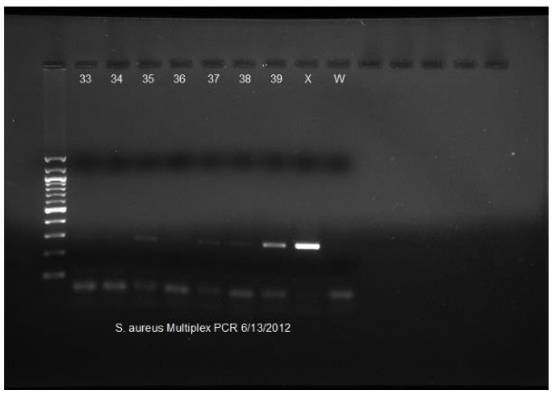
Figure 2: The correlation between spiked milk bacterial concentration and extracted DNA concentration was poor (R^2 = 0.0133 – trendline not depicted). Two PCR-positive results were noted at relatively low bacterial concentrations (4 cfu/mL and 104 cfu/mL), and one PCR-positive sample was noted for a dilution of moderate *S. aureus* concentration. However, consistent detection by multiplex PCR was not noted below a concentration of 53,333 cfu/mL.



(a)



(b)



(c)

Figure 3: Results of the *S. aureus* multiplex PCR from Experiment A are depicted in gel images (a) through (c). The stepwise dilution gradients from 0 cfu/mL to 10^5 cfu/mL were designated as "33" through "39" for Lancaster DHIA labeling purposes. "X" denotes the positive control (pure *S. aureus* suspension) from the Norgen extraction, and "W" represents the water-substituted negative control lane demonstrating that the PCR had not been contaminated. Ladders of 100 bp increments are depicted in the leftmost lane of each image. (a) PCR from the first rendition of the experiment only produced a visible band in

lane "39", corresponding to a milk dilution with 276,667 cfu/mL of *S. aureus*. (b) PCR from the second rendition of the experiment produced visible bands in lanes "33", "38" and "39", corresponding to milk dilutions with 4 cfu/mL, 53,333 cfu/mL and 490,000 cfu/mL of *S. aureus*, respectively. (c) A repeat of the PCR in the second rendition of the experiment produced visible bands in lanes "35", "37", "38" and "39", corresponding to milk dilutions of 104 cfu/mL, 4,433 cfu/mL, 53,333 cfu/mL and 490,000 cfu/mL of *S. aureus*, respectively.

Experiment B: Qiagen Milk Bacterial DNA Extraction

As was the case in Experiment A, the *S. aureus* growth gradient established by spiked milk dilutions did not correspond exactly with the intended 0-10^5 range as calculated by the *S. aureus* suspension inoculum, but an acceptably continuous gradient was created nonetheless. BAP plate counts indicated steadily rising stepwise concentrations differing by factors of approximately 10 (Table 2). Once again, the gradient was intended to establish an estimated threshold of detection for milk bacterial concentration, this time using the Qiagen DNA Mini Kit with subsequent end-point *S. aureus* multiplex PCR.

Intended Dilution Scheme	0 cfu/mL	10^0 cfu/mL	10^1 cfu/mL	10^2 cfu/mL	10^3 cfu/mL	10^4 cfu/mL	10^5 cfu/mL
True Gradient	0 cfu/mL	1 cfu/mL	20 cfu/mL	190 cfu/mL	2,003 cfu/mL	19,867 cfu/mL	218,333 cfu/mL

Table 2: *S. aureus*-spiked milk dilution gradients achieved in Experiment B

Lancaster DHIA's real-time PCR services were called upon once again to corroborate the bacterial concentrations of the spiked milk dilutions calculated from aerobic culture and to confirm the extractability of the *S. aureus* DNA from the milk. On this occasion, the ranges of the semi-quantitative categories dictated by Lancaster DHIA differed slightly from those reported in Experiment A. The range of the Suspect classification shrank to include C_t values between approximately 37 and 40, while the Low classification swelled to capture C_t values between approximately 30 and 37 cycles. The Moderate classification also grew slightly to include C_t values between approximately 24 and 30 cycles, while the High classification's range was reduced to C_t values less than approximately 24 cycles. Again, these intervals were inferred by the author by comparing Lancaster DHIA categorizations with precise C_t values (Figure 4). Dilutions negative for *S. aureus* detection by real-time PCR naturally did not have corresponding C_t values reported by Lancaster DHIA, but for the purposes of graphical representation, they were given a C_t value of 40 (the largest number of cycles run in this real-time PCR). Additionally, because the logarithmic y-axis prevented the depiction of data points with y values of 0 (i.e. with *S. aureus* concentrations of 0 cfu/mL), one data point was altered to have an *S. aureus* concentration of 1 so that it could be shown on the graph (see Figure 4). The bacterial concentrations calculated from plate counts in aerobic culture work exhibited an even stronger inverse relationship with real-time PCR C_t values than in Experiment A; an R^2 of 0.9964 was calculated from an exponential regression of these two variables.

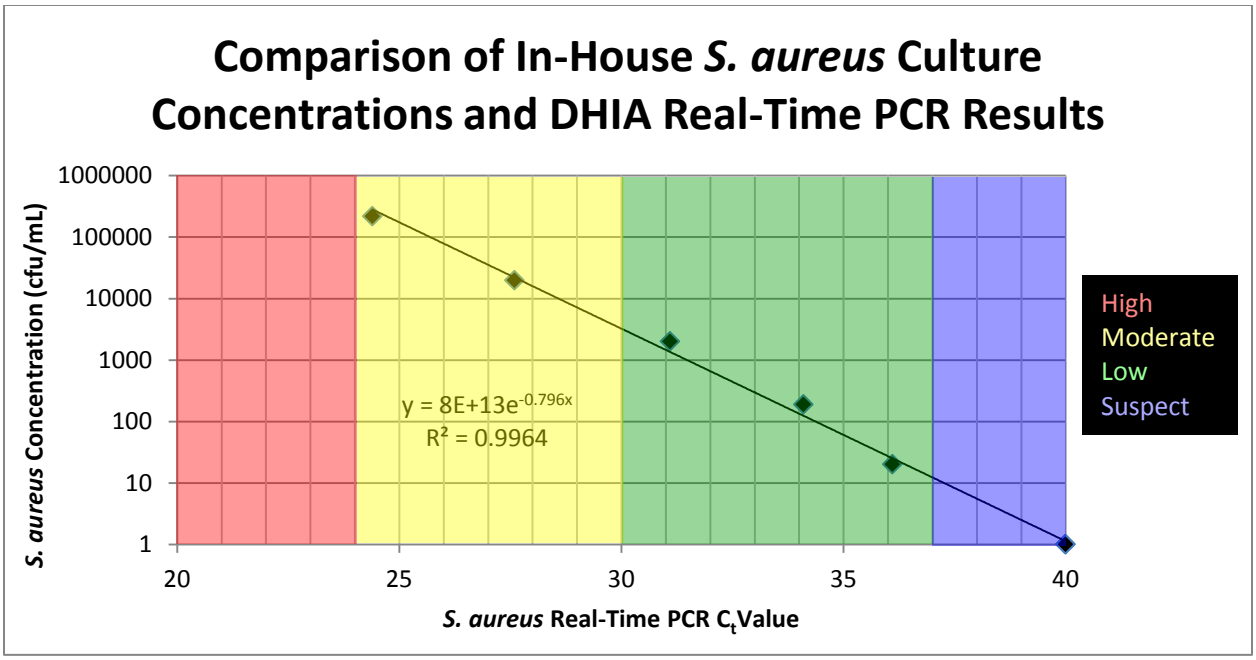


Figure 4: The BAP plate counts of *S. aureus* concentrations in the spiked milk dilutions once again showed a strong inverse relationship with Lancaster DHIA real-time PCR C_t values ($R^2 = 0.9964$). New prevalence categories were inferred by comparing Lancaster DHIA categorization with precise C_t values from the spiked milk dilutions; these ranges differed slightly from those of Experiment A's results. Dilutions negative for S. aureus detection by real-time PCR naturally did not have corresponding C_t values reported by Lancaster DHIA and were consequently given a C_t value of 40 so that they could be included in the graphical representation. Also, the logarithmic y-axis prevented the depiction of data points with y values of 0, so one data point representing *S. aureus*-negative dilutions was altered to have a bacterial concentration of 1 cfu/mL in order to be included on the graph.

The *S. aureus* concentrations of the spiked milk dilutions were compared to the corresponding amount of DNA extracted using the Qiagen kit (Figure 5). A poor relationship was once again exhibited between these two variables – the R^2 value obtained from an exponential regression of the data was 0.1515. The data point amended from 0 cfu/mL to 1 cfu/mL for depiction in Figure 4 was omitted during the construction of Figure 5 so as not to influence the regression's accuracy in describing the data. None of the DNA extracted from this range of *S. aureus*-spiked milk dilutions produced visible bands of the base-pair lengths characteristic of the *nuc*, *blaZ* or *mecA* genes (all of which are present in the *S. aureus* ATCC strain 33591), indicating that none of these genes had been amplified for any sample (Figure 6).

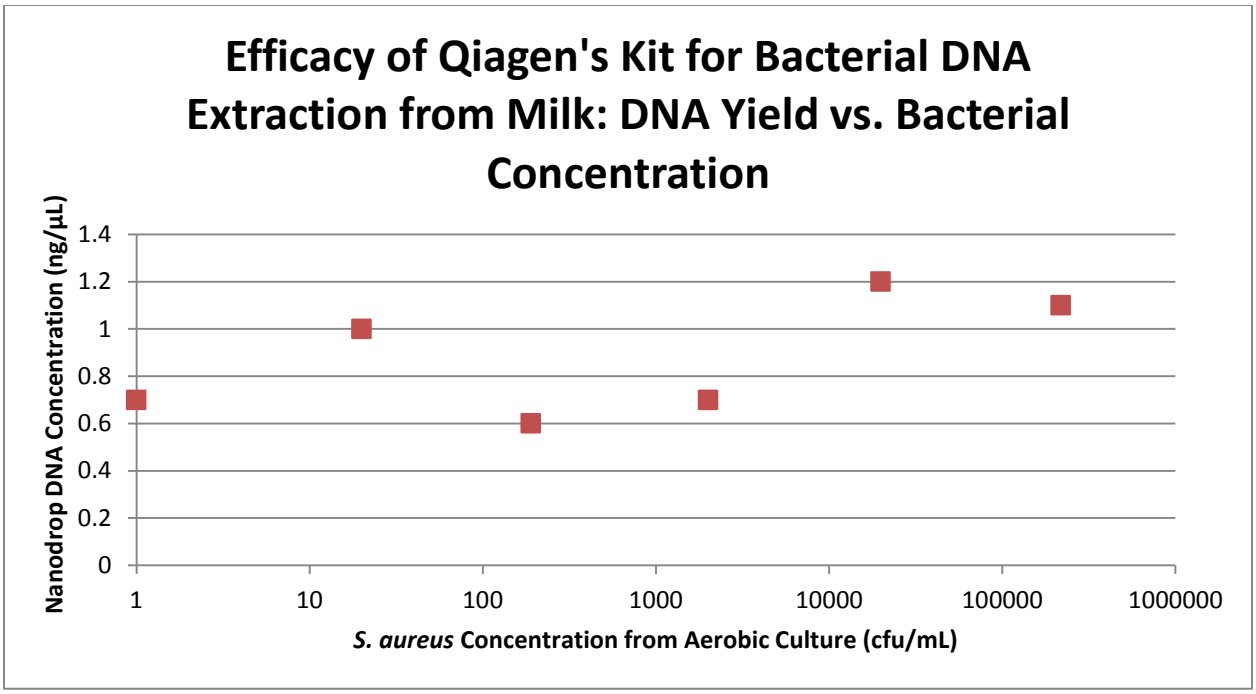


Figure 5: The correlation between spiked milk bacterial concentration and extracted DNA concentration was poor again (R^2 = 0.1515– trendline not depicted). The data point amended from 0 cfu/mL to 1 cfu/mL for depiction in Figure 4 was omitted during the construction of Figure 5 so as not to influence the regression's accuracy in describing the data. None of the *S. aureus* multiplex genes were amplified from DNA extracted using the Qiagen kit.

Figure 6: Results of the *S. aureus* multiplex PCR in Experiment B are depicted in the gel image above. The stepwise dilution gradients from 0 cfu/mL to 10^5 cfu/mL were designated as "60" through "66" for Lancaster DHIA labeling purposes. "X" denotes the positive control (pure *S. aureus* suspension) from the Qiagen extraction, and "W" represents the water-substituted negative control lane demonstrating that the PCR had not been contaminated. Ladders of 100 bp increments are depicted in the leftmost lane of the image. Visible bands corresponding with the *nuc* (middle band in "X"), *blaZ* (top band in "X") and *mecA* (bottom band in "X") genes were not observed in any of the lanes corresponding to the *S. aureus*-spiked milk dilutions.

Experiment C: Skin Swab Transport Media Comparison

Measures of average bacterial yield and average species richness (i.e. the number of different colony morphologies observed) for each transport medium were calculated based upon aerobic culture of bacterial suspensions on BAPs (Table 3). In general, Milk Buffer preserved the highest average number of bacteria, whereas Swab Buffer preserved the lowest average number of bacteria. Regarding teat skin sites, the highest bacterial yields were obtained from TOS samples transported in Milk Buffer, followed by TBS samples transported in Milk Buffer

and TOS samples transported in ddH_2O (Figure 7). The least amount of bacteria was consistently recovered from the SCE. It should be noted, however, that the standard deviations for the average bacterial yields for each transport medium were quite high, generally falling on the same order of magnitude as the averages themselves (if not surpassing them substantially).

Transport Medium	Average Bacterial Yield (cfu/mL)	Standard Deviation of Average Bacterial Yield	Average Species Richness)	Standard Deviation of Average Species Richness
ddH_2O	148.3	141.5	6.42	5.60
Milk Buffer	460.0	576.7	9.17	6.48
Swab Buffer	45.8	119.1	1.83	2.85

Table 3: Average bacterial yield and average species richness (with associated standard deviations) were calculated for each transport medium treatment group. Standard deviations were generally very high when juxtaposed to their corresponding averages.

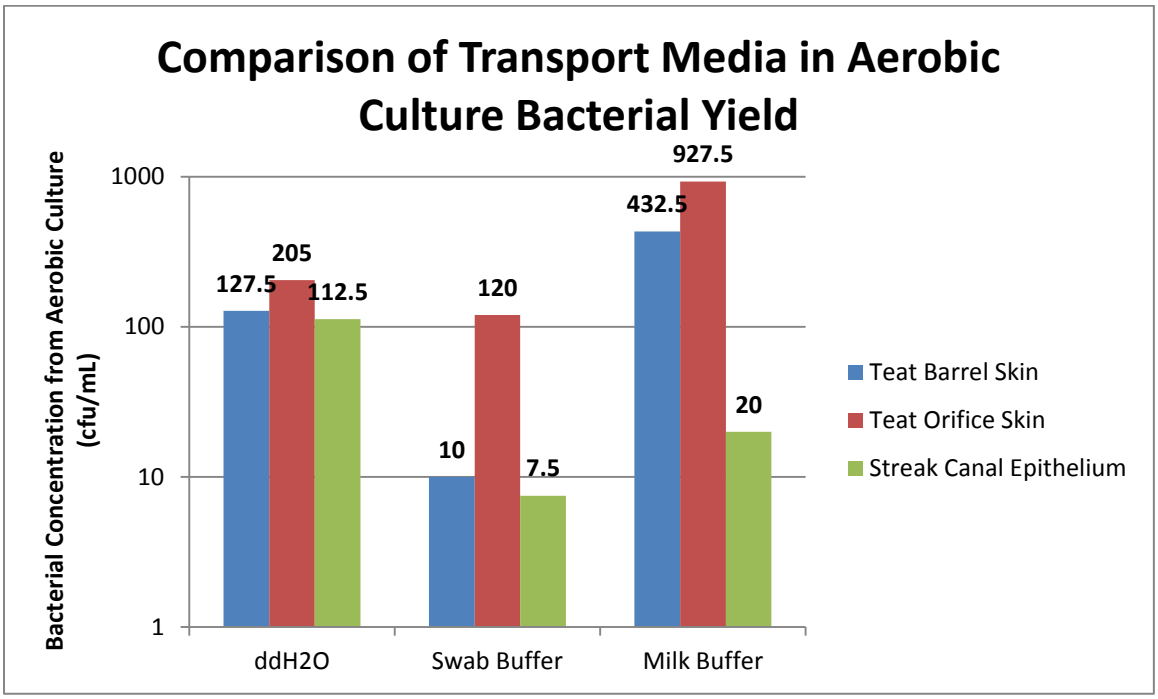


Figure 7: Average bacterial yields calculated from aerobically-cultured plate counts are depicted. Regarding transport media, Milk Buffer yields were generally highest amongst while Swab Buffer yields were generally lowest. Regarding skin sites, TOS yields were typically highest, while SCE yields were typically lowest.

Aerobic culture also revealed a diverse array of microorganisms growing on the teat skin. The highest average species richness was observed in TOS samples transported using Milk Buffer (reaching 15.5 unique colony morphologies), followed again by TBS samples transported in Milk Buffer and TOS samples transported in ddH_2O (Figure 8). Regarding teat skin sites, the most diverse communities were isolated from TOS, while the least diverse communities were isolated from the SCE. Again, though, the standard deviations of the average species richness for each transport medium were often considerably high when compared to their corresponding averages. Table 4 depicts the most prevalent types of bacteria present in the skin swab samples based upon the diagnostic testing measures described in Methods and Materials. CNS species

were almost invariably the major bacterial family recovered from each sample, residing in all three teat skin regions. Gram-positive rod species were present in their highest numbers on the TOS and SCE regions, and one cow's TOS (3593 Mandrake) was populated relatively heavily by a streptococcal species.

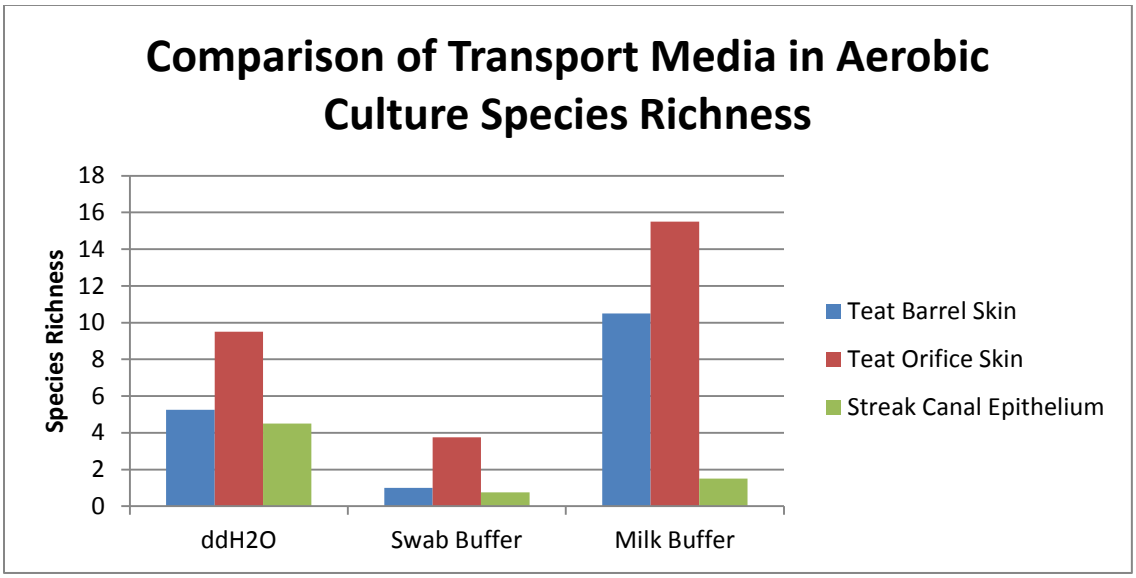


Figure 8: Average species richness values calculated from aerobically-cultured plate counts are depicted. Regarding transport media, Milk Buffer yields were generally the most diverse, while Swab Buffer yields were generally the least diverse. Regarding skin sites, TOS communities were typically the most diverse, while SCE communities were typically the least diverse.

Cow	Sample Site	Transport Medium	Most Prevalent Colony Type
3593	TBS	ddH2O	CNS
3593	TBS	Swab Buffer	N/A
3593	TBS	Milk Buffer	CNS
3593	TOS	ddH2O	CNS
3593	TOS	Swab Buffer	CNS
3593	TOS	Milk Buffer	CNS, Strep. Sp.
3593	SCE	ddH2O	CNS, Gram-Positive Rod sp.
3593	SCE	Swab Buffer	CNS, Gram-Positive Rod sp.
3593	SCE	Milk Buffer	CNS
3540	TBS	ddH2O	CNS
3540	TBS	Swab Buffer	CNS
3540	TBS	Milk Buffer	CNS
3540	TOS	ddH2O	CNS
3540	TOS	Swab Buffer	Gram-Positive Rod sp.
3540	TOS	Milk Buffer	Gram-Positive Rod sp.
3540	SCE	ddH2O	N/A
3540	SCE	Swab Buffer	N/A
3540	SCE	Milk Buffer	CNS

Table 4: CNS species were far and away the most prevalent teat skin colonizers of the cows sampled.

Experiment D: Swab Moisture Comparison

The concentrations of the skin swab bacterial suspensions determined using aerobically-cultured plate counts were compared to the corresponding DNA concentrations extracted from each sample (Figure 9). Those samples that were too numerous to count (**TNTC**) on the BAPs (i.e. containing more than 350 cfus on the plate) at both of the given plating dilutions (neat and 1:10) were designated as having 35,000 cfu/mL (the lowest concentration possible if at least 350 colonies are present on a plate and a 1:10 sample dilution factor is combined with a 1:10 plating dilution factor). Those samples that yielded too little DNA for measurement with the Qubit 2.0 Fluorometer (<0.50 ng/mL of DNA in the Qubit reagent mixture) were designated as having extracted DNA concentrations of 33 ng/mL (a 200 µL Qubit reagent mixture with a DNA concentration of 0.50 ng/mL of DNA contains 0.1 ng of DNA; this mass of DNA was obtained from a 3 µL volume of extracted DNA solution added to the Qubit reagent mixture, so a 1 mL volume of extracted DNA solution with proportional DNA content has 33 ng of DNA). After these data had been amended, an exponential linear regression of bacterial yields and extracted DNA concentrations was conducted to determine the degree of correlation between the two variables. The calculated R^2 value from this regression was only 0.4318, suggesting a very weakly direct relationship between bacterial yield and extracted DNA concentration.

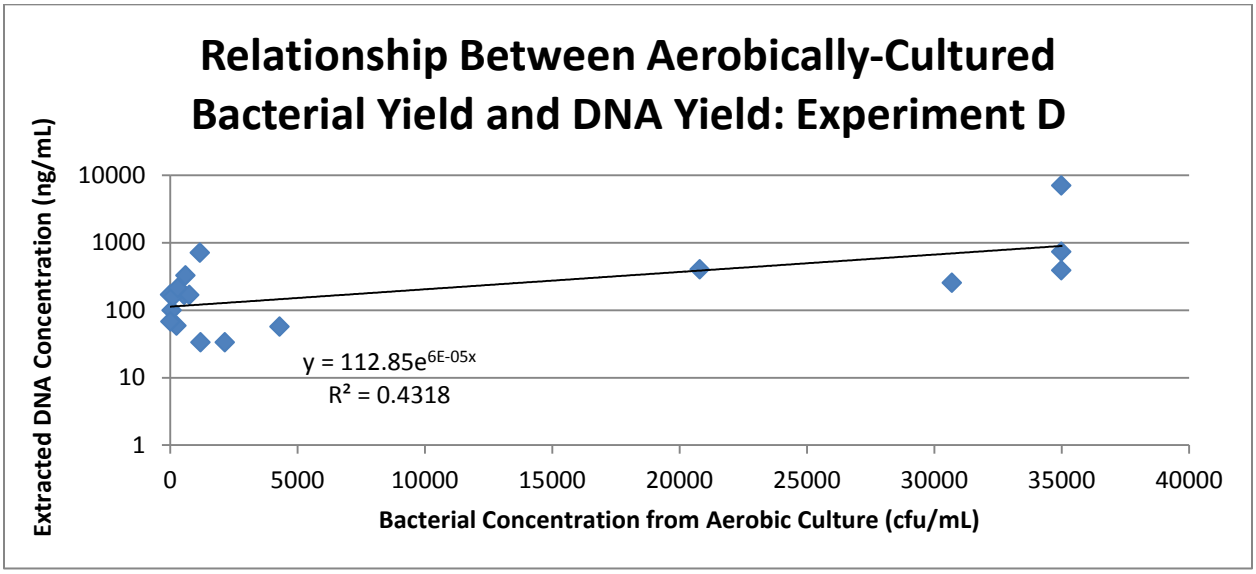


Figure 9: Bacterial concentrations from aerobic culture of the skin swab bacterial suspensions and extracted DNA concentrations from these samples exhibited a direct (albeit very weak) relationship (R^2 = 0.4318). Those samples that were TNTC on the BAPs at both of the given plating dilutions were designated as having 35,000 cfu/mL. Those samples that yielded too little DNA for measurement with the Qubit 2.0 Fluorometer (<0.50 ng/mL of DNA in the Qubit reagent mixture) were designated as having extracted DNA concentrations of 33 ng/mL.

The amount of extracted DNA was furthermore compared across sampling techniques (Figure 10). Once again, DNA concentrations too low for Qubit measurement were adjusted to 33 ng/mL as described above. The distribution of DNA concentrations obtained from dry swabbing was more condensed than that of the DNA concentrations derived from wet swabbing, but the overall mean concentration of extracted DNA from samples obtained from dry swabbing was quite lower than that of samples obtained by wet swabbing (180 ng/mL versus 1046 ng/mL). Though these means seem significantly disparate, it is important to note that the larger wet swab mean was likely skewed by a single value of 7,000 ng/mL obtained from bacteria colonizing the

TOS of 3748 Lima. For this reason, the paired, two-tailed Student's t-Test described in Materials and Methods was employed to determine the significance of this difference in mean DNA concentration. The t-Test produced a *p* value of 0.265, indicating that the difference in average extracted DNA concentration between the two swabbing methods was not statistically significant ($p>0.05$). However, there were four 16S rRNA PCR-positive samples in the Wet treatment group compared to only one in the Dry treatment group (Figure 11). Both dry controls (positive and negative) did not produce visible bands at base-pair fragment lengths characteristic of the 16S rRNA gene, indicating that is was not amplified by the ubiquitous bacterial primer PCR described in Materials and Methods. Conversely, both wet controls (positive and negative) did produce visible bands, thereby demonstrating that the 16S rRNA gene had been amplified from the bacteria on those swabs.

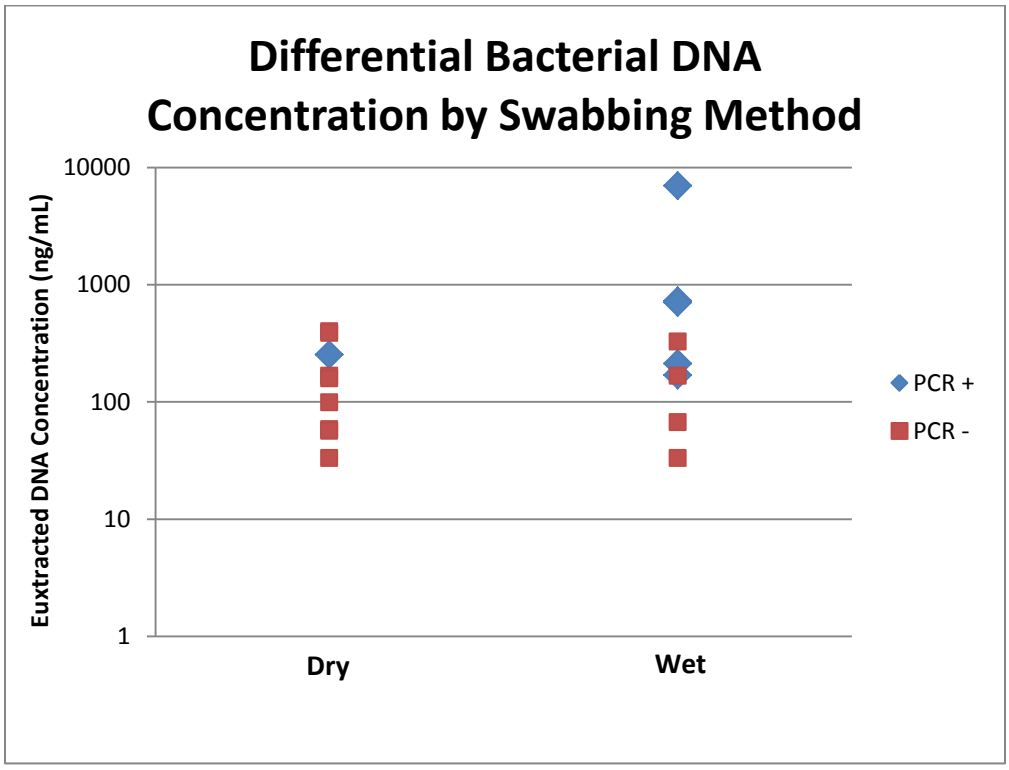


Figure 10: The distribution of the Dry treatment group's extracted DNA concentrations was more condensed than that of the Wet treatment group's values, but the Wet group's mean concentration (1046 ng/mL) was considerably higher than that of the Dry group (180 ng/mL). The difference between these two means was not statistically significant ($p = 0.265$). Those samples that yielded too little DNA for measurement with the Qubit 2.0 Fluorometer (<0.50 ng/mL of DNA in the Qubit reagent mixture) were designated as having extracted DNA concentrations of 33 ng/mL.

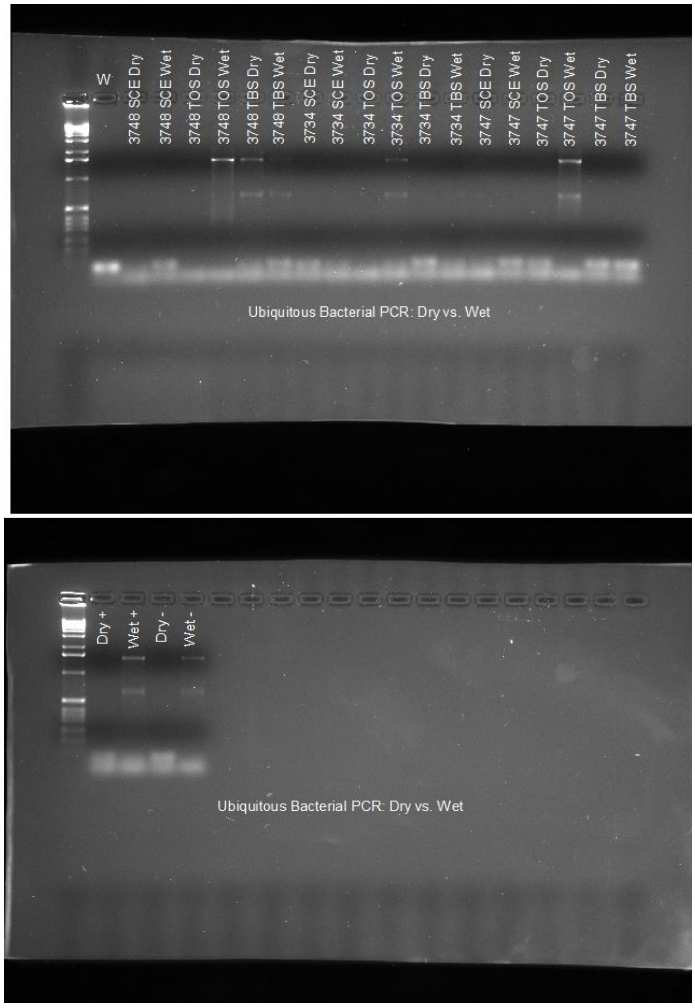


Figure 11: Results of the ubiquitous bacterial primer PCR in Experiment D are depicted in gel images (a) and (b). Ladders of 1 kbp increments are depicted in the leftmost lane of each image. (a) "W" represents the water-substituted negative control lane demonstrating that the PCR had not been contaminated. Banding characteristic of the 16S rRNA gene was observed in five experimental samples (3748 Lima TOS Wet, 3748 Lima TBS Dry, 3748 Lima TBS Wet, 3734 TOS Wet and 3747 TOS Wet). Of these five, four represented the Wet treatment group, while only one represented the Dry treatment group. (b) Neither of the Dry controls (positive and negative) exhibited banding characteristic of the 16S rRNA gene, whereas both of the Wet controls (positive and negative) exhibited such banding.

Experiment E: Swab Agitation Comparison

The concentrations of the skin swab bacterial suspensions determined using aerobically-cultured plate counts were compared to the corresponding DNA concentrations extracted from each sample (Figure 12). Those samples that were TNTC on the BAPs at both of the given plating dilutions (neat and 1:10) were designated as having 35,000 cfu/mL for the reasons previously described. After these data had been amended, an exponential linear regression of bacterial yields and extracted DNA concentrations was conducted to determine the degree of correlation between the two variables. The calculated R^2 value from this regression was a diminutive 0.0936, indicating the virtual absence of a correlation between bacterial yield and extracted DNA concentration from both the stomacher and vortexer treatment groups.

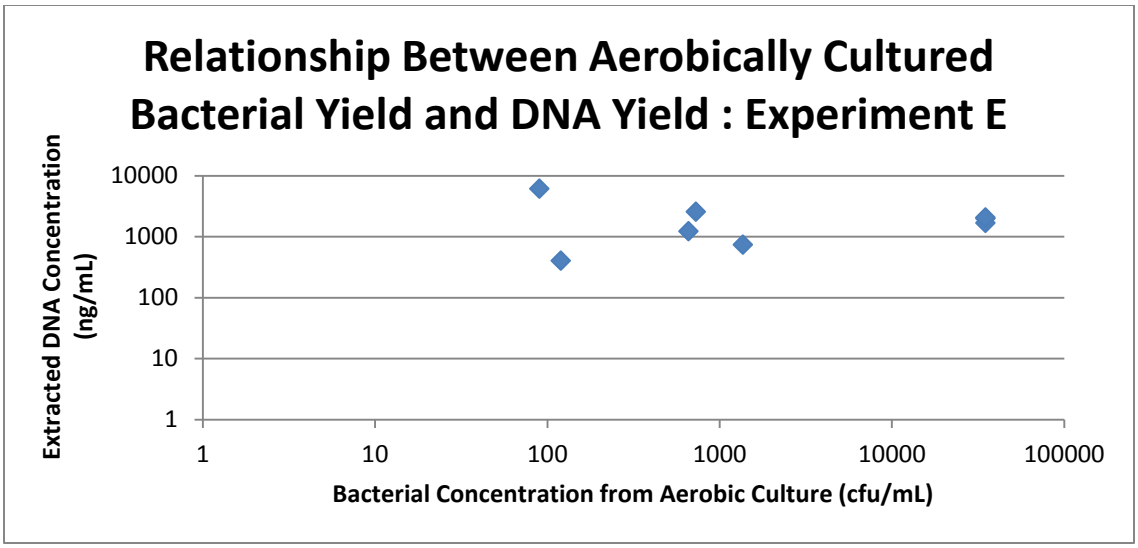


Figure 12: Bacterial concentrations from aerobic culture of the skin swab bacterial suspensions and extracted DNA concentrations from these samples exhibited very little correlation ($R^2 = 0.0936$ – trendline not depicted). Those samples that were TNTC on the BAPs at both of the given plating dilutions were designated as having 35,000 cfu/mL.

The amount of extracted DNA was compared between swab agitation techniques (Figure 13). The distribution of DNA concentrations obtained after vortexer agitation was far more condensed than that of the DNA concentrations obtained after stomacher agitation, but the overall mean concentration of extracted DNA from samples obtained from vortexer agitation was quite lower than that of samples obtained by stomacher agitation (751.5 ng/mL versus 2784 ng/mL). Similar to the corresponding data from Experiment D, this disparity can likely be attributed to the skewing influence by a single data point in the stomacher treatment group: an extracted DNA concentration of 6,090 ng/mL obtained from bacteria colonizing the perineal region of 3747 Faline. For this reason, the two-tailed t-Test assuming unequal variance described in Materials and Methods was employed to determine the significance of this difference. The t-Test produced a *p* value of 0.076, indicating that the difference in average extracted DNA concentration between the two swab agitation methods was not statistically significant ($p>0.05$). However, there were two 16S rRNA PCR-positive samples in the Stomacher treatment group, whereas there were no 16S rRNA PCR-positive samples in the Vortexer treatment group (Figure 14). Time constraints prevented extractions from being performed on all samples, but the Stomacher treatment's negative control was processed and fittingly exhibited both the lowest concentration of extracted DNA in the experiment (72.7 ng/mL) and a negative 16S rRNA PCR result.

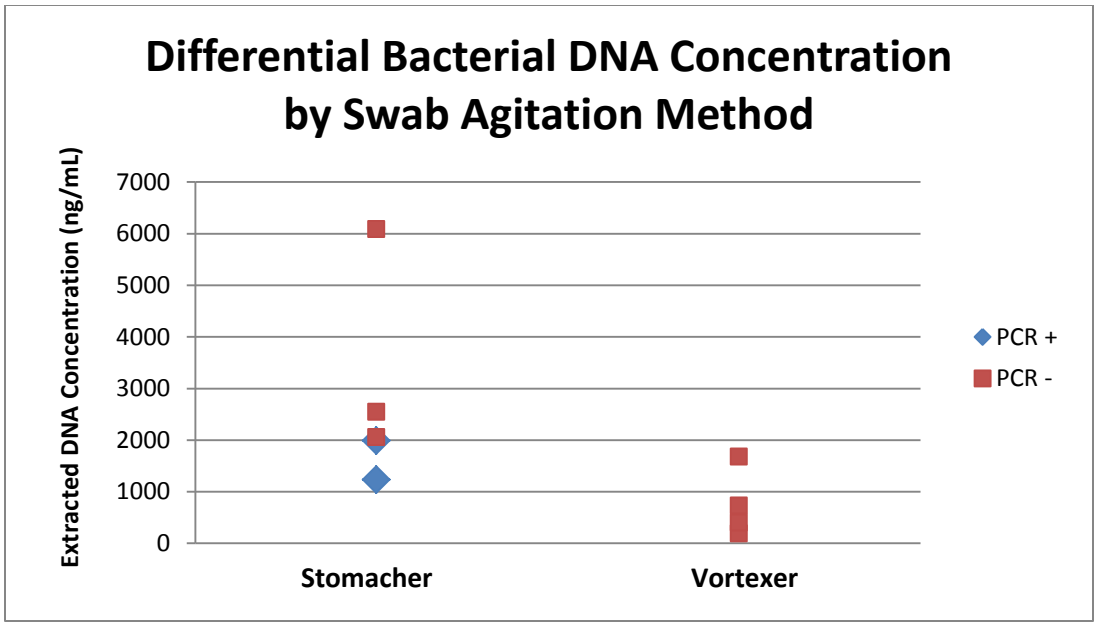


Figure 13: The distribution of the Vortexer treatment group's extracted DNA concentrations was more condensed than that of the Stomacher treatment group's values, but the Stomacher group's mean concentration (2784 ng/mL) was considerably higher than that of the Vortexer group (751.5 ng/mL). The difference between these two means was not statistically significant ($p = 0.076$).

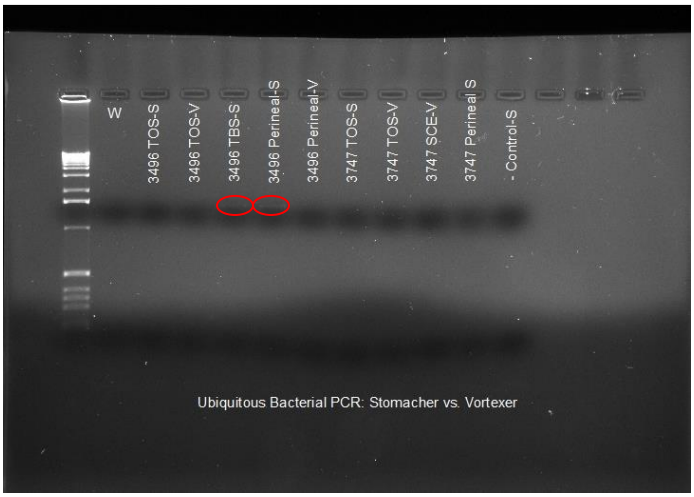


Figure 14: Results of the ubiquitous bacterial primer PCR in Experiment E are depicted in the gel image above. A ladders of 1 kbp increments is depicted in the leftmost lane of the image. "W" represents the water-substituted negative control lane demonstrating that the PCR had not been contaminated. Banding characteristic of the 16S rRNA gene was observed faintly (circled red) in two experimental samples (3496 Siobhan TBS-Stomacher and 3496 Perineal-Stomacher). All of these 16S rRNA-positive samples represented the Stomacher treatment group. The lone control lane (negative Stomacher in the far right lane) did not exhibit banding.

DISCUSSION

Experiments A and B: DNA Extraction from Milk

Experiments A and B were designed to evaluate the efficacy of two commercial kits (the Norgen Milk Bacterial DNA Extraction Kit and the Qiagen QIAamp DNA Mini Kit) in extracting bacterial DNA from milk. Non-mastitic (SCC ≤ 2) cows were chosen for sample collection so that milk was not contaminated by transient pathogenic species of bacteria.

Extracted DNA from non-spiked samples would have been considered representative of normal flora residing on the teat cistern epidermis. Both kits employ silica-based column extractions, which are widely used for the recovery of nucleic acids (i.e. both DNA and RNA) from a vast array of sampling environments (Tan & Yiap, 2009). The electrical attraction between negatively charged DNA and positively-charged silica particles in the filter of a spin column cause rapid binding, thus precipitating DNA molecules out of solution from extraction buffers whose high salt concentrations enhance this binding interaction by interfering with hydrogen bonding between H_2O molecules and the silica bed (Tan & Yiap, 2009). It is critical, then, that the column filters be washed (often by rapid centrifugation, as in the Norgen and Qiagen kits) to remove all contaminants that may hinder their binding to DNA (Tan & Yiap, 2009).

Milk contains a number of substances (fats, enzymes, proteins, polysaccharides, Ca^{2+} ions, etc.) that have been known to interfere with DNA polymerase binding in PCR reactions (Marianelli et al., 2008), so it is probable that these substances also impede DNA-silica ionic interactions and reduce DNA yields from spin column extractions when present in sufficient concentrations. Centrifugation of milk separates the colloid into distinct fractions that include a sedimented pellet of heavy cellular and proteinacious debris, a liquid solution of whey and a lipid-dense cream layer; in many studies, the cream and whey fractions were discarded (Gao, A. et al., 2007), purportedly to eliminate the numerous interfering substances present within them. This practice was reflected in the manufacturer's instructions for the Norgen kit. However, when this kit failed to produce reliably amplifiable DNA for low milk bacteria concentrations (the threshold of consistent detection was 53,333 cfu/mL – see Figures 2 and 3 in Results), the Qiagen kit was selected for evaluation due to its inclusion of all milk fractions in the lysis step. Indeed, multiple studies (Gao et al., 2005; Angen et al., 2007; Gao, A. et al., 2007; Graber et al., 2007) recommended pooling the cream and pellet fractions of centrifuged milk due to preferential sequestration of some organisms (e.g. *S. aureus*, *M. avium* subsp. *paratuberculosis*) in the cream fraction. Moreover, the extraction protocol utilized by Lancaster DHIA for recovery of real-time PCR templates employs the QIAamp DNA Mini Kit (albeit with additional proprietary buffers not disclosed to the public). Nevertheless, DNA yields from this second kit were similarly low and failed to produce amplicons from *S. aureus*-specific multiplex PCR (see Figures 5 and 6 in Results). The presence of the whey fraction, which has been documented to interfere with certain extraction reagents (Gao et al, 2005), in the lysis mixture may be responsible for this inefficacy. Additionally, differential heat treatment across milk fractions before lysis may have benefited this procedure, for Gao et al. (2005) indicated that heating the cream fraction after centrifugation of raw milk and subsequently pooling cream and pellet fractions produced higher yields of *M. avium* subsp. *paratuberculosis* DNA and resulted in more sensitive PCR detection.

Another crucial step in spin column extraction procedures that likely factored into the poor DNA yields from Experiments A and B is cell lysis (Tan & Yiap, 2009). Both kits made use of buffer solutions, which increase DNA-silica binding affinity a previously described (Tan & Yiap, 2009), and proteinase K, a protease stimulated by the denaturing agents commonly found in lysis solutions (Hilz et al., 1975); additionally, the Norgen kit's lysis buffer included lysozyme, an enzyme that degrades peptidoglycan in bacterial cell walls (Murphy, 2012), and lysostaphin, an enzyme that specifically cleaves the cross-linking pentaglycine cross-bridges in the cell walls of staphylococci (Wu et al., 2003). In short, chemical and enzymatic cellular disrupters were well represented in these solutions. What was not included in either case, however, was a physical disruption step. For example, Gao et al. (2011) enhanced isolation of

protozoal DNA from *Prototheca zopfii* in milk by adding glass beads to extraction mixtures and vortexing them for 20 minutes to mechanically shear cells and release their inner contents. This line of reasoning prompted the Vermont Cancer Center DNA Analysis facility to incorporate a FastPrep abrasion step in their extractions on skin swab-derived bacterial pellets in Experiments D and E. Direct comparison between the DNA yields of the milk kit extractions and the skin swab extractions was not performed due to the different measurement devices employed between the two classes of experiments. The Nanodrop 2000c Spectrophotometer was originally used to measure extracted DNA concentrations from milk samples, but it was determined that, given the extremely low DNA yields obtained with the kits, salt components of the elution buffers known to absorb UV light and hinder measurement of true DNA absorption (Sukumaran, 2011) were rendering the NanoDrop readings quite inaccurate. This was apparent when comparing DNA measurements of skin swabs samples from the Nanodrop with those obtained from the Qubit (data not shown), which functions by detecting fluorescence of a double-stranded DNA binding reagent (Foley et al., 2011) and thus selectively omits interfering fluorescence from superfluous substances in the elution buffer. Future studies should take care to use consistent DNA measurement techniques to describe such differences between extraction protocols.

Alternative strategies for DNA extraction beyond kit-based protocols were considered following the failure of the Norgen kit to recover sufficient concentrations of bacterial DNA. Several studies conducted phenol-chloroform-isoamyl extractions (Romero & Lopez-Goñi, 1999; Lafarge et al., 2004; Delbès et al., 2007; Marianelli et al., 2008) in which DNA suspended in a phenol:chloroform:isoamyl mixture (25:24:1) was separated into an aqueous phase from which it could be precipitated with the addition of ethanol or isopropanol (Tan & Yiap, 2009). However, the highly toxic reagents used in this procedure and its rigorous learning curve detracted substantially from its appeal despite reports of high sensitivity. The Qiagen kit was chosen instead to restrict the scope of the investigation to column-based systems.

Experiments C, D and E: Skin Swab Collection and Processing

In regards to bovine skin swabbing, there is a marked paucity of explicit methodological comparisons of swabbing and sample processing techniques in the existing literature. Experiments C, D and E were consequently designed to judge different methods of sample collection and to identify which of these methods are most useful for application in culture-independent studies of skin microbiota (i.e. which methods yield DNA that is both high in concentration and representative of a skin microbiome's true composition).

Descriptions of teat apex swabbing by De Vliegher et al. (2003) and Braem et al. (2012) are rather vague in their explanation of swab "transportation" from the field site to the laboratory and as such were the subject of this study's first inquiry, Experiment C. The first medium selected was plain ddH_2O, representing a simple liquid in which bacterial cells from swab tips could be suspended following sampling. The second and third media (Swab Buffer and Milk Buffer) were adapted from those described by Verdier-Metz et al. (2012).

Amongst transport media, Milk Buffer exhibited both the highest average aerobically-cultured bacterial yield and the highest average species richness (see Table 3 in Results). This observation may possibly be attributed to the fact that some bacterial species preferentially bind to milk fats (Gao et al., 2007; Graber et al., 2007) and may consequently be more effectively pulled into suspension from the surface of the swab tip when immersed in a liquid containing a sufficient concentration of these organic compounds. Based upon this line of reasoning, Milk Buffer was employed for the remaining skin swab investigations (Experiments D and E). However, one should approach the Milk Buffer's high averages with extreme caution, for this

treatment group also exhibited the highest standard deviation of any transport medium for both average bacterial yield and average species richness. It is therefore difficult to have confidence in the significance of these data despite the temptation to infer Milk Buffer's superiority. What is more, a prospective bacterial DNA extraction procedure still had not been chosen when this experiment was conducted, so data of extracted DNA concentrations could not be included in this study. As a result, it was necessary to rely upon the results of culture-based methods to infer the efficacy of the swab media in supporting high bacterial DNA yields and maintaining microbial diversity. The inclusion of actual extracted DNA data would have been more helpful in describing the transport media's true utility in culture-independent analysis; for example, though Swab Buffer consistently yielded the least number of aerobically-cultured organisms, it is possible that osmotic imbalances created by the medium's ionic components caused cells to lyse, thus releasing large amounts of readily-accessible DNA. These dead cells would not grow up in culture, but their DNA could still be harvested. Future renditions of this experiment should include a DNA extraction component to account for such possibilities. Nevertheless, the prevalence of CNS species at multiple teat environments across several cows is consistent with previous culture -independent investigations of this microbiome's species profile (Braem et al., 2012; Verdier-Metz et al., 2012; Braem et al., 2013).

Fortunately, a revised DNA extraction protocol was available for Experiments D and E. The data generated from this protocol in Experiment D demonstrated a weak relationship between aerobically-cultured bacterial counts and extracted DNA concentrations (exponential regression $R^2 = 0.4318$ – see Figure 9 in Results) in the comparison of swab moisture treatments. Although wet swabbing was tentatively established as having relatively higher bacterial DNA extract yields, the data from this treatment group also suffered from high degrees of variability (see Figure 10 in Results). The paired, two-tailed t-Test executed from these data confirmed the lack of a statistically significant difference between extracted DNA yields from the two treatment groups ($p = 0.265$). Moreover, the presence of 16S rRNA amplicons from the wet swab negative control raised concerns of increased aerosol contamination of wet swabs in the barn environment. Dairy barns have been reported to exhibit high levels of bacterial aerosol contamination from hay and straw (Duchaine et al., 1999), so it is plausible that the generally higher bacterial DNA yields and four PCR-positive results obtained from wet swab samples could have resulted from the accidental amplification of the 16S rRNA genes of aerosol contaminant species. This problem of aerosol is more likely in investigations of livestock skin microbiota, for studies of human skin microbiota have the convenient option of moving subjects to sterile sampling environments (Paulino et al., 2006). Ultimately, all of these factors prevented the naming of a definitively superior swabbing method, but the slight advantage in average extracted DNA yield exhibited by the Wet treatment was still used as justification for performing wet swabbing during sample collection for Experiment E.

This final investigation sought to compare stomacher agitation of skin swabs with vortexer agitation. Both procedures were intended to physically shake swab tips to release more bacteria into the transport media. Vortexing achieved this by rapidly spinning swabs within the transport media in a circular direction, whereas stomaching beat swabs repeatedly with the apparatus' internal paddles. Vortexing conditions were adapted from Braem et al. (2012), while stomaching conditions were adapted from Verdier-Metz et al. (2012). Aerobic culture of the bacterial suspensions exhibited an even weaker relationship with extracted DNA concentrations than in Experiment D (exponential linear regression $R^2 = 0.0936$ – see Figure 12 in Results), further emphasizing the potential disparities between aerobically-cultured and molecular

measures of sample yield. It is possible that the somewhat violent manner of agitation in the stomacher apparatus actually killed considerable numbers of cells in this treatment group, thus leading to the relatively low number of cultured organisms observed within this treatment group. The physical contact of the stomacher paddles with the Whirl-Paks may have been sufficient to break open cells, which would translate to the reduced populations of viable organisms observed in culture. If this were true, the damaged cells would theoretically release more readily-extracted DNA into the transport media. This rationale may explain the overall larger average DNA extract concentrations from stomached samples, although a high degree of variability about the mean in this treatment group once again detracts from the significance of this difference. The two-tailed t-Test assuming unequal variance asserted a lack of significance as well ($p = 0.076$). For this reason, a superior swab agitation method cannot be named definitively. Nevertheless, it should be noted that the only two 16S rRNA PCR-positive samples from this experiment belonged to the Stomacher treatment group (see Figure 14 in Results).

General Concerns

Sample size was invariably a concern across all skin swab experiments. Only two cows were included in Experiment C, resulting in a total sample population of just 18 swabs. Experiment D's sample population was slightly more robust at 36 swabs, but only six cows were represented. Finally, Experiment E's sample population was restricted to just nine swabs distributed unevenly between two cows. Future experiments performed in the field should therefore seek to capture a larger population of cows to mitigate the high degree of variation about mean bacterial DNA yields. However, more expansive on-farm sampling may not even represent the most effective study design for accomplishing this purpose. A major flaw encountered in this study was that the use of multiple swabs at the same teat skin site introduced bias based upon which swab treatment was sampled first. In Experiments C and D, the order in which swab treatments were sampled was not recorded; in Experiment E, Stomacher samples were consistently collected before Vortexer samples. This quandary was not particularly important for TBS sites, which afforded a more expansive surface area to swab, but the relatively smaller TOS and SCE sites were likely depleted of microbes by the second or third swabbing. Other on-farm study designs might increase cow numbers and sample different treatments across different cows, but inter-animal variation would be introduced as a result. Similarly, sampling different treatments across different quarters on the same cow trades the prospect of inter-animal variation for that of intra-animal variation. An attractive alternative model would be an excised teat study design, which is most frequently associated with evaluations of sanitizing teat dips (Boddie et al., 2002). Excised teats from slaughtered dairy cattle could be housed in a contaminant-free laboratory setting and coated with bacterial suspensions of known concentration; samples could then be swabbed with cleaning and re-coating between treatment groups to prevent the gradual decline of bacterial yields experienced in this study.

Of additional concern were the multiple bands observed for a number of samples (3748 Lima TBS Dry, 3748 Lima TBS Wet, 3734 TOS Wet and 3747 TOS Wet, Wet Positive Control, Wet Negative Control – see Figure 11 in Results) following amplification of the 16S rRNA gene with primers 27F and 1492R. These observations were troubling because the 27F/1492R 16S rRNA primer set generally only amplifies one gene sequence corresponding to the nearly complete 16S rRNA gene in the majority of bacterial species (Lane, 1991). However, an instance was documented in which an amplification artifact of approximately 1,500 bp in length was observed in conjunction with normal amplicons from this primer set (Osbourne et al., 2005). The additional band observed in the Experiment D reactions were larger in size than this artifact,

but the potential for variable artifact length has been described (Osbourne et al., 2005). Other reverse primers isolating 16S rRNA gene with 27F (e.g. 519r, 1525R) were consequently recommended for use in downstream applications of PCR products (Osbourne et al., 2005). Therefore, future culture-independent investigations might want to consider other 16S rRNA primer combinations. It should be noted that no double-banded patterns were observed for the PCR-positive samples from Experiment E (see Figure 14 in Results).

CONCLUSIONS

The two commercial milk bacterial DNA extraction kits (the Norgen Milk Bacterial DNA Isolation Kit and the Qiagen QIAamp DNA Mini Kit) were generally ineffectual in recovering amplifiable bacterial DNA extracts from low concentrations of bacteria. As a result, they should be avoided for culture-independent analysis of bacterial communities in non-mastitic milk unless their protocols are amended with improved milk fraction treatment and more rigorous cellular lysis conditions. The comparison of skin swab transport media demonstrated Milk Buffer to foster, on average, the growth of the largest and most diverse bacterial communities in aerobic culture, but the large standard deviations exhibited by aerobically-cultured plate counts detracted from the significance of this finding. A sound DNA extraction method was not available to confirm the superiority of Milk Buffer as an effective transport medium, so its value to culture-independent analysis can only be loosely inferred. Similarly, neither of the swabbing methods (wet and dry) exhibited a statistically significant advantage in associated bacterial DNA extract concentration; a slight edge by wet swabbing was weakened by possible aerosol contamination from the barn environment. Finally, the slightly higher bacterial DNA extract concentrations associated with stomaching (as opposed to vortexing) skin swabs was not statistically significant. Repeated analyses of skin swab sample collection and processing methods should incorporate a larger number of samples or make use of the proposed excised teat model.

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