IDENTIFICATION AND CHARACTERIZATION OF CANDIDATE *STAPHYLOCOCCUS AUREUS* ANTIGENS FOR INCLUSION IN A MULTIVALENT BOVINE MASTITIS VACCINE

by

Danielle Scarbrough

A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in Biomolecular Sciences

Boise State University

December 2020

BOISE STATE UNIVERSITY GRADUATE COLLEGE

DEFENSE COMMITTEE AND FINAL READING APPROVALS

of the thesis submitted by

Danielle Scarbrough

Thesis Title: Identification and Characterization of Candidate *Staphylococcus Aureus* Antigens for Inclusion in a Multivalent Bovine Mastitis Vaccine

Date of Final Oral Examination: 23 October 2020

The following individuals read and discussed the thesis submitted by student Danielle Scarbrough, and they evaluated their presentation and response to questions during the final oral examination. They found that the student passed the final oral examination.

Juliette Tinker, Ph.D.	Chair, Supervisory Committee
Ken Cornell, Ph.D.	Member, Supervisory Committee
Brad Morrison, Ph.D.	Member, Supervisory Committee

The final reading approval of the thesis was granted by Juliette Tinker, Ph.D., Chair of the Supervisory Committee. The thesis was approved by the Graduate College.

ACKNOWLEDGMENTS

Thank you to Dr. Juliette Tinker for insight and guidance, to Dr. Shin Pu for performing mass spectrometry and database searches, and to Cierra Wheeler for her work classifying antigens by function and subcellular localization. This work, including bioinformatics training at the National Center for Genome Resources, was supported by NIH Grant Nos. P20GM103408 and P20GM109095, National Science Foundation Grant Nos. 0619793 and 0923535, the MJ Murdock Charitable Trust, the Idaho State Board of Education, and the J.R. Simplot Company.

ABSTRACT

Bovine mastitis, inflammation typically caused by bacterial infection, is the most prevalent disease affecting the global dairy industry. *Staphylococcus aureus* remains one of the most important pathogens implicated in the disease and can persist within herds at subclinical levels. A preventative *S. aureus* bovine mastitis vaccine would substantially lessen costs associated with treatment and restore revenue lost due to decreased milk production. One such experimental vaccine is the IsdA-CTA$_2$/B + ClfA-CTA$_2$/B vaccine, containing the *S. aureus* antigens IsdA and ClfA , each fused to the nontoxic A$_2$/B subunits of cholera toxin, which serves as an adjuvant. Previous clinical studies have indicated protective, but not fully preventative, effects following vaccination in cows. Additional antigens are required to increase vaccine efficacy. To identify priority candidate vaccine antigens, a combined proteomics and transcriptomics approach was implemented. *S. aureus* was cultured in traditional LB broth and, to replicate biological conditions in the bovine udder, skim milk. *S. aureus* extracts were subsequently analyzed by mass spectrometry and Next Generation RNA sequencing to measure unique surface proteins and upregulated genes in skim milk culture conditions. Selected candidates were further prioritized by subcellular localization to the cell periphery and by adhesin probability. Top candidate vaccine antigens identified include: fibrinogen-binding protein (Fib), bifunctional autolysin (Atl), thermonuclease (Nuc), autolysin/adhesin (Aaa), secretory antigen SsaA-like protein, and zinc ABC transporter substrate-binding protein.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AdsA	Adensosine Synthase A
AT	Hemolysin-1 Mutant (α-toxin)
ABC	ATP-binding Cassette
cDNA	Complementary DNA
CFU	Colony Forming Units
ClfA	Clumping Factor A
Coa	Coagulase
CP5	Capsular Polysaccharide Type 5
CP8	Capsular Polysaccharide Type 8
EsxA	Type VII Secretion System Extracellular Protein A
EsxB	Type VII Secretion System Extracellular Protein B
EV	Extracellular Vesicles
FBS	Fetal Bovine Serum
GO	Gene Ontology
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
IFN-γ	Interferon gamma
IL-6	Interleukin 6
IL-10	Interleukin 12
IsdA	Iron-regulated Surface Determinant Protein A

IsdB	Iron-regulated Surface Determinant Protein B
LB	Luria Broth
LIM	Low Iron Media
MntC	Manganese Transport Protein C
MSA	Mannitol Salt Agar
OSP	Opsonophagocytic
PCR	Polymerase Chain Reaction
PBMCs	Peripheral Blood Mononuclear Cells
RIN	RNA Integrity Number
RPKM	Reads per Kilobase (Feature) per Million (Sequencing Reads)
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SdrD	Serine Aspartate Repeat Containing Protein D
SdrE	Serine Aspartate Repeat Containing Protein E
SKM	5% Skim Milk
SpA	Staphylococcal Protein A

CHAPTER ONE: INTRODUCTION

Bovine mastitis, an inflammatory reaction in the udder that is most often in response to microorganism infection, is a problematic disease affecting the worldwide dairy industry. It is costly due to reduced milk production and thereby revenue loss, and due to expenses associated with treatment of the disease[1,2]. Bovine mastitis is characterized by the immunological response to pathogen infection and colonization of the udder, resulting in inflammation. Infection may be clinical or exist undetected at subclinical levels. This disease is caused by several bacterial species and, while variable depending on locale, the responsible pathogens include: *Escherichia coli*; *Streptococcus* species, notably *S. agalactiae* and *S. uberis*; *Corynebacterium bovis*; and, most commonly, *Staphylococcus* species such as *S. aureus*[1,3,4,5,6].

Staphylococcus aureus Pathogenesis in the Development of Bovine Mastitis

S. aureus is a non-commensal, contagious pathogen responsible for the development of bovine mastitis and particularly associated with subclinical, persistent infection. The main virulence factors implicated in pathogenesis include: biofilm factors, enterotoxins, toxic shock syndrome toxin, hemolysins, and leukotoxins[7,8]. *S. aureus* secretes cytolytic toxins that permeabilize membranes of the host cells leading to tissue damage, bacterial invasion, and colonization. The pathogen can resist the innate immune system and neutrophil phagocytic uptake by expression of anti-opsonic proteins and the polysaccharide capsule, and can express superantigens which may lead to

immunosuppression[9]. It has also been reported that *S. aureus* may persist intracellularly in bovine mammary epithelial cells, alveolar cells, and macrophages[10,11].

Staphylococcus aureus Vaccine Development

S. aureus vaccine research has primarily focused on prevention of human infection, particularly hospital-acquired infection of multi-drug resistant strains. While no vaccine currently exists that is approved for clinical use, substantial progress has been made. The most promising experimental vaccines include subunit vaccines that incorporate several *S. aureus* surface proteins and an adjuvant. An early vaccine that incorporated the conserved surface proteins IsdA, IsdB, SdrD, and SdrE was determined more protective combined than as individual components in mice[12]. Vaccine concepts that have moved forward to clinical trials include protein subunit vaccines combined with capsular polysaccharides: ClfA/MntC/CP5/CP8 and CP5/CP8/AT/ClfA[13,14,15]. Current preclinical vaccines include antigens such as EsxA, EsxB, SpA, AdsA, and Coa[16]. A new vaccine concept of interest is extracellular vesicles (EV) containing detoxified cytolysins produced by and purified from. *S. aureus* mutants, and reported to be immunogenic in mice[17]. EVs could be designed to package and deliver multiple antigens of interest, and simultaneously act as an adjuvant[18].

Staphylococcus aureus Bovine Mastitis Vaccines

While modern preventative practices have substantially reduced the prevalence of clinical bovine mastitis caused most frequently by *E.coli,* subclinical mastitis remains a threat to the dairy industry at a global scale[19,20]. Antibiotic therapy is commonly used as treatment for bovine mastitis, however, resurgence of infections within a herd, dangers of antibiotic resistance, and overall cost for treatment remain challenges in management of

the disease. In addition, *S. aureus* is especially challenging as the pathogen responds poorly to antibiotic treatment[21,22]. A protective vaccine would be invaluable to minimize the impact of mastitis on the dairy industry.

Several vaccines are currently in development, and while successful at minimizing severity of the disease, none are fully preventative[23,24,25]. One such experimental *S. aureus* bovine mastitis vaccine under development is the IsdA-CTA$_2$/B + ClfA-CTA$_2$/B vaccine. This vaccine consists of two *S. aureus* antigens, each fused to the nontoxic A$_2$/B subunits of cholera toxin, which serves as an adjuvant. This vaccine has now been studied in two clinical trials[26,27]. While the results have been promising, with vaccinated cows demonstrating induced immune responses and reduced infection, complete prevention of infection has not been achieved (Appendix A). Due to genetic diversity and differential expression of virulence factors among strains, a robust *S. aureus* bovine mastitis vaccine will require several conserved antigens[28,29].

Identifying Vaccine Antigens

Additional conserved and immunogenic *S. aureus* antigens need to be identified for inclusion in an effective, multivalent *S. aureus* bovine mastitis vaccine. Reverse vaccinology remains a popular method for bacterial vaccine antigen discovery. Reverse vaccinology is a computational approach that identifies antigens by filtering genomic open reading frame sequences by categories such as subcellular localization, adhesin, and epitope predictions, conservation among strains, and similarity to host proteins[30]. Another popular method is immunoproteomics, which uses mass spectrometry proteomics approaches in combination with immunogenic antigen selection by probing with

antibodies[31]. Some studies have also utilized transcriptomic approaches to identify key genes involved in pathogenesis, that could represent good vaccine candidate antigens[32,33].

Initial work has been completed to identify two *S. aureus* bovine mastitis vaccine candidate antigens, EsxA and IsdC (Appendix B). The study detailed here further expands upon this effort. Proteomics, transcriptomics, and antigenicity predictions using reverse vaccinology tools were utilized to identify additional candidates. Aiming to replicate growth conditions within the bovine udder, and to identify important genes and surface proteins expressed specifically during pathogenesis, *S. aureus* strain Newbould 305 was cultured in 5% skim milk (SKM) in direct comparison to traditional Luria broth (LB) media. Surface proteins were harvested from cultured *S. aureus*, representing the surfaceome, and analyzed by mass spectrometry. In addition, total RNA was isolated from *S. aureus* cultures, submitted for RNA sequencing, and investigated by differential gene expression analysis. Candidates identified by these approaches were characterized further by subcellular localization and adhesin probability, resulting in several promising candidate antigens to include in a robust, multivalent bovine mastitis vaccine.

CHAPTER TWO: MATERIALS AND METHODS

Bacterial Cell Culture

Staphyloccocus aureus strain Newbould 305 was cultured on mannitol salt agar plates (MSA) overnight at 37°C. For experimentation, *S. aureus* Newbould 305 was cultured in Luria Broth (LB) or in 5% skim milk (SKM). LB broth is the traditional bacterial culture medium; it is nutritionally rich, including tryptone (peptides; 50%), yeast extract (vitamins; 25%), and sodium chloride (electrolytes; 25%). SKM media serves to replicate the biological conditions within the bovine udder. While use of whole milk would enable replication of fat concentrations *in vivo*, sterilization of whole milk is difficult and can result in Maillard reactions with protein denaturation and changes in pH[34,35]. SKM media contains primarily lactose (50%) and protein (35%) and minerals such as calcium, iron, and magnesium. Luria broth (LB) or 5% skim milk (SKM) cultures were inoculated with a single colony and grown for twelve hours at 37 °C and 200 rpm. General experimental design is presented in Figure 1.

Surfaceome Protein Isolation and Mass Spectrometry

Three biological replicates were prepared per culture medium. Bacterial cultures were harvested by centrifugation at 3500 x g for ten minutes. Pellets were washed with 1X PBS, centrifuged, and then resuspended in enzyme digest solution (5 µg/mL trypsin in 30% sucrose/1X PBS, pH 7.4) as reported by Rodriguez-Ortega, 2018[36]. Pellets were digested for 30 minutes at 37 °C to release surface proteins. Following digestion, samples were centrifuged at 3500 x g for ten minutes, and supernatant containing surfaceome

extract was filtered with 0.2 µm filters. Protein extracts were prepared for LC-ESI-MS/MS analysis. Extracts were dried under vacuum and reconstituted in 5% acetonitrile, 0.1% formic acid. A total of 5 µl was injected onto a C18 reverse-phase column (10 cm x 75 µm, 3 µm, 120 Å), and a linear gradient with two mobile phases at a flow rate of 300 nL/min separated peptide mixtures. Full scan MS spectra were acquired from m/z 300-2000. Proteins were identified by searching the UniProtKB/Swiss-Prot protein database for *S. aureus*.

RNA Extraction and RNA Sequencing

Four biological replicates were prepared per culture medium. RNA was extracted from the bacterial cell pellets as reported by Qin et al[37]. Briefly, two volumes RNAprotect Bacteria Reagent (Qiagen) was combined to one volume *S. aureus* culture after twelve-hour incubation. The mixtures were vortexed and incubated at room temperature for five minutes. Following incubation, the cells were centrifuged at 5000 xg for ten minutes. The supernatant was removed, and cell pellets resuspended in TE buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing lysozyme (20 mg/mL), lysostaphin (12.5 µg/mL), and proteinase K. Pellets were digested for one hour at 37°C, with vortexing every 10 minutes. Following digestion, RNA was extracted using the Qiagen RNeasy Maxi Kit according to the manufacturer's instructions. Genomic DNA was removed using TURBO™ DNase (Invitrogen) and purified with the Zymo Research RNA Clean & Concentrator kit. RNA concentration was measured by NanoDrop, and quality confirmed by agarose gel electrophoresis. Total RNA was submitted to Novogene Corporation, Inc. (Sacramento, CA) for RNA sequencing. Novogene confirmed RNA quality using the Agilent 2100 Bioanalyzer, constructed prokaryotic sequencing libraries

following ribosomal RNA depletion, and performed paired-end 150 bp RNA sequencing using the Illumina Novaseq 6000.

Differential Gene Expression and GO Analysis

S. aureus reference genome (subsp. *aureus* NCTC 8325) and annotation files were downloaded from NCBI. Bioinformatics was performed using the CyVerse Discovery Environment software. Sequencing quality was assessed using FastQC and MultiQC. Sequencing reads were then mapped to the reference genome with HISAT2. Aligned reads were counted using FeatureCounts, and differential gene expression analysis was performed with DESeq2. Normalized gene expression values in RPKM (Reads per Kilobase feature per Million reads) were calculated per gene. GO analysis was performed using the ClueGO Cytoscape application.

RT-qPCR

Total RNA was extracted from LB and SKM cultured Newbould 305, DNase treated, and purified as previously described. RNA was converted to cDNA with the RevertAid First Strand cDNA Synthesis kit (Thermo). Resulting cDNA was diluted 1:40 prior to RT-qPCR. Primer sequences specific to a select few genes of interest (*ssaA*, *isaA*, *lysM*) and reference gene *16s* are presented in Table 1. RT-qPCR reaction conditions are presented in Table 2 and Table 3. Relative gene expression was calculated by 2^-ΔCq, where ΔCq represents $Cq_{target} - Cq_{reference}$. Fold change was determined by the expression of SKM divided by LB, and then $\log_2$ transformed.

Antigenicity Assessment

Candidate antigens of interest were chosen from unique surfaceome proteins and upregulated genes expressed in skim milk cultured *S. aureus*. This list was further

characterized for antigenicity by cellular localization and adhesin probability using PSORTb Subcellular Localization tool (v3.0.2; psort.org/psortb) and Vaxign (violinet.org/vaxign).

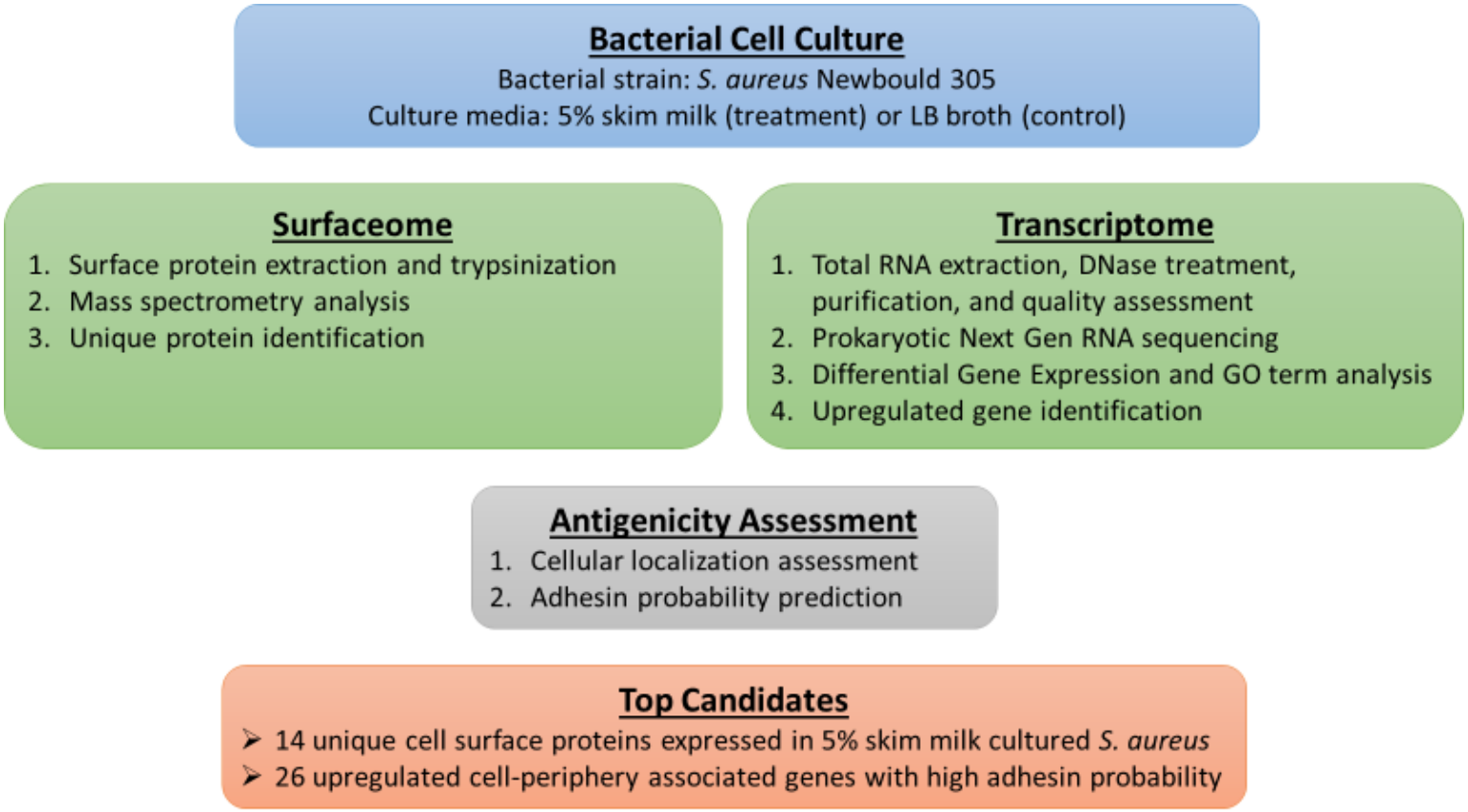

Figure 1. General Experimental Design.

S. aureus strain Newbould 305 was cultured in 5% skim milk (treatment) and in LB broth (control). Uniquely expressed surface proteins in skim milk were identified by mass spectrometry and upregulated genes expressed in milk were identified by Next Gen RNA sequencing and differential gene expression analysis. Candidates were further prioritized by subcellular localization and adhesin probability, resulting in a final list of 40 candidate antigens.

Table 1. RNA Sequencing Validation Gene of Interest Primer Sequences.

Gene	Primer Sequence (5' to 3')	Amplicon Size (bp)
ssaA	**FW** GGTCAAGCACATCATGCAG	316
	RV CATATGCAACGTGACC	
isaA	**FW** CAGCTCCAATCAAAGATGGTGC	436
	RV GAAGCACCTGATGGGTTG	
lysM	**FW** CATCAATCGCATGCCGCAG	330
	RV GTTAAAGTTACGTGCTGCC	
16s rRNA	**FW** AGAGTTTGATCCTGGCTCAG	530
	RV ATTACCGCGGCTGCTGGC	

Table 2. RNA Sequencing Validation RT-qPCR Reaction Conditions.

Reagent	Final Concentration	Volume
2X Maxima SYBR Green (Thermo)	1X	10 µl
Forward Primer 10 µM	0.3 µM	0.6 µl
Reverse Primer 10 µM	0.3 µM	0.6 µl
cDNA Template	varies	4 µl
Nuclease-free water		4.8 µl

Table 3. RNA Sequencing Validation RT-qPCR Cycling Conditions.

Temperature (°C)	Time (s)	Cycles
95 °C	600 s	1
95 °C	15 s	40
55 °C	30 s	
72 °C	30 s	

CHAPTER THREE: RESULTS

S. aureus Surfaceome

Protein extraction was designed to harvest extracellular and surface-associated proteins from *S. aureus* cultured in LB or in SKM. Surface proteins unique to SKM cultured *S. aureus* represent vaccine candidate antigens that may be of particular importance to bacterial invasion and colonization of the udder. A total of 1,006 proteins were identified in LB cultured *S. aureus* surfaceome extracts. In SKM cultured *S. aureus* surfaceome extracts, 481 proteins were identified. Out of the 481 proteins identified in SKM, 203 proteins were unique to SKM and not found in LB. Of these 203 unique proteins, 45 were present in a minimum of two out of three biological replicates. The 45 unique proteins were subsequently narrowed down by percent protein coverage (>10%), and predicted subcellular localization (cell membrane, cell wall, or extracellular) to a priority list of 14 proteins as presented in Table 4.

Table 4. Unique Surface Proteins Expressed in Milk-Cultured S. aureus Newbould 305.
Proteins were expressed in a minimum of two biological replicates and were not expressed in LB-cultured S. aureus samples.

Gene	Protein	Average Coverage (%)	Function[1]	Subcellular Location[2]	Adhesin Probability[3]
fib	Fibrinogen-binding protein	27	Complement binding, pathogenesis	extracellular	0.52
mnhF1	Na(+)/H(+) antiporter subunit F1	27	Sodium:proton antiporter activity	cytoplasmic membrane	0.17
rimP	Ribosome maturation factor	22	Ribosomal small subunit biogenesis	extracellular	0.32
metN2	Methionine import ATP-binding protein	19	ATPase activity, translocase	cytoplasmic membrane	0.10
atl	Bifunctional autolysin	18	Amidase activity, cell wall organization	cytoplasmic membrane	0.79
nuc	Thermonuclease	16	Nuclease activity	extracellular	0.78
crtO	Glycosyl-4,4'-diaponeurosporenoate acyltransferase	15	Transferase activity, carotenoid biosynthetic process	extracellular	0.13
mnhG2	Putative antiporter subunit	15	Antiporter activity	cytoplasmic membrane	0.20
ilvD	Dihyroxy-acid dehydratase	14	Metal ion binding, isoleucine and valine biosynthetic process	extracellular	0.26
oppF	Putative oligopeptide transport ATP-binding protein	12	ATPase activity, protein transport	cytoplasmic membrane	0.05
isdF	Probable heme-iron transport system permease protein	11	Transmembrane transporter activity	unknown	0.29

dapB	4-hydroxy-tetrahydrodipicolinate reductase	11	Oxidoreductase activity, diaminopimelate biosynthetic process	cytoplasmic membrane	0.27
Gene	Protein	Average Coverage (%)	Function[1]	Subcellular Location[2]	Adhesin Probability[3]
hutG	Formimidoylglutamase	11	Histidine catabolic process	cytoplasmic membrane	0.35
lacD	Tagatose 1,6-diphosphate aldolase	11	Lactose catabolic process	cytoplasmic membrane	0.31

[1]Uniprot.org
[2]Psort.org/psortb/
[3]Violinet.org/vaxign

Total RNA and RNA Sequencing Quality Analysis

Next Generation RNA sequencing was utilized to identify genes upregulated in SKM cultured *S. aureus*. Highly expressed, and highly upregulated, genes may indicate good targets for inclusion in a comprehensive bovine mastitis vaccine. Next Generation RNA sequencing requires high quality RNA for optimal results. Total RNA quality, following DNase treatment and purification, was determined by agarose gel electrophoresis to be consistently high across all samples (Figure 2). Quality was confirmed by 260/280 ratios (2.0-2.2) as measured by NanoDrop™ Spectrophotometer (Thermo Scientific) prior to shipment to Novogene. RNA integrity number (RIN) was measured by the Agilent Bioanalyzer by Novogene upon arrival. Six samples were reported as excellent quality (>8.0) and, although two (SKM-2, SKM-3) were below ideal quality thresholds, all samples were deemed suitable for library construction and sequencing (Table 5).

RNA sequencing quality was consistently high, both per sequence and per base across the sequencing read, across all replicates as determined by FastQC and MultiQC analysis (Figures 3 and 4). Sequencing quality is represented by the phred score. Phred scores are assigned by the software by reading the sequence chromatogram. A phred score of 10 indicates a 10% probability that the nucleotide base may be called wrong, a score of 20 indicates a 1% probability, a score of 30 indicates a 0.1% probability, and a score of 40 indicates a 0.01% probability[38,39]. Scores above 30 are considered high quality base calls.

Approximately 20 million reads were generated per sample and aligned to the reference genome. Read mapping was variable between samples, with between 32% and

96% of reads mapping to the reference genome (Table 6). However, the percentage of uniquely mapped reads (unique mapped reads per total number of mapped reads) was more consistent, ranging between 54% and 82%. Uniquely mapped reads align to only one location in the reference genome, whereas multi-mapped reads align nonspecifically to more than one location in the genome. The uniquely mapped reads were used for subsequent differential gene expression analysis.

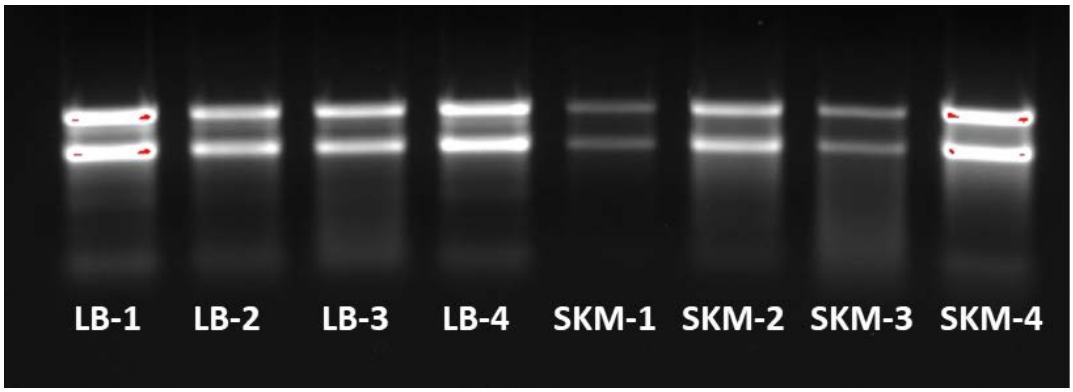

Figure 2. RNA Quality Determined by Agarose Gel Electrophoresis. All total RNA samples remained high quality following DNase treatment and purification, prior to sequencing submission.

Table 5. Total RNA Quality as Determined by Nanodrop and Bioanalyzer. All total RNA samples were confirmed high quality following DNase treatment and purification by 260/280 ratios. While all samples were determined acceptable quality by RIN score assessment to proceed with sequencing, SKM-2 and SKM-3 were reported as below ideal quality thresholds (RIN>8.00).

	Performed at Boise State University		Performed at Novogene
Sample	Concentration (ng/µl)	Quality (260/280)	Quality (RIN)
LB-1	222	2.17	10.00
LB-2	182	2.19	10.00
LB-3	157	2.19	10.00
LB-4	190	2.17	9.90
SKM-1	85	2.2	10.00
SKM-2	180	2.14	5.00
SKM-3	132	2.07	7.00
SKM-4	266	2.09	9.70

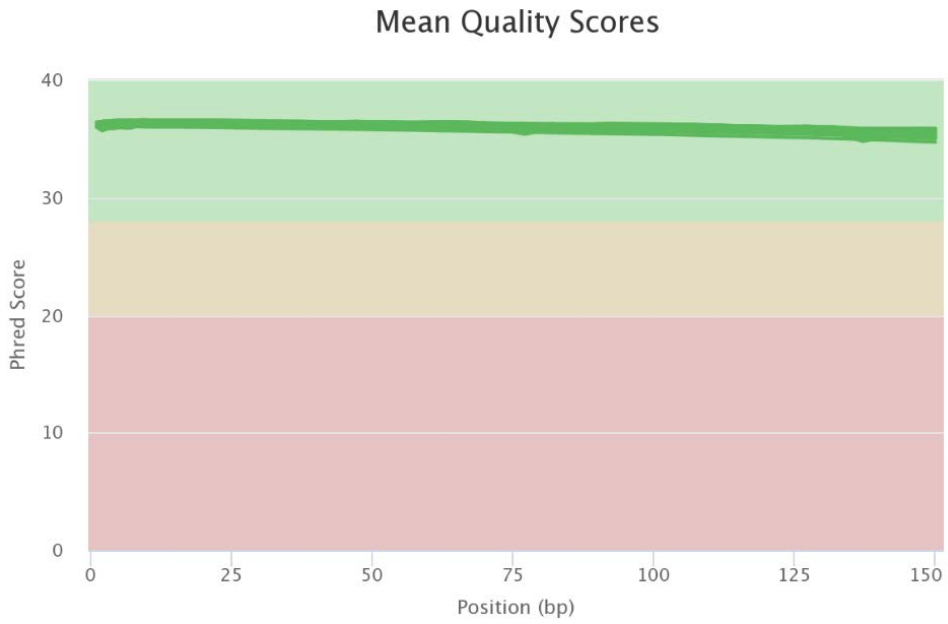

Figure 3. Sequence Quality Histogram. Mean Quality per Base across the Sequencing Read.
Created using FastQC and MultiQC. Per base quality scores (Phred scores) were consistently high across all samples.

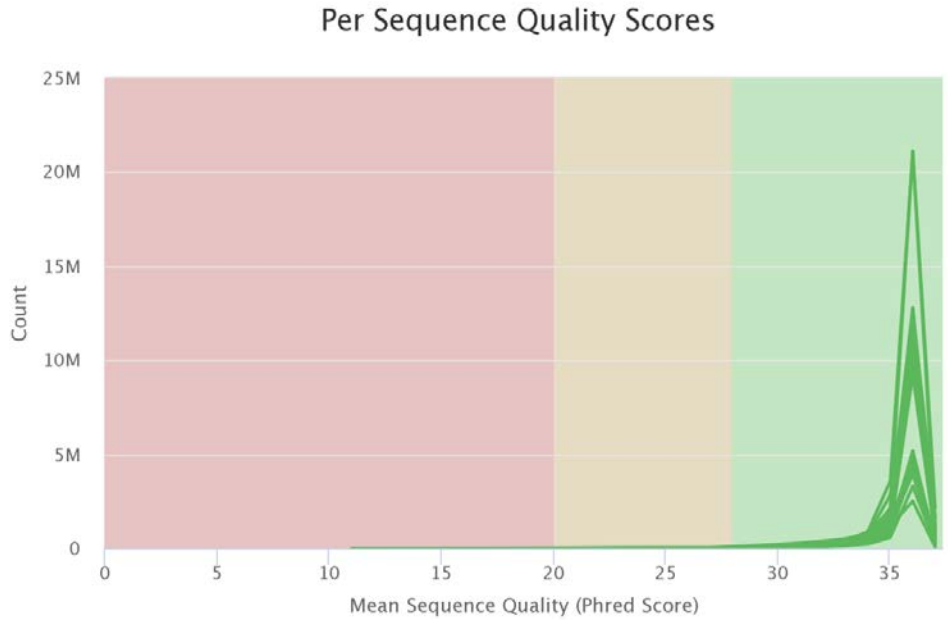

Figure 4. Sequence Quality Histogram. Mean per Sequence Quality Score.
Created using FastQC and MultiQC. Per sequence quality scores (Phred scores) were consistently high across all samples.

Table 6. Sequencing Read Mapping Quality.
Total sequencing reads per sample and overall percent reads mapped to the reference genome, and the number of uniquely mapped reads. LB represents S. aureus cultured in LB broth, and SKM represents S. aureus cultured in 5% skim milk.

Sample Name	Total Read Count	Mapped Read	Uniquely Mapped Reads	Mapped Reads (%)	Uniquely Mapped Reads (%)
LB 1	21407937	20154109	12142111	94	60
LB 2	19337330	18121528	10523420	94	58
LB 3	21156113	19776846	11632755	93	59
LB 4	20856540	20007058	10893904	96	54
SKM 1	27918308	22973837	16621001	82	72
SKM 2	17022124	8711353	6801804	51	78
SKM 3	21329079	9199618	7585226	43	82
SKM 4	22158448	7086566	5391291	32	76

Differential Gene Expression and RT-qPCR

Principal component analysis (PCA) was performed to assess sample similarity. PCA and the associated cluster dendrogram demonstrated acceptable biological replicate clustering and biological condition separation, with LB cultured samples most comparable to each other, and SKM cultured samples comparable to each other (Figure 5). The entire transcriptomic dataset can be visualized with the MA and Volcano plots presented in Figure 6. The MA plot provides $\log_2$ fold change values of SKM vs. LB and the average normalized read counts across all samples, with each gene represented by a dot. The Volcano plot provides the $\log_2$ fold change values and the adjusted p-values, with each gene again represented by a dot, demonstrating significantly differentially expressed features.

Following quality assessment, differentially expressed genes were inspected. A total of 2383 features are annotated in the *S. aureus* reference genome. There were 1268 differentially expressed genes, with 659 upregulated and 609 downregulated in SKM vs. LB ($p_{adj}<0.05$, Figure 7). When further selecting by identifying genes with an absolute

$\log_2$ fold change >2, a total of 601 genes were differentially expressed in SKM vs. LB. A total of 332 genes were upregulated, and 269 genes were downregulated. Select genes (*ssaA*, *isaA*, *lysM*) were chosen for RNA sequencing and differential gene expression analysis validation by RT-qPCR. All three genes were determined, by differential gene expression analysis, to be upregulated in SKM vs. LB. Upregulated of gene expression in SKM vs. LB was confirmed by RT-qPCR (Figure 8).

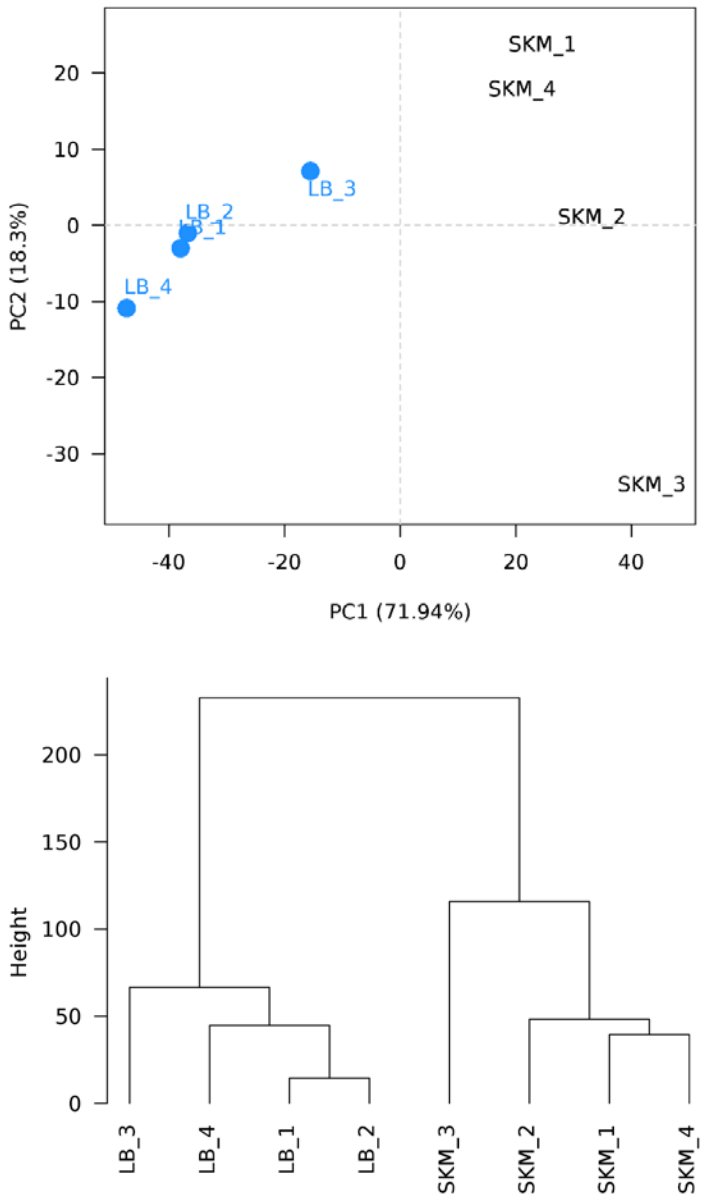

Figure 5. Principal Component Analysis and Cluster Dendrogram.
LB represents S. aureus cultured in LB broth, and SKM represents S. aureus cultured in 5% skim milk. LB cultured samples and SKM cultured samples cluster separately.

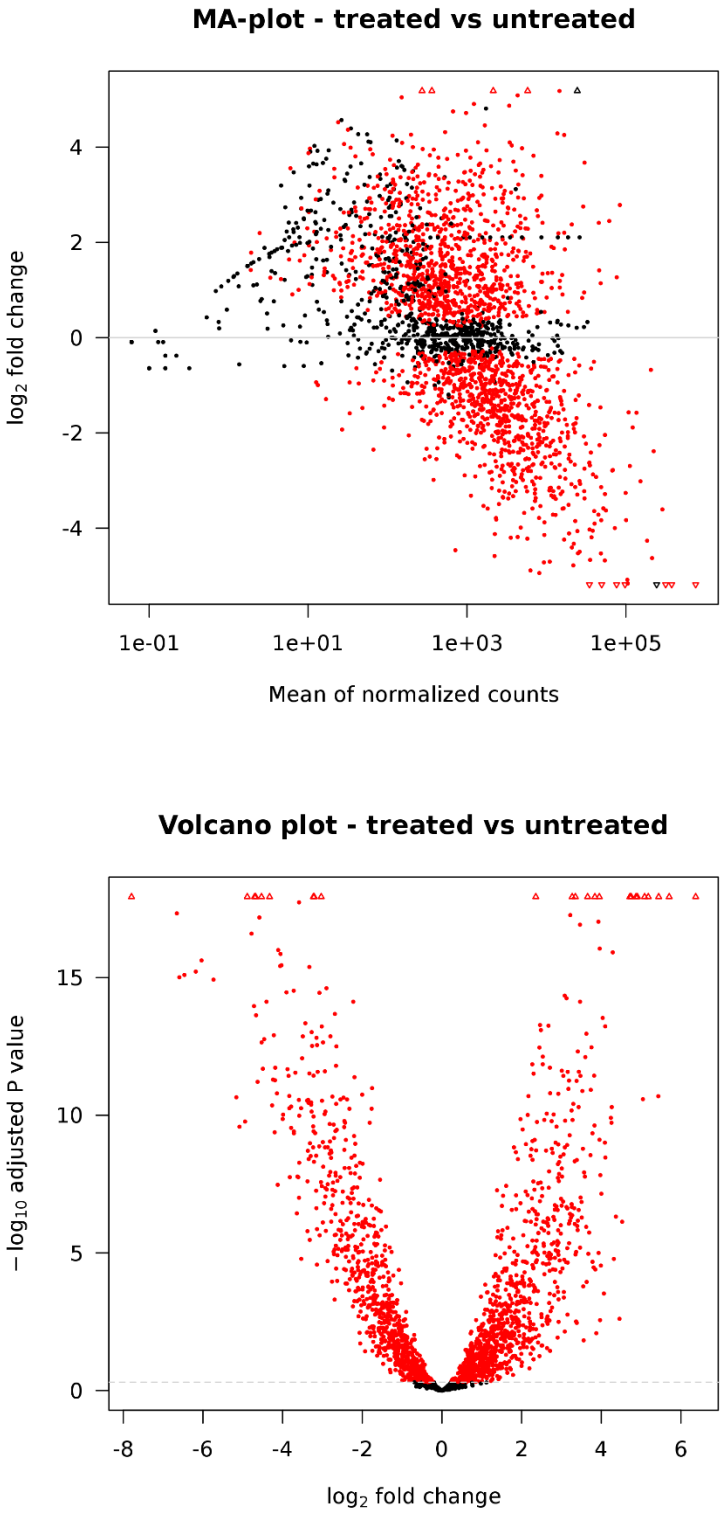

Figure 6. MA and Volcano Plots in Treated (SKM) vs. Untreated (LB) S. aureus Newbould 305 Cultures.

Significantly differentially expressed features (padj<0.05) are colored in red, and remaining features are colored in black.

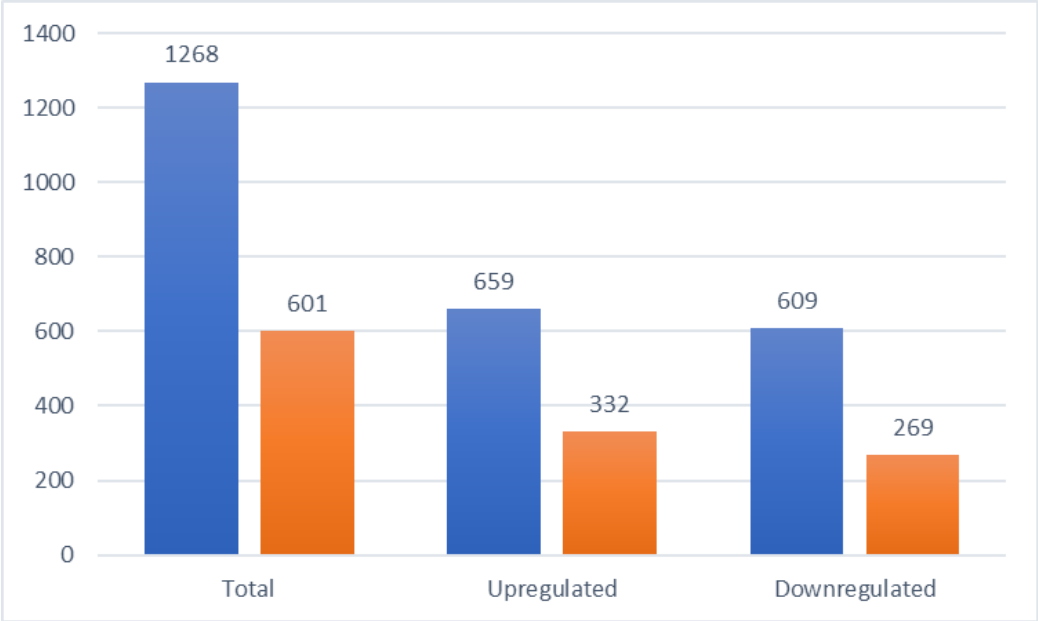

Figure 7. Number of Differentially Expressed Genes in SKM vs. LB Cultured S. aureus Newbould 305.

The number of differentially expressed genes were selected by padj<0.05 (blue bars) or by Log2 Fold Change>2 and padj<0.05 (orange bars).

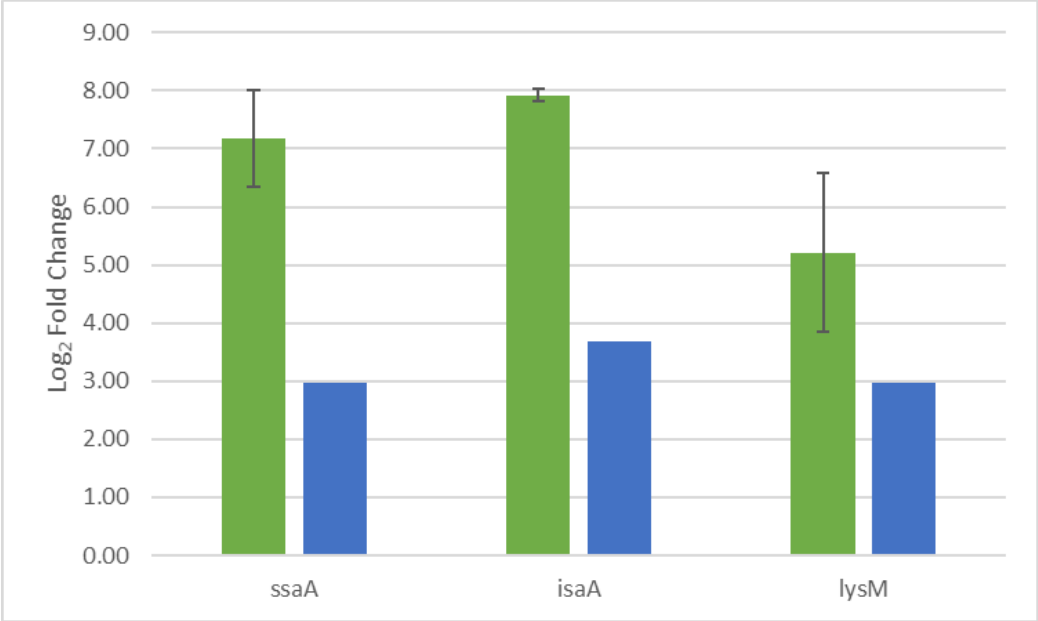

Figure 8. RNA Sequencing Validation by RT-qPCR.

Gene expression of select genes (ssaA, isaA, and lysM) was measured by RT-qPCR. Upregulation of each gene as determined by RNA sequencing and differential gene expression (blue) was confirmed by RT-qPCR (green).

Gene Ontology (GO) Term Analysis

The Gene Ontology classifies genes according to molecular functions, cellular locations, and biological processes. GO term analysis allows for broader interpretation of biological pathways impacted by experimental treatments. Genes highly upregulated in SKM vs. LB represent potential vaccine candidate antigens of interest and were subjected to GO term analysis. Excess ribosomal RNA or tRNA identified in the significantly upregulated genes (Absolute Log_2 Fold Change >2, p_{adj} <0.05) were manually removed and resulting genes were further assessed by Biological and Cellular GO analysis.

Significantly enriched ($p<0.05$) biological processes were evaluated to assess comprehensive biological changes due to SKM vs. LB culture conditions. Enriched pathways included inosine monophosphate (IMP) metabolic process, diaminopimelate biosynthetic process, alpha-amino biosynthetic process, cellular macromolecule biosynthetic process, and amide transport (Figure 9). Cellular GO analysis was utilized to reveal subcellular localization classifications of all significantly upregulated genes. Upregulated genes were found to be primarily associated with the cell periphery, such as plasma membrane, external encapsulating structure, and cell envelope (Figure 10). The largest cellular GO term classification was ABC transporter complex, representing over 30% of upregulated genes that had associated GO terms.

Antigenicity Assessment

Unique surfaceome proteins and upregulated genes in SKM cultured *S. aureus* samples were further characterized by potential antigenicity, leading to finalized candidate vaccine antigen lists as presented in Table 4 and Table 7. Characteristics evaluated included subcellular localization (cell membrane, cell wall, or extracellular)

prediction by PSORTb, and adhesin probability prediction by Vaxign. Fifteen proteins identified as unique to SKM cultured *S. aureus* were predicted to be associated with the cell periphery, and three of these were also predicted adhesins (probability>0.5): fibrinogen binding-protein, bifunctional autolysin, and thermonuclease. The majority of highly upregulated genes were classified by the Gene Ontology as associated with the cell periphery, and this was confirmed by PSORTb predictions. Of these surface-related genes, 26 were also predicted as probable adhesins.

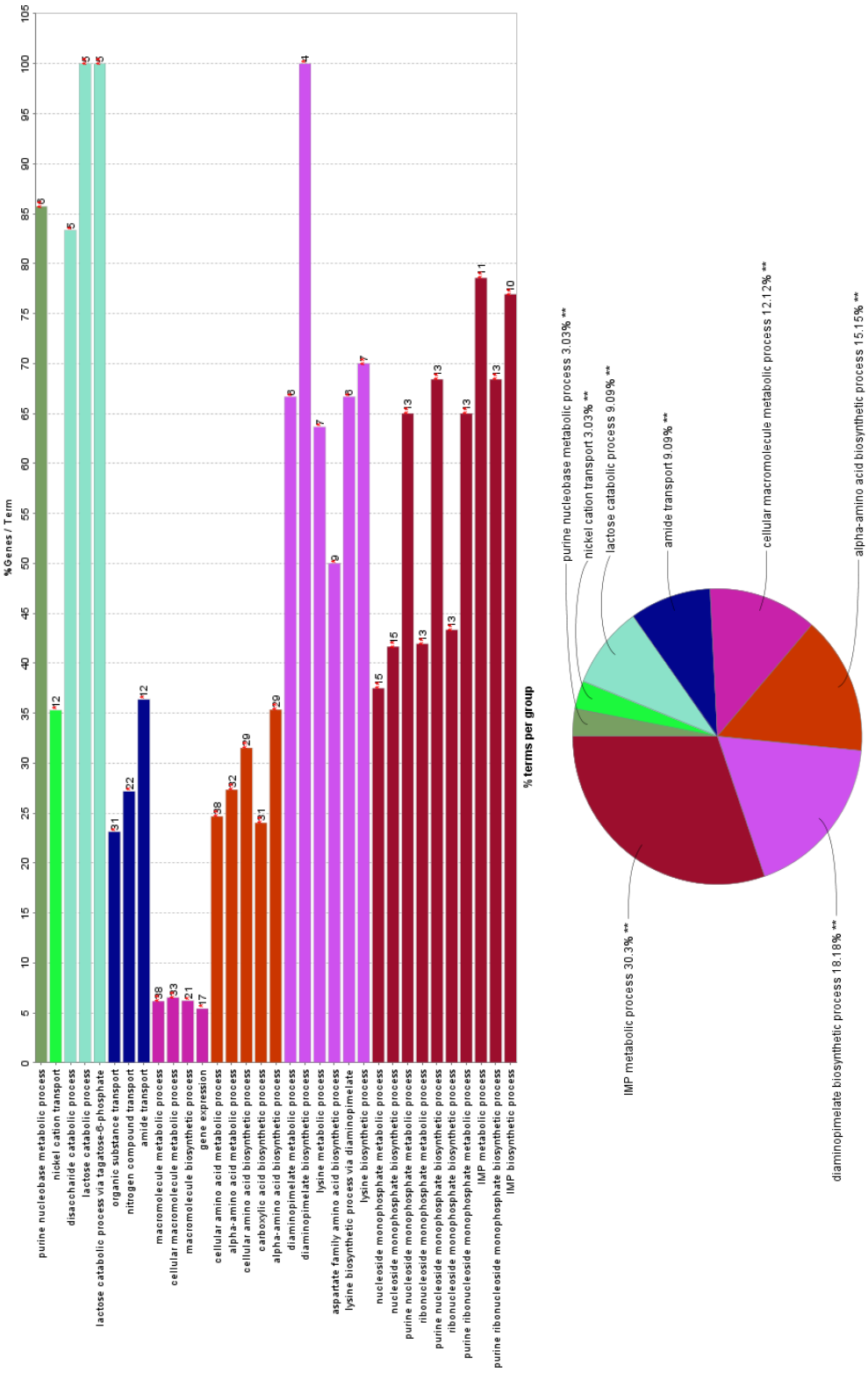

Figure 9. Biological Process GO Terms Associated with Significantly Upregulated Genes.
Significantly enriched pathways ($p<0.05$) for upregulated genes ($Log_2FC>2$; $p_{adj}<0.05$) in skim-milk cultured S. aureus are presented.

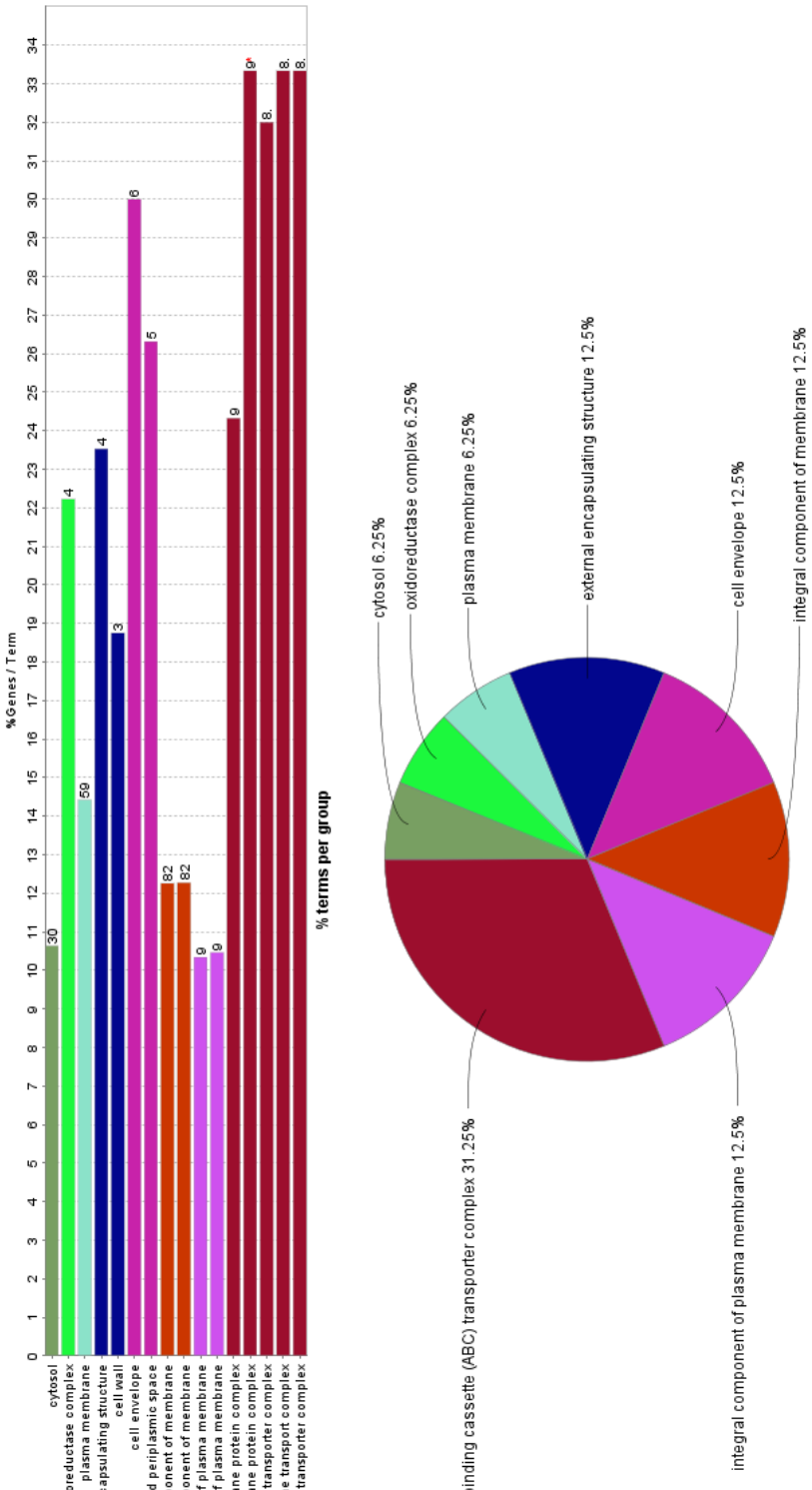

Figure 10. Cellular Process GO Terms Associated with Significantly Upregulated Genes.
The majority of upregulated genes (Log2FC>2; padj<0.05) in SKM cultured S. aureus are localized to the cell periphery.

Table 7. Upregulated Genes in SKM cultured S. aureus Newbould 305 Associated with the Cell Periphery and with High Adhesin Probability.

Significantly (Log2FC>2; padj<0.05) upregulated genes were selected by subcellular localization to the cell periphery (extracellular, cell wall, or cell membrane), and for adhesin probability (>0.5). RPKM SUM represents the combined RPKM across all eight samples.

Gene Name	Gene Symbol	Log_2 Fold Change	RPKM SUM	Gene Description[1]	Location[2]	Adhesin probability[3]
asp1	SAOUHSC_02988	3.83	323	accessory Sec system protein Asp1	Extracellular	0.73
aaa	SAOUHSC_00427	3.82	8391	autolysin/adhesin Aaa	Cell wall	0.70
-	SAOUHSC_00284	3.78	638	5'-nucleotidase, lipoprotein e(P4) family	Extracellular	0.54
sarR	SAOUHSC_02566	3.47	8077	HTH-type transcriptional regulator SarR	Extracellular	0.68
cntL	SAOUHSC_02769	3.43	188	D-histidine (S)-2-aminobutanoyltransferase CntL	Extracellular	0.51
-	SAOUHSC_02943	3.41	409	CitMHS family transporter	Extracellular	0.51
-	SAOUHSC_02316	3.40	6925	DEAD/DEAH box helicase	Extracellular	0.73
-	SAOUHSC_02886	3.26	362	GNAT family N-acetyltransferase	Extracellular	0.72
-	SAOUHSC_02764	3.05	191	ABC transporter ATP-binding protein	Extracellular	0.59
-	SAOUHSC_00671	2.98	12066	LysM peptidoglycan-binding domain-containing protein	Extracellular	0.67
sdpC	SAOUHSC_02611	2.92	2070	CPBP family intramembrane metalloprotease SdpC	Extracellular	0.51
isdA	SAOUHSC_01081	2.91	520	LPXTG-anchored heme-scavenging protein IsdA	Extracellular	0.58
-	SAOUHSC_01084	2.82	145	hypothetical protein	Extracellular	0.57
-	SAOUHSC_02115	2.72	99	hypothetical protein	Extracellular	0.59
dacA	SAOUHSC_00646	2.67	766	D-alanyl-D-alanine carboxypeptidase	Extracellular	0.61
feoA	SAOUHSC_02865	2.63	214	ferrous iron transport protein A	Extracellular	0.71
-	SAOUHSC_00349	2.61	7736	bacteriophage L54a single-stranded DNA binding protein	Extracellular	0.56

esaA	SAOUHSC_00258	2.54	258	type VII secretion protein EsaA	Extracellular	0.56
-	SAOUHSC_02266	2.50	689	hypothetical protein	Extracellular	0.57
Gene Name	Gene Symbol	Log_2 Fold Change	RPKM SUM	Gene Description[1]	Location[2]	Adhesin probability[3]
-	SAOUHSC_02741	2.48	1566	ABC transporter permease	Extracellular	0.52
essA	SAOUHSC_00259	2.37	309	type VII secretion protein EssA	Extracellular	0.58
-	SAOUHSC_02848	2.32	695	PTS system glucose-specific transporter subunit II	Extracellular	0.55
-	SAOUHSC_00052	2.27	235	tandem-type lipoprotein	Extracellular	0.57
-	SAOUHSC_00818	2.23	1321	thermonuclease family protein	Extracellular	0.58
-	SAOUHSC_00634	2.20	19431	zinc ABC transporter substrate-binding protein	Extracellular	0.62
-	SAOUHSC_00253	2.13	382	hypothetical protein	Extracellular	0.63

[1]NCBI
[2]Psort.org/psortb/
[3]Violinet.org/vaxign

CHAPTER FOUR: DISCUSSION

The biological adaptation of *S. aureus* to culture in milk is not well studied. For these studies, *Staphylococcus aureus* bovine strain Newbould 305 was cultured in SKM to replicate the bovine udder environment. Milk contains compounds that sequester metals required for bacterial growth, such as lactoferrin, calprotectin, citrate, and casein[40]. In such a metal-limiting environment, *S. aureus* may directly import these compounds or produce chelators to compete for metal acquisition. It has also been reported that bovine *S. aureus* isolates increase biofilm formation by upregulated production of biofilm-associated proteins such as polysaccharide intercellular adhesin proteins (*icaA*, *icaB*, *icaC*, and *icaD*) following addition of lactose or milk[41]. Unique surfaceome proteins, and upregulated genes, in SKM cultured *S. aureus* are likely key to survival in a high lactose, metal limiting environment, and may be considered of particular interest for candidate *S. aureus* bovine mastitis vaccine antigens.

Biological GO term analysis of differentially expressed genes revealed that significantly enriched pathways included peptide biosynthetic process, transport, inosine monophosphate (IMP) metabolic process, and organonitrogen compound biosynthetic process. IMPDH, a *de novo* purine biosynthesis enzyme, is conserved in prokaryotes and has also been determined to be a transcription factor, regulating the cell cycle and cell profileration[42]. *De novo* purine biosynthesis was reported to be essential for intracellular proliferation of *E. coli* in bladder epithelial cells[43]. Upregulation of this pathway may similarly allow *S. aureus* to replicate intracellularly in bovine cells. In addition, the *de*

novo purine nucleotide biosynthesis pathway is a requirement for biofilm production in the model organism *Pseudomonas fluorescens*[44]. As milk is a more nutrient limiting environment in comparison to LB, upregulation of IMP metabolic processes could indicate responsive *S. aureus* biofilm formation. Cellular GO term analysis indicated activation of ABC transporter expression. ATP-binding cassette (ABC) transporters are ubiquitous, integral membrane proteins that facilitate ATP-driven transport of various substrates and one such transporter in *S. aureus* has been described to secrete cytotoxins, leading to host antimicrobial peptide resistance[45,46]. Considering milk as a metal limiting and high lactose environment, ABC transporters may be particularly important in the transport of sugars and metal ions in and out of the cell.

Widely considered the most important feature of a vaccine antigen is cell periphery-associated subcellular localization. To initially narrow down the list of candidates identified by mass spectrometry and RNA sequencing, subcellular localization was predicted with PSORTb. PSORTb 3.0 is the most recent version of the protein subcellular localization prediction tool. It has been reported to have the highest precision, recall, and accuracy for both gram-positive and gram-negative bacteria in comparison to PSORTb 2.0 and to other subcellular localization prediction tools[47]. Fifteen unique surfaceome proteins were identified in SKM cultured samples and were predicted to be associated with the cell periphery. Similarly, most significantly upregulated genes were predicted by PSORTb to be associated with the cell periphery, and this was further supported by cellular GO term analysis.

Adhesins are considered good vaccine targets to prevent bacterial infection and colonization[48]. Adhesin probability was calculated per candidate using Vaxign, a web-

based vaccine target prediction software that integrates several open source tools, including the optimized adhesin probability tool SPAAN[49,50]. Three of the unique surfaceome proteins that are likely adhesins include fibrinogen-binding protein (Fib), bifunctional autolysin (Atl), and thermonuclease (Nuc). Fib has been reported to assist *S. aureus* avoidance of the innate immune system by blocking host neutrophil phagocytic uptake[51]. Atl has been implicated in biofilm development[52]. Nuc acts to degrade host DNA and RNA, and, in conjunction with additional secreted enzymes, destroy tissues to aid in colonization[9]. Of the significantly upregulated genes determined by RNAseq, 26 were predicted to be adhesins.

Notably, the most highly upregulated gene associated with the cell periphery and with adhesin probability >0.5 was *asp1*. Asp1 is part of the accessory Sec protein system, and is essential for transport of adhesin SraP, a highly conserved surface glycoprotein, to the cell surface[53]. It has been reported to be highly conserved in *S. aureus*[54]. Of the 26 predicted adhesins, all upregulated in SKM, six were particularly highly expressed (RPKM SUM > 5000): autolysin/adhesin (Aaa), HTH-type transcriptional regulator (SarR), DEAD/DEAH box helicase, LysM peptidoglycan-binding domain-containing protein, bacteriophage L54a single-stranded DNA binding protein, and zinc ABC transporter substrate-binding protein. Aaa has been confirmed by immunofluorescence microscopy to localize to the cell surface, and has been reported to bind extracellular matrix proteins and interact with fibrinogen, fibronectin, and vitronectin[55,56]. SarR is a member of the SarA protein family and is involved in the complex regulatory network associated with virulence factor and secreted protease production. SarR induction is reported to induce protease loci *spl*, *scp*, and *ssp*[57]. DEAD/DEAH box helicase is an ATP

dependent RNA helicase, an important player in general gene expression regulation[58]. Proteins containing LysM domains may be secreted or localized to the outer membrane or cell wall and, in bacteria, such proteins are generally peptidoglycan hydrolases[59]. The particular LysM containing protein upregulated in SKM is also more specifically annotated as secretory antigen SsaA-like protein, which may have autolysin activities[60]. Closely related SsaA has been reported to be highly immunogenic and a virulence factor[61]. Current study concerning bacteriophage L54a single-stranded DNA binding protein, which was upregulated in SKM, is limited. A zinc-specific ABC transporter was notably upregulated in SKM, possibly a result of increased levels of zinc in milk.

CHAPTER FIVE: CONCLUSION AND FUTURE DIRECTIONS

A combined proteomics and transcriptomics approach was utilized to identify candidate *S. aureus* bovine mastitis vaccine antigens, which were further characterized by reverse vaccinology tools. Candidates of interest were unique to, or highly expressed and upregulated in, *S. aureus* cultured in milk, and therefore potential vaccine candidates to prevent infection of the bovine udder. Candidates were also predicted to be associated with the cell periphery and probable adhesins. The candidates of most interest include fibrinogen-binding protein, bifunctional autolysin, thermonuclease, autolysin/adhesin Aaa, secretory antigen SsaA-like protein, and zinc ABC transporter substrate-binding protein.

Additional work is required to screen these candidates further. Protein specificity, conservation, antigenicity, and immunogenicity must all be considered. To ensure specificity, candidate antigens need to be unique to *S. aureus*, and not expressed in other bacteria species. In addition, candidates will be assessed for conservation in multiple *S. aureus* strains. Antigenicity is described as the ability of a protein to be recognized by lymphocytes during early infection, whereas immunogenicity is the ability of a protein to induce a T cell and B cell response following immunization[62]. Both antigenicity and immunogenicity are required in development of a prophylactic vaccine. Antigenicity was explored by subcellular localization and by adhesin probability, but the characterization of these antigens will need to be extended in the future to include epitope analysis and the measurement of immunoreactivity of milk and serum collected from infected cows.

Immunogenicity and vaccine efficacy will need to be evaluated by incorporation of the candidates into CTA_2/B chimeras, or another vaccine platform, and assessment in animal models.

APPENDIX A

Current *Staphylococcus aureus* Bovine Mastitis Vaccine Efficacy

Introduction

A recent trial evaluated efficacy of the experimental cholera toxin-based *Staphylococcus aureus* vaccine[27]. The bovine mucosal vaccine design includes the two *S. aureus* antigens IsdA and ClfA, each fused to the CTA_2/B nontoxic portion of the cholera toxin. Trial design included three vaccinated cows, and three mock-vaccinated, control cows. Each cow was vaccinated on day 1 and day 14. Following vaccination, on day 20, all cows were subjected to intramammary challenge with 400 CFU *S. aureus* Newbould 305. Challenge period progressed from day 20 to day 30, followed by treatment day 30 through day 40. Milk and blood samples were collected from all cows on day 1, day 4, and twice daily days 20-30. Vaccine efficacy was evaluated by bacterial shedding, somatic cell count, rectal temperature, and antibody responses (*manuscript in preparation/submission*). In support of this study, anti-IsdA IgG ELISA was performed to screen animals for entry into the study, *Staphylococcus aureus* levels in milk were quantified by qPCR, antibody presence in serum was evaluated by opsonophagocytic (OSP) assay, and bovine cytokine gene expression in peripheral blood mononuclear cells (PBMCs) was assessed by RT-qPCR.

Materials and Methods

Milk anti-IsdA IgG ELISA

Milk was collected from individual cows, each provided with a unique four-digit ID number, for screening by ELISA. Wells of a 96-well plate were coated with 50 µl purified IsdA (10 µg/mL) and incubated at 4°C overnight. Plates were washed three times with 0.05% Tween-20 in 1X PBS with shaking, and then blocked with 1% skim goat milk in 1X PBS for two hours at 37°C. Washes were repeated an additional three times.

Curves were prepared using 1:2 serial dilutions of each serum sample. Following addition of serum, the plates were incubated at 4°C overnight. The plates were washed three times with 0.05% Tween-20 in 1X PBS with shaking. Following washes, the wells were coated with HRP-conjugated rabbit anti-bovine IgG antibody (1:10,000 in blocking buffer). Plates were incubated for one hour at 37°C. After incubation with the secondary antibody, plates were washed six times with 0.05% Tween-20 in 1X PBS with shaking. Tetramethylbenzidine (Promega) was added and incubated for 10 minutes at room temperature with shaking. Absorbance was measured at 370 nm with a spectrophotometer.

Staphylococcus aureus DNA Quantification in Milk

DNA was isolated from milk samples collected day 1 and days 20-30 (PM collections only). Milk collected from each individual quarter was pooled per cow prior to DNA isolation. Following pooling, milk samples (1 mL) were centrifuged at 13,000 xg for 10 minutes at 4°C. The supernatant was removed, and the pellet resuspended in 500 µl enzyme digestion buffer (500 µg lysozyme, 6 U mutanolysin, 12 U lysostaphin in 10 mM Tris-HCL, 50 mM EDTA, pH 8.0). Samples were incubated for 60 minutes at 37°C, with vortexing every 10 minutes. Samples were then transferred to tubes pre-filled with 750 mg 0.1 mm Zirconia silica beads. Samples were disrupted by bead beating for one minute using the FastPrep® bead-beater. Resulting lysate was transferred to a new tube, and DNA isolated by the EZ Blood/Cell DNA Isolation Kit (EZ Bioresearch) following manufacturer's protocol.

Quantitative PCR was performed using *Staphylococcus aureus* specific *isdA* primers and reference *16S* primers (Table 8) and according to the parameters presented in

Tables 9 and 10. *Staphyloccocus aureus* presence was determined by $2^{-(\Delta Cq_{sample} - \Delta Cq_{calibrator})}$ where ΔCq represents $Cq_{target} - Cq_{reference}$ and the calibrator is *Staphylococcus aureus* genomic DNA. Statistical analysis was performed by two-group t-test between vaccinated and unvaccinated control using JMP software (Cary, NC).

Table 8. *S. aureus* Detection in Milk qPCR Primer Sequences.

Gene	Primer Sequence (5' to 3')	Amplicon Size (bp)
isdA	**FW** CGGTTCAACCAAAACCTGCT	380
	RV GCGAAGGCAACTGTGCTAAT	
16s rRNA	**FW** AGAGTTTGATCCTGGCTCAG	530
	RV ATTACCGCGGCTGCTGGC	

Table 9. qPCR and RT-qPCR Reaction Conditions.

Reagent	Final Concentration	Volume
2X Maxima SYBR Green (Thermo)	1X	10 µl
Forward Primer 10 µM	0.3 µM	0.6 µl
Reverse Primer 10 µM	0.3 µM	0.6 µl
Template (gDNA or cDNA)	varies	2 µl
Nuclease-free water		6.8 µl

Table 10. qPCR and RT-qPCR Cycling Conditions.

Temperature (°C)	Time (s)	Cycles
95 °C	600 s	1
95 °C	15 s	40
58 °C	30 s	
72 °C	30 s	

Opsonophagocytic Assay

OSP assay was performed using serum collected from vaccinated and unvaccinated cows on days -2, 1, 14, 20, and 30. Protein concentration was measured in serum samples using the Pierce™ BCA Protein Assay kit (Thermo Scientific). Samples

were subsequently diluted to 1 µg/µl in sterile 1X PBS. Serum samples were heat inactivated by incubation at 56°C for 30 minutes, and then stored at -20°C prior to use.

PBMCs were isolated from commercial cow's blood (Hardy Diagnostics). Blood was equilibrated at room temperature, and then diluted 1:2 with sterile 1X PBS. PBMCs were separated from the diluted blood samples by layering onto Histopaque®-1077 (Sigma-Aldrich) and subsequent centrifugation for 30 minutes at 800 xg at room temperature (9,1 acceleration, deceleration). Following separation, PBMCs were carefully removed from the solution and washed 3x by gentle resuspension in Hank's buffered salt solution (HBSS) and centrifugation at 800 xg for 10 minutes (9,9 acceleration and deceleration). PBMCs were then resuspended in 500 µl 1X HBSS and cell counts measured with a hemocytometer. Cells were diluted to $2x10^6$ cells/mL in RPM1 1640 medium containing HEPES and 10% FBS. PBMCs (100 µl) were plated onto Nunc™ MicroWell™ 96-well plates (Thermo Scientific). Cells were incubated at 37°C, 5% CO_2 overnight.

S. aureus strain Newbould 305 was cultured by inoculating 4 mL low iron media (LIM) with a single colony cultured on MSA plates and incubating overnight at 37°C and 200 RPM. Immediately before use, *S. aureus* concentration was measured by optical density measurements, and diluted to $4x10^6$ cells/mL in RPM1 1640 medium containing HEPES and 10% FBS. Cow serum (50 µl) and *S. aureus* (50 µl) was added directly to the PBMCs. Controls included PBMCs (100 µl), *S. aureus* (50 µl), and 1X PBS (50 µl) only. Plates were incubated for 90 minutes at 37°C. Following incubation, reactions were diluted 1:1,000 and 100 µl plated onto MSA plates. MSA plates were incubated at 37°C overnight and resulting *S. aureus* colonies counted. Statistical analysis was performed by

two-group t-test between vaccinated and unvaccinated control using JMP software (Cary, NC).

Bovine PBMC Cytokine Gene Expression

Whole blood was collected from cows on Day 20 prior to intramammary challenge and shipped overnight. PBMCs were isolated from whole blood samples immediately upon arrival using Histopaque®-1077 (Sigma-Aldrich) as previously described. Total RNA was extracted from PBMCs using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was confirmed by agarose gel electrophoresis. Genomic DNA was removed by treatment with TURBO™ DNase (Invitrogen) and cDNA was made with the RevertAid First Strand cDNA Synthesis kit (Thermo). cDNA was diluted 1:2 with nuclease-free water prior to qPCR. Primers specific to bovine cytokines of interest were selected from the literature (Table 11). Bovine GAPDH served as the reference gene. RT-qPCR reaction and cycling conditions are presented in Table 9 and Table 10. Relative gene expression was calculated by 2^-ΔCq, where ΔCq represents $Cq_{target} - Cq_{reference}$. Statistical analysis was performed by two-group t-test between vaccinated and unvaccinated control using JMP software (Cary, NC).

Table 11. Bovine Cytokine RT-qPCR Primer Sequences.
Primers specific to bovine cytokine genes, and to reference gene GAPDH, were selected from the literature.

Gene	Primer Sequence (5' to 3')	Amplicon Size (bp)	Source
gapdh	**FW** GCATCGTGGAGGGACTTATGA	67	[63]
	RV GGGCCATCCACAGTCTTCTG		
IL-10	**FW** CTTGTCGGAAATGATCCAGTTTT	66	[64]
	RV TCAGGCCCGTGGTTCTCA		
IFN-γ	**FW** CAGAAAGCGGAAGAGAAGTCAGA	72	[63]
	RV TGCAGGCAGGAGGACCAT		
IL-6	**FW** GGCTCCCATGATTGTGGTAGTT	64	[64]
	RV GCCCAGTGGACAGGTTTCTG		

Results

Milk anti-IsdA IgG ELISA

Cows were initially screened for evidence of *S. aureus* infection prior to study. ELISA was used to measure levels of anti-IsdA IgG in milk, indicating infection. Three cows, ID numbers 2771, 2776, and 2781, had significantly elevated anti-IsdA IgG titers, and were eliminated from the study (Figure 11). Remaining animals did not appear to have previous infections and moved forward to clinical trialing.

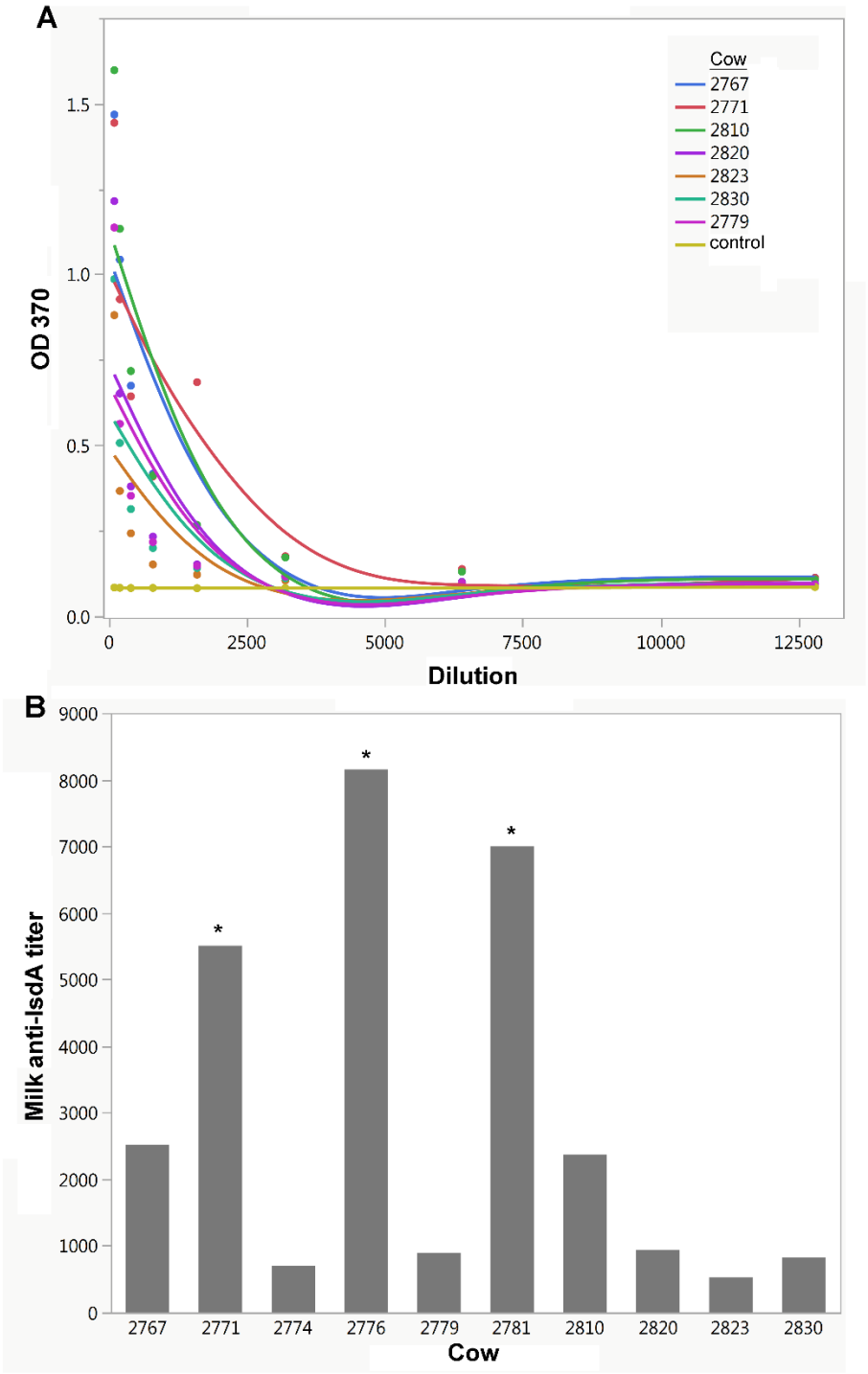

Figure 11. Milk Anti-IsdA IgG ELISA Screening.

Cows were screened to eliminate animals with previous S. aureus infections. Absorbance values (370 nm) per dilution per cow are presented in (A), and anti-IsdA IgG titers are presented in (B). Cows 2771, 2776, and 2781 had significantly elevated titers, and were removed from the study.

Staphylococcus aureus DNA Quantification

The presence of *S. aureus* in milk collected from vaccinated and unvaccinated cows on day 1 and days 20-30 was evaluated by qPCR. Primers specific to *isdA* detect *S. aureus*, whereas primers specific to *16S* detect all bacteria species. A *S. aureus* genomic DNA sample was included as a calibrator to determine the ratio of *S. aureus* to other bacteria present in milk. No significant difference was measured between unvaccinated cows and vaccinated cows at all days analyzed. While not significant, *S. aureus* presence was reduced in vaccinated cows at Day 30 (p=0.15) which is consistent with observed reductions in colony counts toward the end of the challenge period (Figure 12).

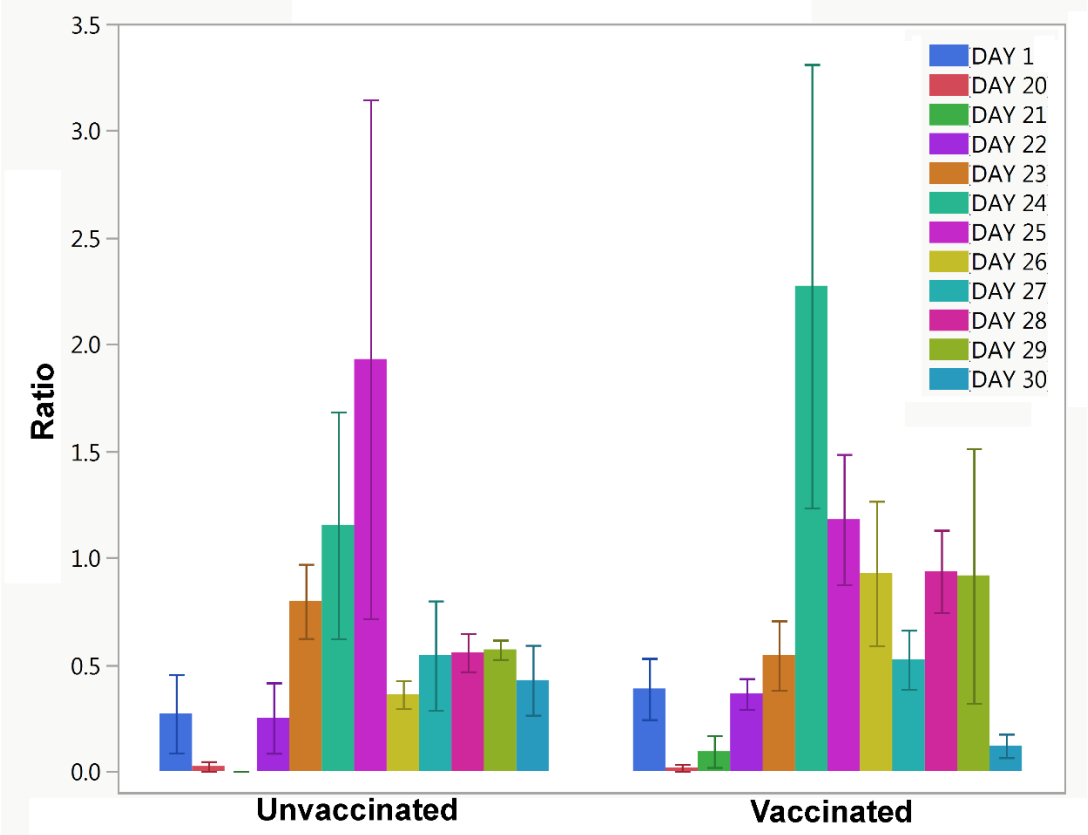

Figure 12. S. aureus Quantification in Vaccinated and Unvaccinated Cow Milk. Values are presented as 2^-(ΔCqsample- ΔCqcalibrator) where ΔCq represents Cqtarget – Cqreference and the calibrator is S. aureus genomic DNA. Although not significant, presence of S. aureus was reduced in vaccinated versus unvaccinated cows on Day 30 (p=0.15).

Opsonophagocytic Assay

To evaluate immunogenic responses in vaccinated versus unvaccinated cows, OSP assays were performed on serum collected on Days -2, 1, 14, 20, and 30. Serum that contain antibodies specific to *S. aureus* are expected to coat the bacteria and allow naïve bovine PBMC cells to recognize and phagocytize them. Serum from vaccinated cows is expected to contain specific antibodies and result in fewer *S. aureus* colony forming units (CFU) compared to serum from unvaccinated cows following OSP assay. Percent killing, or the reduction in CFU compared to no-serum control, is presented in Figure 13. CFU was slightly reduced, and percent killing increased, in the vaccinated group versus the unvaccinated group on Day 30, consistent with differences observed in *S. aureus* shedding from milk on this day. However, no significant differences were detected.

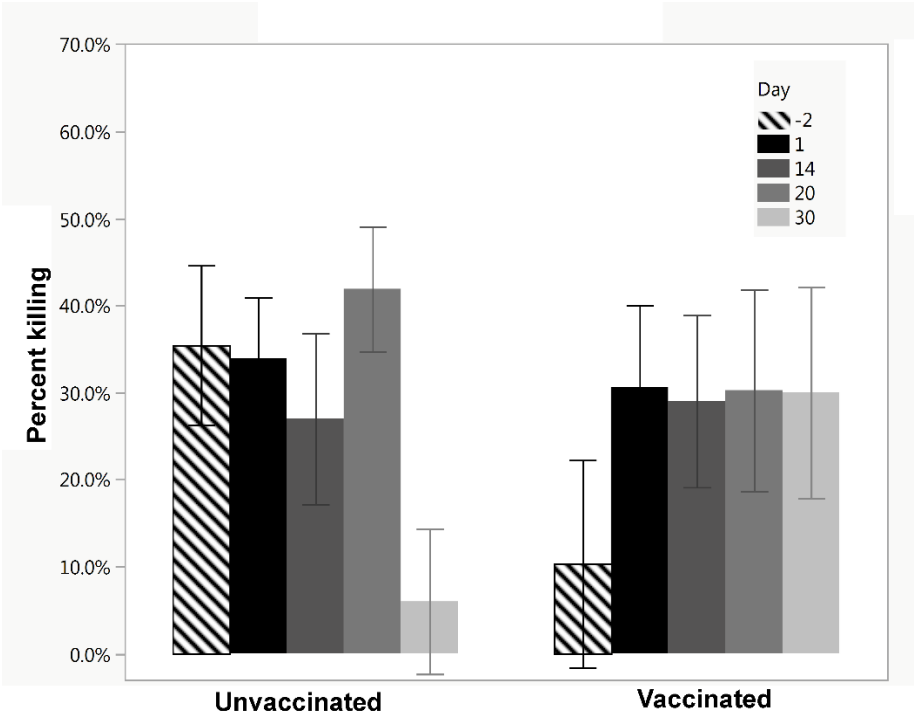

Figure 13. Percent Killing Following Opsonophagocytic Assay using Serum from Vaccinated and Unvaccinated Cows.

Bovine PBMC Cytokine Gene Expression

To assess induction of the immune system following vaccination, bovine PBMC cytokine gene expression was measured. IL-10, IL-6, and IFN-γ gene expression was measured in PBMCs collected from vaccinated and unvaccinated cows, post vaccination but prior to *S. aureus* intramammary challenge at Day 20 of the trial. No significant difference was observed between vaccinated and unvaccinated cows for IL-10 and IL-6 cytokine gene expression. In contrast, IFN-γ expression was significantly ($p<0.05$) elevated in vaccinated versus unvaccinated cows (Figure 14). IFN-γ upregulation may indicate macrophage activation and increased antigen presentation, whereas no, or limited, changes in IL-10 (anti-inflammatory) and IL-6 (pro-inflammatory) suggest no impact on the inflammatory balance.

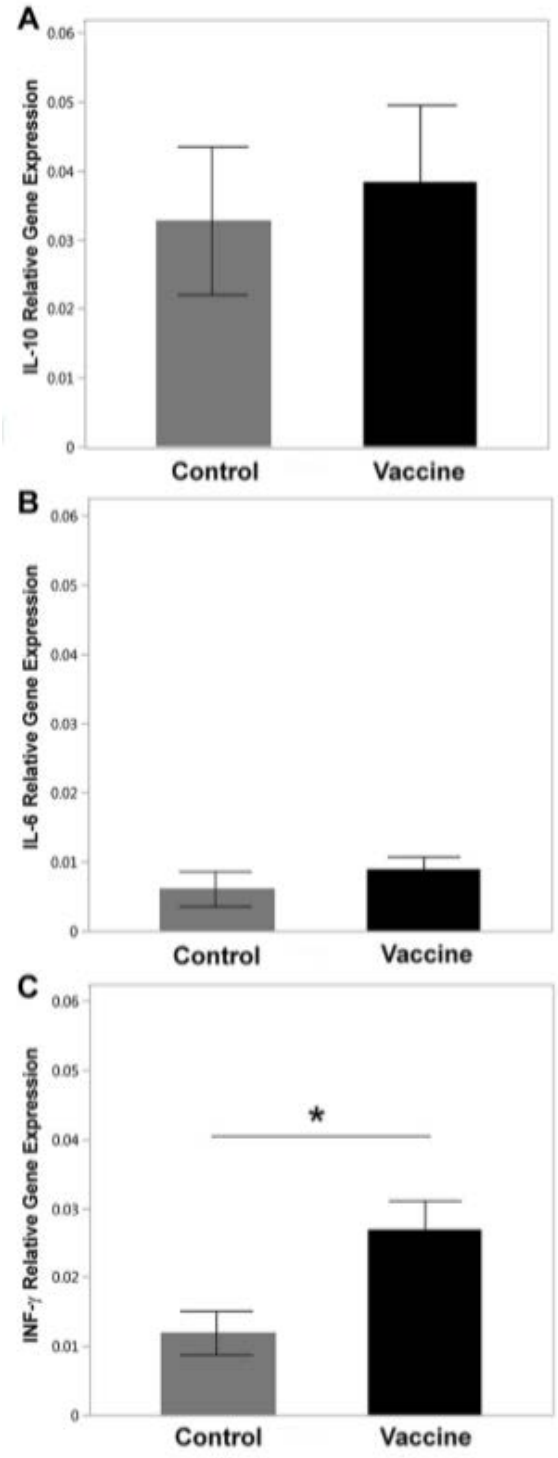

Figure 14. Peripheral Blood Mononuclear Cell Cytokine Gene Expression in Vaccinated versus Unvaccinated Cows[27].

Cows were vaccinated at Day 1 and Day 14, and whole blood was collected for PBMC isolation and RT-qPCR at Day 20. (A) IL-10, (B) IL-6, and (C) IFN-γ gene expression is presented relative to bovine GAPDH (2^-ΔCq) in vaccinated and control cows (n=3 per group; * denotes $p<0.05$).

Discussion and Conclusion

The experimental vaccine, IsdA+ClfA-CTA2/B, was determined to induce specific immune responses and reduce the somatic cell counts (SCC) of infected cows, however, despite observed reductions in CFU by day 30, it did not prevent colonization of *S. aureus* following intramammary challenge. Quantitative PCR measurements of *S. aureus* specific *isdA* in DNA isolated from milk was variable and not found to be consistent with CFU analysis until potentially Day 30. DNA may be present much longer than live cells and may not ultimately be a reliable predictor of infection, especially for low bacterial counts. OSP assay results were also variable, but consistent on Day 30 with observed *S. aureus* specific antibody presence in serum collected from vaccinated animals following vaccination and challenge. These studies will need to be repeated using IgG purified from milk for confirmation. Gene expression analysis by RT-qPCR indicated no difference in IL-10 and IL-6 expression between vaccinated and unvaccinated cows, while a significant increase in IFN-γ gene expression was observed in vaccinated versus unvaccinated cows and is indicative of immune system induction. Together, these results support the conclusion that the current vaccine design induces immune responses and is protective, but not preventative, and will require additional antigens to increase efficacy.

APPENDIX B

Conservation of Two Candidate *Staphylococcus aureus* Antigens Expressed in Bovine Milk During Mastitis

Introduction

A previous study identified potential candidate *Staphylococcus aureus* antigens by a novel immunoproteomics method[65]. *S. aureus* was cultured in low iron media to induce the expression of important cell-periphery associated proteins. Proteins were subsequently isolated and fragmented by trypsin digestion. Resulting protein extracts were separated by two-dimensional gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed using mastitic bovine milk. Any positive spots were picked from the gel and prepared for and analyzed by mass spectrometry. The top two proteins of interest selected for further characterization, due to factors including high coverage, association with cell periphery, and adhesin probability, were *esxA* and *isdC*. As part of this study, these genes were evaluated for conservation across multiple *bovine S. aureus* strains.

Materials and Methods

Bovine *S. aureus* isolates were provided by Udder Health Systems Inc. (Meridian, ID), and Larry Fox (Washington State University, Pullman, WA). Bovine isolates were cultured in LB media overnight at 37°C with shaking prior to genomic DNA isolation. Genes *esxA* and *isdC* were PCR amplified from the eight provided *S. aureus* strains (LF1, 2, 4, 5, 7, 8, 9, and UH9) with the primers described in Table 12 and reaction conditions described in Table 13 and Table 14. PCR products were visualized by agarose gel electrophoresis, PCR purified with the GeneJET PCR Purification kit (Thermo) and submitted for sequencing (Idaho State Molecular Research Core, Pocatello, ID). Resulting sequences were translated into amino acid sequences, and aligned using Clone Manager (Sci-Ed, Denver, CO).

Table 12. Primers Specific to Candidate *S. aureus* Antigen Genes *esxA* and *isdC*.

Gene	Primer Sequence (5' to 3')	Amplicon Size (bp)
esxA	FR GGCAATGATTAAGATGAGTCC	287
	RV GCAAACCGAAATTATTAG	
isdC	FR GAGTATCGAAGGACATAAAG	384
	RV GCTAAGGATGCAACTGG	

Table 13. PCR Reaction Conditions.

Reagent	Final Concentration	Volume
2X GoTaq® Master Mix (Promega)	1X	12.5 µl
Forward Primer 10 µM	0.2 µM	0.5 µl
Forward Primer 10 µM	0.2 µM	0.5 µl
DNA template	50 ng	1 µl
Nuclease-free water		10.5 µl

Table 14. PCR Cycling Conditions.

Temperature (°C)	Time (s)	Cycles
95 °C	120 s	1
94 °C	60 s	30
50 °C	60 s	
72 °C	60 s	

Results

Gene presence was confirmed in all strains, excluding LF8 which was determined *isdC* positive and *esxA* negative. Amino acid alignments determined high conservation of both genes across strains (Figure 15). The region of *isdC* selected for PCR amplification

included the NEAT domain and sortase. Each strain demonstrated between 97% and 100% identity to the reference *S. aureus* strain Newbould 305 IsdC protein sequence. The region of *esxA* selected for PCR amplification included WXG and sortase. While strain LF8 did not contain the *esxA* gene, the remaining strains demonstrated 100% protein identity with the reference EsxA sequence.

Discussion and Conclusion

Proteins IsdC and EsxA were determined immunogenic by presence of specific antibodies in mastitic cow milk and selected as priority vaccine candidates due to high percent coverage by mass spectrometry, subcellular localization to the cell periphery, and adhesin probability > 0.4. Here, the conserved regions of IsdC and EsxA were successfully amplified and sequenced from eight bovine *S. aureus* isolates. High sequence similarity of IsdC and EsxA found amongst *S. aureus* strains support these two candidates for further study.

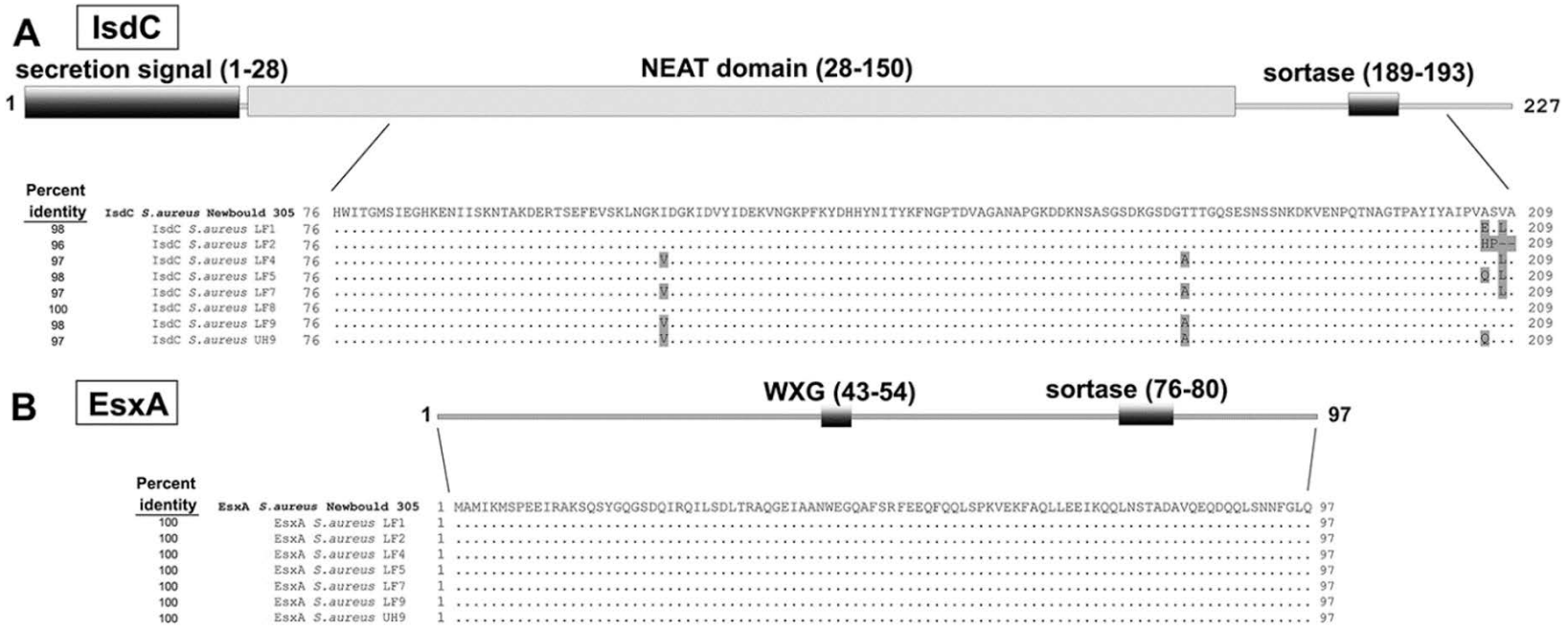

Figure 15. Amino Acid Alignments of Conserved Protein Domains of IsdC and EsxA[65].

Conserved regions of candidate antigens IsdC and EsxA were PCR amplified and sequenced from genomic DNA of eight bovine S. aureus isolates. Nucleotide sequences were translated and aligned to confirm conservation between strains. S. aureus isolate LF8 did not contain esxA.

REFERENCES

1. Wilson, D. J., Gonzalez, R. N. & Das, H. H. Bovine Mastitis Pathogens in New York and Pennsylvania: Prevalence and Effects on Somatic Cell Count and Milk Production. *J. Dairy Sci.* **80**, 2592–2598 (1997).

2. Petrovski, K. R., Trajcev, M. & Buneski, G. A review of the factors affecting the costs of bovine mastitis. *J. S. Afr. Vet. Assoc.* **77**, 52–60 (2006).

3. Bradley, A. J. Bovine mastitis: An evolving disease. *Vet. J.* **164**, 116–128 (2002).

4. Bi, Y. *et al.* Prevalence of bovine mastitis pathogens in bulk tank milk in China. *PLoS One* **11**, 1–13 (2016).

5. Hegde, R. *et al.* Incidence of Subclinical Mastitis and Prevalence of Major Mastitis Pathogens in Organized Farms and Unorganized Sectors. *Indian J. Microbiol.* **53**, 315–320 (2013).

6. Sumathi, B. R., Veeregowda, B. M. & Gomes, A. R. Prevalence and antibiogram profile of bacterial Isolates from clinical bovine mastitis. *Vet. World* **1**, 237–238 (2008).

7. Castro Pérez, V. K. *et al.* Relationship between virulence factors and antimicrobial resistance in Staphylococcus aureus from bovine mastitis. *J. Glob. Antimicrob. Resist.* **22**, 792–802 (2020).

8. Artursson, K., Söderlund, R., Liu, L., Monecke, S. & Schelin, J. Genotyping of Staphylococcus aureus in bovine mastitis and correlation to phenotypic characteristics. *Vet. Microbiol.* **193**, 156–161 (2016).

9. Foster, T. J. Immune evasion by staphylococci. *Nat. Rev. Microbiol.* **3**, 948–958 (2005).

10. Hébert, A., Sayasith, K., Sénéchal, S., Dubreuil, P. & Lagacé, J. Demonstration of intracellular Staphylococcus aureus in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. *FEMS Microbiol. Lett.* **193**, 57–62 (2000).

11. Almeida, P. A., Matthews, K. R., Cifrian, E., Guidry, A. J. & Oliver, S. P. Staphylococcus aureus Invasion of Bovine Mammary Epithelial Cells. *J. Dairy Sci.* **79**, 1021–1026 (1996).

12. Stranger-Jones, Y. K., Bae, T. & Schneewind, O. Vaccine assembly from surface proteins of Staphylococcus aureus. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 16942–16947 (2006).

13. Giersing, B. K., Dastgheyb, S. S., Modjarrad, K. & Moorthy, V. Status of vaccine research and development of vaccines for Staphylococcus aureus. *Vaccine* **34**, 2962–2966 (2016).

14. Frenck, R. W. *et al.* Safety, tolerability, and immunogenicity of a 4-antigen Staphylococcus aureus vaccine (SA4Ag): Results from a first-in-human randomised, placebo-controlled phase 1/2 study. *Vaccine* **35**, 375–384 (2017).

15. Levy, J. *et al.* Safety and immunogenicity of an investigational 4-component Staphylococcus aureus vaccine with or without AS03B adjuvant: Results of a randomized phase I trial. *Hum. Vaccines Immunother.* **11**, 620–631 (2015).

16. Ansari, S., Jha, R. K., Mishra, S. K., Tiwari, B. R. & Asaad, A. M. Recent advances in staphylococcus aureus infection: Focus on vaccine development. *Infect. Drug Resist.* **12**, 1243–1255 (2019).

17. Wang, X., Thompson, C. D., Weidenmaier, C. & Lee, J. C. Release of Staphylococcus aureus extracellular vesicles and their application as a vaccine platform. *Nat. Commun.* **9**, (2018).

18. Jiang, L., Schinkel, M., van Essen, M. & Schiffelers, R. M. Bacterial membrane vesicles as promising vaccine candidates. *Eur. J. Pharm. Biopharm.* **145**, 1–6 (2019).

19. Tiwari, J. G. *et al.* Trends in therapeutic and prevention strategies for management of Bovine Mastitis: An overview. *J. Vaccines Vaccin.* **4**, 1–11 (2013).

20. Ruegg, P. L. A 100-Year Review: Mastitis detection, management, and prevention. *J. Dairy Sci.* **100**, 10381–10397 (2017).

21. Owens, W. E., Ray, C. H., Watts, J. L. & Yancey, R. J. Comparison of Success of Antibiotic Therapy during Lactation and Results of Antimicrobial Susceptibility Tests for Bovine Mastitis. *J. Dairy Sci.* **80**, 313–317 (1997).

22. Turutoglu, H., Ercelik, S. & Ozturk, D. Antibiotic resistance of Staphylococcus aureus and coagulase-negative staphylococci isolated from bovine mastitis. *Bull. Vet. Inst. Pulawy* **50**, 41–45 (2006).

23. Merrill, C. *et al.* Immunological responses and evaluation of the protection in dairy cows vaccinated with staphylococcal surface proteins. *Vet. Immunol. Immunopathol.* **214**, (2019).

24. Piepers, S. *et al.* Immune response after an experimental intramammary challenge with killed Staphylococcus aureus in cows and heifers vaccinated and not vaccinated with Startvac, a polyvalent mastitis vaccine. *J. Dairy Sci.* **100**, 769–782 (2017).

25. Schukken, Y. H. *et al.* Efficacy of vaccination on Staphylococcus aureus and coagulase-negative staphylococci intramammary infection dynamics in 2 dairy herds. *J. Dairy Sci.* **97**, 5250–5264 (2014).

26. Misra, N. *et al.* Immunogenicity of a Staphylococcus aureus-cholera toxin A2/B vaccine for bovine mastitis. *Vaccine* **36**, 3513–3521 (2018).

27. Hussain Alabdullah, Danielle Scarbrough, Elise Overgaard, Janet Williams, Omid Mohammad Mousa, Gary Dunn, Laura Bond, Mark McGuire, J. T. Evaluation of the efficacy of a cholera toxin-based Staphylococcus aureus vaccine against bovine intramammary challenge. *Not Publ.* 3–29 (2020).

28. Klein, R. C., Fabres-Klein, M. H., Brito, M. A. V. P., Fietto, L. G. & Ribon, A. de O. B. Staphylococcus aureus of bovine origin: Genetic diversity, prevalence and the expression of adhesin-encoding genes. *Vet. Microbiol.* **160**, 183–188 (2012).

29. Monistero, V. *et al.* Staphylococcus aureus isolates from bovine mastitis in eight countries: Genotypes, detection of genes encoding different toxins and other virulence genes. *Toxins (Basel).* **10**, (2018).

30. Dalsass, M., Brozzi, A., Medini, D. & Rappuoli, R. Comparison of open-source reverse vaccinology programs for bacterial vaccine antigen discovery. *Front. Immunol.* **10**, 1–12 (2019).

31. Tjalsma, H., Schaeps, R. M. J. & Swinkels, D. W. Immunoproteomics: From biomarker discovery to diagnostic applications. *Proteomics - Clin. Appl.* **2**, 167–180 (2008).

32. Atchou, K. *et al.* Comparative Transcriptomics of the Bovine Apicomplexan Parasite Theileria parva Developmental Stages Reveals Massive Gene Expression Variation and Potential Vaccine Antigens. *Front. Vet. Sci.* **7**, 1–16 (2020).

33. Kurz, S. *et al.* Transcriptome-based antigen identification for Neisseria meningitidis. *Vaccine* **21**, 768–775 (2003).

34. Boekel, M. A. J. S. Van. Effect of heating on Maillard reactions in milk. *Food Chem.* **62**, 403–414 (1998).

35. Gliguem, H. & Birlouez-Aragon, I. Effects of sterilization, packaging, and storage on vitamin C degradation, protein denaturation, and glycation in fortified milks. *J. Dairy Sci.* **88**, 891–899 (2005).

36. Rodriguez-Ortega, M. J. 'Shaving' live bacterial cells with proteases for proteomic analysis of surface proteins. **1**, 22–23 (2018).

37. Qin, N. *et al.* RNA-Seq-based transcriptome analysis of methicillin-resistant staphylococcus aureus biofilm inhibition by ursolic acid and resveratrol. *Sci. Rep.* **4**, 1–9 (2014).

38. Ewing, B. & Green, P. Base-calling of automated sequencer traces using phred. I. Error probabilities. *Genome Res.* **8**, 186–194 (1998).

39. Ewing, B. & Green, P. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.* **8**, 186–194 (1998).

40. Carlson, S. K., Erickson, D. L. & Wilson, E. Staphylococcus aureus metal acquisition in the mastitic mammary gland. *Microb. Pathog.* **144**, 104179 (2020).

41. Xue, T., Chen, X. & Shang, F. Short communication: Effects of lactose and milk on the expression of biofilm-associated genes in Staphylococcus aureus strains isolated from a dairy cow with mastitis. *J. Dairy Sci.* **97**, 6129–6134 (2014).

42. Kozhevnikova, E. N. *et al.* Metabolic Enzyme IMPDH Is Also a Transcription Factor Regulated by Cellular State. *Mol. Cell* **47**, 133–139 (2012).

43. Shaffer, C. *et al.* Purine Biosynthesis Metabolically Constrains Intracellular Survival of Uropathogenic Escherichia coli. *Infect. Immun.* 1–14 (2017).

44. Yoshioka, S. & Newell, P. D. Disruption of de novo purine biosynthesis in Pseudomonas fluorescens Pf0-1 leads to reduced biofilm formation and a reduction in cell size of surface-attached but not planktonic cells. *PeerJ* **2016**, 1–24 (2016).

45. Hollenstein, K., Dawson, R. J. & Locher, K. P. Structure and mechanism of ABC transporter proteins. *Curr. Opin. Struct. Biol.* **17**, 412–418 (2007).

46. Cheung, G. Y. C. *et al.* Antimicrobial Peptide Resistance Mechanism Contributes to Staphylococcus aureus Infection. *J. Infect. Dis.* **217**, 1153–1159 (2018).

47. Yu, N. Y. *et al.* PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* **26**, 1608–1615 (2010).

48. Wizemann, T. M., Adamou, J. E. & Langermann, S. Adhesins as targets for vaccine development. *Emerg. Infect. Dis.* **5**, 395–403 (1999).

49. He, Y., Xiang, Z. & Mobley, H. L. T. Vaxign: The first web-based vaccine design program for reverse vaccinology and applications for vaccine development. *J. Biomed. Biotechnol.* **2010**, (2010).

50. Sachdeva, G., Kumar, K., Jain, P. & Ramachandran, S. SPAAN: A software program for prediction of adhesins and adhesin-like proteins using neural networks. *Bioinformatics* **21**, 483–491 (2005).

51. Kuipers, A. *et al.* The Staphylococcus aureus polysaccharide capsule and Efb-

dependent fibrinogen shield act in concert to protect against phagocytosis. *Microbiol. (United Kingdom)* **162**, 1185–1194 (2016).

52. Bose, J. L., Lehman, M. K., Fey, P. D. & Bayles, K. W. Contribution of the Staphylococcus aureus Atl AM and GL Murein hydrolase activities in cell division, autolysis, and biofilm formation. *PLoS One* **7**, (2012).

53. Siboo, I. R., Chaffin, D. O., Rubens, C. E. & Sullam, P. M. Characterization of the accessory sec system of Staphylococcus aureus. *J. Bacteriol.* **190**, 6188–6196 (2008).

54. Siboo, I. R., Chambers, H. F. & Sullam, P. M. Role of SraP, a Serine-Rich Surface Protein of. *Society* **73**, 2273–2280 (2005).

55. Heilmann, C., Hartleib, J., Hussain, M. S. & Peters, G. The multifunctional Staphylococcus aureus autolysin Aaa mediates adherence to immobilized fibrinogen and fibronectin. *Infect. Immun.* **73**, 4793–4802 (2005).

56. Hirschhausen, N., Schlesier, T., Peters, G. & Heilmann, C. Characterization of the modular design of the autolysin/adhesin aaa from Staphylococcus aureus. *PLoS One* **7**, (2012).

57. Gimza, B. D., Larias, M. I., Budny, B. G. & Shaw, L. N. Mapping the Global Network of Extracellular Protease Regulation in Staphylococcus aureus . *mSphere* **4**, 1–17 (2019).

58. Iost, I., Bizebard, T. & Dreyfus, M. Functions of DEAD-box proteins in bacteria: Current knowledge and pending questions. *Biochim. Biophys. Acta - Gene Regul. Mech.* **1829**, 866–877 (2013).

59. Buist, G., Steen, A., Kok, J. & Kuipers, O. P. LysM, a widely distributed protein motif for binding to (peptido)glycans. *Mol. Microbiol.* **68**, 838–847 (2008).

60. Osipovitch, D. C., Therrien, S. & Griswold, K. E. Discovery of novel S. aureus autolysins and molecular engineering to enhance bacteriolytic activity. *Appl. Microbiol. Biotechnol.* **99**, 6315–6326 (2015).

61. JebaMercy, G., Prithika, U., Lavanya, N., Sekar, C. & Balamurugan, K. Changes in Caenorhabditis elegans immunity and Staphylococcal virulence factors during their interactions. *Gene* **558**, 159–172 (2015).

62. Van Regenmortel, M. H. V. Antigenicity and immunogenicity of synthetic peptides. *Biologicals* **29**, 209–213 (2001).

63. Gonzales, V. K. *et al.* Platelet-rich plasma can replace fetal bovine serum in human meniscus cell cultures. *Tissue Eng. - Part C Methods* **19**, 892–899 (2013).

64. Coussens, P. M., Verman, N., Coussens, M. A., Elftman, M. D. & Mcnulty, A. M. Cytokine Gene Expression in Peripheral Blood Mononuclear Cells and Tissues of Cattle Infected with. *Microbiology* **72**, 1409–1422 (2004).

65. Misra, N., Pu, X., Holt, D. N., McGuire, M. A. & Tinker, J. K. Immunoproteomics to identify Staphylococcus aureus antigens expressed in bovine milk during mastitis. *J. Dairy Sci.* **101**, 6296–6309 (2018).