5-2016

A Novel and Rapid Staphylococcus aureus Bacterial Identification Method Utilizing ImmunoMagnetic Beads and Single Cell Laser-Light Scattering

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IDENTIFICATION METHOD UTILIZING IMMUNOMAGNETIC BEADS AND
SINGLE CELL LASER-LIGHT SCATTERING

By

Kaylagh Hollen

THESIS

Submitted to
Northern Michigan University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE

Office of Graduate Education and Research

May 2016

SIGNATURE APPROVAL FORM

A NOVEL AND RAPID *STAPHYLOCOCCUS AUREUS* BACTERIAL
IDENTIFICATION METHOD UTILIZING IMMUNOMAGNETIC BEADS AND
SINGLE CELL LASER-LIGHT SCATTERING

This thesis by <u>Kaylagh Hollen</u> is recommended for approval by the student's Thesis
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ABSTRACT

A NOVEL AND RAPID *STAPHYLOCOCCUS AUREUS* BACTERIAL

IDENTIFICATION METHOD UTILIZING IMMUNOMAGNETIC BEADS AND

SINGLE CELL LASER-LIGHT SCATTERING

By

Kaylagh Hollen

Staphylococcus aureus (Gram-positive cocci) is the most commonly isolated
human associated bacterial pathogen. It plays an important role in skin and soft-tissue
infections, pneumonia, endocarditis, osteomyelitis, foreign-body infections, and sepsis. *S.
aureus* diagnosis and treatment includes identification, susceptibility testing, screening
for methicillin resistance, and glycopeptide resistance, which requires a minimum of 24-
48 hours depending upon the method. With this in mind, previous studies suggest that
faster pathogen identification has been linked to improved patient outcomes. Improved
patient outcomes including a reduction in hospitalization time, decreased risk of
nosocomial infections, and decreased in medical costs. The impact of faster identification
on patient outcome has led us to develop an alternative method of *S. aureus* identification
via ImmunoMagnetic Separation and laser-light scattering identification technology.
With this method, we hypothesized that anti-Protein A conjugated to magnetic
DynaBeads (also referred to as, anti-Protein A DynaBeads) could bind to surface Protein
A on *S. aureus* from swab sample and facilitate their isolation upon exposure to a
magnetic field within a 4-8 hour procedure. *S. aureus* cells isolated by IMS would then
be accurately identified using laser-light scattering technology in less than 5 minutes.

Prior to the development of these methods, MIT identification accuracy analysis was conducted and displayed that both laboratory and clinical *Staphylococcus* species strains identified at a rate greater than 95% and negative control strains identified at a rate less than 1%. After confirming MIT accuracy, we developed IMS capture methods in order to bypass the lengthy step of growing bacteria on agar plates. We then evaluated these methods for specificity and capture efficiency for *S. aureus.* Our *S. aureus* IMS methods displayed statistically significant (P < 0.001) specificity for *S. aureus* and capture efficiency greater than 80%. After IMS capture, an enrichment step was developed prior to laser-light identification, in order to obtain the necessary number of bacteria cells within a sample for proper laser-light scattering identification (1,000 bacteria cells per milliliter). Optimal conditions for IMS capture, enrichment, and laser-light identification methods were established and utilized to isolate and identify *S. aureus* from both pure and mixed cultures in 4-8 hours. With these methods we were able to successfully capture and identify *S. aureus* in less than 8 hours. In typical wound infections, specimens are collected from these types of infection sites on sterile swabs. Furthermore, we utilized our methods on swab collected specimens, where we demonstrated that we could successfully capture and identify *S. aureus* in less than 8 hours. The combination of IMS and laser-light identification gives a rapid and accurate identification in less than 8 hours, which is significantly less than traditional culture based identification methods.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS OR ABBREVIATIONS

AOAC RI PTM – Association of Analytical Communities Research Institute Performance Tested Methods
BHI – brain heart infusion
BSA – bovine serum albumin
CA-MRSA – community-acquired Methicillin-resistant *Staphylococcus aureus*
CBPs – collagen binding proteins
CDC – Centers for Disease Control and Prevention
CE – capture efficiency
CFU/ml – colony forming units per milliliter
CWA – cell wall-anchored
DNase – deoxyribonuclease
FnbpA and FnbpB – fibronectin-binding proteins
HA-MRSA – hospital-acquired Methicillin-resistant *Staphylococcus aureus*
IMS – ImmunoMagnetic Separation
LB – lysogeny broth
MIT – Micro Identification Technologies
MRSA – Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs – microbial surface components recognizing adhesive matrix molecules
MSSA – Methicillin-sensitive *Staphylococcus aureus*
NEAT – near iron transporter
PBP2a – Penicillin Binding Protein 2a
PBS – phosphate buffered saline
PSMs – phenol-soluble modulins
PVL – Panton-Valentine leucocidin
SEB – *Staphylococcal* enterotoxin B
SCC – *Staphylococcal* cassette chromosome
SEC – *Staphylococcal* enterotoxin C
SFP – *Staphylococcal* food poisoning
SSSS – *Staphylococcal* scalded skin syndrome
TSB – tryptic soy broth
TSS – toxic shock syndrome
TSST – toxic shock syndrome toxin

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Staphylococcus aureus

Staphylococcus aureus is the most commonly isolated human associated bacterial

pathogen. It plays an important role in skin and soft-tissue infections, pneumonia,

endocarditis, osteomyelitis, foreign-body infections, and sepsis [21,69]. *S. aureus* is

characterized as a large Gram-positive coccus (1 µm in diameter) that grows in grape-like

clusters [21]. Its colonies are pigmented yellow or golden but some colonies may also be

white. Unlike many other species of the *Staphylococcus* genus, *S. aureus* secretes an

enzyme, coagulase, which clots plasma. *S. aureus* is best distinguished from other

Staphylococcus species by the presence of coagulase and mannitol fermentation. *S.

aureus* is one of the most resilient of the non-spore forming bacteria and

characteristically survives on dry, inanimate objects for up to 7 days to 7 months [47,51].

Humans are the main reservoir for *S. aureus*. Approximately 30% of healthy

individuals are colonized by *S. aureus* and up to 90% of people are colonized during

one's lifetime. It is believed that 20% are persistent carriers, 60% are intermittent

carriers, and 20% are non-carriers who rarely harbor the bacteria [32]. *S. aureus* commonly

colonizes in the nares of humans; however, it can also be found on the skin, oropharynx,

vagina, and in feces. Due to its ability to produce lipases and glycerol ester hydrolases, *S.

aureus* has the ability to grow at high salt and lipid concentrations enabling it to colonize

the skin. In addition to human colonization, it can also survive on domestic animals,

livestock, and inanimate surfaces and objects such as clothing, bedding, and other hard

surfaces [21,69].

S. aureus as a pathogen

S. aureus is the most common pus producing (pyogenic) bacteria that can cause human disease. *S. aureus* can generate a wide range of infections including: abscesses at any site of the body, pneumonia, osteomyelitis, endocarditis, toxic shock syndrome (TSS), *Staphylococcal* scalded skin syndrome (SSSS), and *Staphylococcal* food poisoning (SFP) [21,69]. *S. aureus* is spread from person to person, usually through direct contact or upper respiratory aerosols during bacterial infections. *S. aureus* generally does not penetrate into deep tissues unless there is damage to the skin. Skin damage such as burns, cuts, lacerations, insect bites, or surgical intervention may result in the entry of *S. aureus* into the mucosal membranes of the host. If *S. aureus* is present in large concentrations, there is a potential for spontaneous entry and infection. Poor hygiene and prolonged skin moisture may increase the growth rate of *S. aureus* and lead to spontaneous infection. It is not known if *S. aureus* spontaneously penetrates the skin or if it enters through damaged skin [69]. If *S. aureus* does penetrate the skin and enter into deep tissues, there are several factors that could contribute to its survival including: the concentration of entering bacteria, the site of entry, the speed of the hosts immune response, and the immune system strength of the host [69]. *S. aureus* infections are usually stopped when the initial inoculum is small and an individual's immune system is competent. If an infection does occur, the damage most commonly results in abscesses. Abscesses are collections of pus. Abscesses in the skin are referred to as boils or furuncles and multiple interconnected abscesses are called carbuncles [69].

Methicillin-resistant *S. aureus* (MRSA)

In 1961, soon after the introduction of the antibiotic methicillin, the first β-lactamase-resistant penicillin strains of *S. aureus* were also found to be resistant to methicillin [21,46]. From the 1960s to the early 1970s, methicillin-resistant *S. aureus* (MRSA) infections were largely found in hospital-acquired settings (HA-MRSA). HA-MRSA strains are largely isolated from immunocompromised individuals or individuals exposed to health care settings. HA-MRSA strains tend to cause pneumonia, bacteremia, and invasive infections [21]. Today, MRSA causes the majority of nosocomial infections worldwide [36]. HA-MRSA infections are defined as a patient whose MRSA isolate was cultured more than 48 hours after admission and who had a history of hospitalization, surgery, dialysis, or residence in a long-term healthcare facility within 6 months prior to the culture date, or who had an indwelling intravenous line, catheter, or any other percutaneous medical device present at the time the culture was taken [56]. HA-MRSA carry a large *Staphylococcal* chromosomal cassette (SCC) *mec* which contains the signature *mecA* gene (type I, II, or III). *MecA* is a 2.1 kb gene that encodes for a transpeptidase, which results in 78-kDa cell-wall protein called Penicillin Binding Protein 2a (PBP2a). PBP2a mediates continued peptidoglycan synthesis even when in the presence of β-lactams. β-lactam antibiotics bind to PBPs that are present on the surface of *S. aureus,* however PBP2a has evolved to have low binding affinity for these antibiotics, resulting in the continued growth of these strains [23,69]. HA-MRSA *mecA* gene types vary in size and function, SCC*mec* I (34.3 kb) results in β-lactam resistance while, SCC*mec* II (53.0 kb) and SCC*mec* III (66.9 kb) result in resistance to multiple classes of antibiotics [23]. HA-MRSA are often resistant to many classes of non-β-lactam antibiotics and rarely

carry the gene for Panton-Valentine leukocidin (PVL), which functions in neutrophil lysis [21].

Prior to the 1990s, MRSA infections were confined largely to patients and within health care settings; however, the rate of community-acquired MRSA (CA-MRSA) infections has increased immensely. A study conducted in 2005 found that greater than 80% of all MRSA infections were attributed to CA-MRSA strains, as opposed to HA-MRSA strains [54]. CA-MRSA infections are defined as strains isolated in an outpatient setting, or isolated from patients within 48 hours of hospital admission. These patients must have no medical history of MRSA infection or colonization, and no medical history in the past year of either hospitalization, admission to a nursing home, or dialysis [56]. These strains tend to be more virulent than HA-MRSA strains and CA-MRSA has begun to replace HA-MRSA in health care settings [44,50]. It has been found that CA-MRSA patients often lacked risk factors known for patients with HA-MRSA infections. Those risk factors include, recent hospitalization, dialysis, nursing-home residence, or other co-morbid conditions [44]. CA-MRSA strains carry smaller SCC*mec* elements (type IV or V) compared to the HA-MRSA (type I, II, or III). The CA-MRSA SCC*mec* types vary in size which are, SCC*mec* IV (20.9-24.3 kb) and SCC*mec* V (28 kb). They also carry the *mecA* gene and are believed to be more genetically mobile. In addition, they also are resistant to fewer non-β-lactam antibiotics than HA-MRSA and tend to carry the gene for PVL. Groups that are at risk for CA-MRSA infections in the United States include: neonates, children beyond the neonatal period (daycare centers), athletes, household contacts of MRSA patients, emergency department patients, indigenous populations,

detainees in jail or prison, cystic fibrosis patients, military personnel, HIV patients, and veterinarians and livestock handlers [21].

S. aureus virulence

In a highly regulated manner, under appropriate conditions, *S. aureus* produces numerous virulence factors such as exotoxins and cell surface proteins. These virulence factors result in increased pathogenicity and survival within host cells [4,69]. *S. aureus* has the ability to colonize the skin and mucosal surfaces by way of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which enable *S. aureus* to bind to a wide variety of host surface proteins [69]. The most prevalent group of cell wall-anchored proteins of *S. aureus* are the MSCRAMMs. MSCRAMMs are defined by their tandemly linked IgG-like folded domains and function in many diverse ways [28]. Their functions include colonization of host tissues and the evasion of host defenses [28]. Notable MSCRAMMs include: fibronectin-binding proteins, collagen binding proteins, and fibrinogen binding proteins. Fibronectin-binding proteins (FnbpA and FnbpB) are found on the surface of *S. aureus* and allow the bacteria to invade epithelial and endothelial cells. Fnbps also attach to exposed fibronectin in wounds leading to deep tissue infections [69]. Collagen binding proteins (CBPs) function by binding collagen-rich tissues and prevent the classical pathway of complement activation [28]. Clumping factors A and B function in fibrinogen binding and result in clot formation and endocarditis. Clumping factor A function in adhesion to immobilized fibrinogen resulting in immune evasion by binding soluble fibrinogen. Clumping factor B function in the adhesions to desquamated epithelial cells and result in nasal colonization [28]. In addition to MSCRAMMs, other cell wall-anchored (CWA) proteins include those from the near iron

transporter (NEAT) motif family, the G5-E repeat family, the Three-helical bundle (Protein A), and structurally uncharacterized proteins.

Protein A, regulated by the s*pa* gene, is expressed on the surface of nearly all *S. aureus* strains and occupies approximately 7% of the *S. aureus* cell surface [30]. Protein A is involved in the avoidance of phagocytosis of the host's defense system. More specifically, Protein A binds the Fc regions of IgG thus preventing opsonization and subsequent phagocytosis [21]. Protein A is expressed in both the secreted and membrane bound form so it can be released into the environment surrounding bacterial growth where it can bind free IgG antibodies [21,69]. This protein possesses two Ig-binding activities: each of its five domains can bind either the Fc or Fab regions of IgG antibodies. The Fc region naturally functions in the binding of antigens for the complement system and the Fab region is responsible for antigen recognition [7]. More specifically, Protein A binds the Fc site via the CH2 and CH3 interfaces and can also bind a variable region of the Fab region of the IgG heavy chain [33].

The result of Protein A binding IgGs is the activation of tumor necrosis factor within the host and this can induce inflammation after triggering B-cell proliferation [21]. Studies display that *S. aureus* strains lacking Protein A are phagocytized more efficiently *in vitro* and have overall decreased virulence in murine models of septic arthritis and pneumonia [59]. Protein A is encoded by the gene *Spa. Spa,* a growth stage dependent gene, is upregulated during the exponential growth phase and is down-regulated during the post-exponential phase of growth [59]. There are numerous regulatory elements that have been shown to directly and indirectly regulate *spa* gene expression including Agr (RNAIII), Arl, MgrA, SarU, SarA, SarS, SarT, and XdrA. *Spa* regulation is not only

associated with Protein A but many other networks that regulate a multitude of virulence factors in *S. aureus,* such as, exotoxins and β-lactam resistance [59].

S. *aureus* secretes many enzymes and toxins that function in the battle with host immune cells [69]. One of those members is catalase, which functions by converting hydrogen peroxide to water, counteracting neutrophils' ability to kill bacteria by the production of oxygen free radicals [69]. Another *S. aureus* enzyme, coagulase, converts fibrinogen to fibrin preventing phagocytosis. In addition to enzymatic immune system evasion, secreted toxins are also utilized to aid in immune system avoidance by *S. aureus.*

S. *aureus* possesses a group of toxins that targets cytoplasmic membranes of its host. These membrane-damaging toxins include four hemolysins (α, β, γ, and δ) and leukocidins. These toxins penetrate membranes causing the formation of pores within the host's membrane. Two types of the membrane-damaging toxins are receptor-mediated toxins and non-receptor mediated toxins. Receptor mediated toxins show higher cell specificity than toxins not mediated by receptors. Receptor mediated toxins often play a role in lysis of red (hemolysin) and white blood cells (leukocidin) [65]. Specific receptor-mediated toxins include the α- and γ-toxins, PVL, and leukocidins LukED and LukAB. Hemolysin, or α-toxin, is lytic to red blood cells and multiple leukocytes, but not to neutrophils. The α-toxin is a secreted β-barrel pore-forming toxin that functions by binding to ADAM10 receptors of host cells resulting in apoptosis of those cells [28,65]. The γ-toxin plays a role in the pathogenesis of TSS [63]. PVL is a two-component pore-forming protein that can lyse neutrophils. PVL can be easily spread strain to strain by horizontal gene transfer via bacteriophages, which drastically increases the virulence of *S. aureus* strains. PVL is closely linked to CA-MRSA strains and it is estimated that 60-100% of

CA-MRSA strains carry the PVL genes [21]. *S. aureus* also possesses leukocidins, which function in neutrophil killing and escape, resulting in the promotion of its survival within the host. LukAB is a leukocidin that contributes to neutrophil killing by both external and internal interactions. LukAB can cause damage to neutrophils by directly binding to its cell surface. *S. aureus* can also cause damage to neutrophils from within the immune cell by secreting LukAB [61]. LukED is a major *S. aureus* virulence factor involved in the promotion of disease progression during septic infection [3]. LukED is produced during the course of human infection and has been linked to *S. aureus* associated impetigo and diarrhea [3,34]

In addition to receptor-mediated hemolysins and leukocidins, there are also toxins that are not regulated by receptors. These toxins are often less specific but still contribute to host cell membrane damage. The δ-toxin, part of a family of secreted peptides called the phenol-soluble modulins (PSMs), functions in non-specific cytolytic activity and the triggering of inflammatory responses [52]. The role of β-toxin is unclear; however, it is believed to play an important role in biofilm formation, especially during endocarditis infections [69]. In addition to membrane-damaging toxin, *S. aureus* also possesses enterotoxins, which interfere with receptor function. Enterotoxins are secreted toxins (20-30 kDa) that interfere with intestine function causing diarrhea and vomiting [40]. These enterotoxins are also considered to be superantigens. Superantigens trigger T cell activation and proliferation without the need for antigen processing by allowing non-specific interaction with T cell receptors [65]. The mechanism for cytokine release for these toxins is not well known but the resulting conclusion is cell death by apoptosis for host immune cells [53]. The most prevalent enterotoxins that *S. aureus* possesses are

Staphylococcal enterotoxin B (SEB), *Staphylococcal* enterotoxin C (SEC), and toxic shock syndrome toxin (TSST). Superantigen SEB causes cellular cytotoxicity by inducing inflammatory cytokine release with the potential to result in septic shock [53]. Superantigen SEC has been linked to endocarditis, sepsis, and kidney damage when the SEC gene is found in CA-MRSA strains [75]. TSST, the superantigen that causes TSS stimulates the release of IL-1, IL-2, TNF-α, and other cytokines which function as signaling molecules to mediate and regulate immunity and inflammation [63]. In addition to the previously mentioned toxins, *S. aureus* is also equipped with other enzymes and toxins that function in the interference with the host's immune system. Overall, *S. aureus* virulence and survival revolves around its ability to both damaging host cells and avoid rapid killing by host neutrophils.

S. aureus diagnosis and treatment

Recommendations for *S. aureus* diagnosis and treatment include identification, susceptibility testing, screening for MRSA, and glycopeptide resistance. Identification of *S. aureus* is traditionally achieved by coagulase tests or latex agglutination tests; however, identification can also be achieved by detection of Protein A production and heat-stable nucleases [8]. Coagulase tests can be conducted in a test tube or on a slide. Test tube coagulase tests are used to identify the presence of bound or free coagulase within a sample. Slide coagulase tests are used to identify the presence of cell-bound clumping factor. Slide agglutination tests are very rapid but up to 15% of *S. aureus* strains are negative, so slide tests need to be confirmed with tube agglutination tests. [83] *Staphylococcus schleiferi, Staphylococcus lugdunensis,* and *Staphylococcus intermedius* may give positive results in coagulase and clumping factor tests [89]. Latex agglutination

tests detect for the presence of Protein A and/or clumping factor, and various surface antigens. There are additional biochemical test kits and automated instruments that are used to identify *S. aureus;* however, these methods are generally slower and more expensive than traditional agar-based identification methods. In contrast, other biochemical methods, including Staphychrom II and CHROMagar have been found to work better than tube coagulase identification tests [9,27]. Staphychrom II is a two hour chromogenic test that uses human prothrombin and protease inhibitors, as well as a color indicator (yellow) to identify *S. aureus* [27]. CHROMagar identifies *S. aureus* by growing the isolated strain on the medium. Colonies that appear pink are identified as *S. aureus* [9]. In addition to traditional identification tests, *S. aureus* can be identified by deoxyribonuclease (DNase) tests and molecular tests. Diagnostic laboratories use of molecular tests for *S. aureus* identification is unlikely unless there is a high suspicion of a MRSA infection. Molecular tests are often conducted for confirmation or to determine susceptibility to methicillin/oxacillin; the combination of *S. aureus* identification and susceptibility reading allows for rapid identification of MRSA isolates [8]. The majority of molecular based tests are PCR based and have species-specific targets. For MRSA strains, traditional targets are nuclease (*nuc*), coagulase (*coa*), Protein A (*spa*), *femA, femB, Sa442,* 16s rRNA and other surface-associated fibrinogen-binding protein genes [31,58,86].

For *S. aureus* skin infections, incision and drainage remains the primary therapy. In addition to incision and drainage, antimicrobial coverage for MRSA may also be necessary [10]. *S. aureus* infections can be treated with antibiotics that target pathways essential for survival including: cell-wall synthesis, folic acid metabolism, and bacterial

protein synthesis. *S. aureus* tends to be resistant to penicillin and semi-sensitive to semisynthetic penicillins, such as nafcillin (methicillin-sensitive *S. aureus,* or MSSA). When determining the course of treatment, antibiotic administration should be guided by the susceptibility profile of the strain and the type of infection. The Centers for Disease Control and Prevention (CDC) recommends clindamycin to treat serious *S. aureus* infections; however, a D-zone test should be performed in order to identify inducible clindamycin resistance in erythromycin-resistant *S. aureus* strains [11]. D-zone tests are performed by disk diffusion by placing an erythromycin disk (15 µg) in proximity to a clindamycin disk (2 µg) on an agar plate that has been inoculated with a *Staphylococcus* species and incubated overnight. A flattening of the zone of inhibition around the clindamycin disk proximal to the erythromycin disk (producing a D shaped zone of inhibition) is considered a positive result. This indicates that erythromycin has induced clindamycin resistance [2]. Tetracyclines, such as doxycycline and minocycline are also used to treat *S. aureus* skin infections, but are not recommended during pregnancy or for children under the age of eight. Rifampin is also used to treat *S. aureus* but only in combination with other agents. Linezolid can be utilized to treat complex skin infections, including MRSA; however, consulting an infectious disease specialist is suggested. When treating MRSA strains, these strains are resistant to all available β-lactam antibiotics (penicillins and cephalosporins). Fluoroquinolones and macrolides are also not optimal treatments for MRSA skin and soft tissue infections due to resistance or the potential of resistance development [11].

Other microbes utilized in this study

Many infections can be polymicrobial, and it is rare to find pathogenic organisms isolated from the human body in pure culture from the initial infection site. Negative control organisms that were used in this project were carefully picked with the intention of mimicking real-life conditions of mixed bacterial samples. Both skin infections and other potential contaminants were chosen in order to represent a realistic mixed sample. The following organisms were chosen in order to recapitulate a skin sample that might contain multiple bacteria: *Staphylococcus epidermidis, Streptococcus pyogenes,* and *Micrococcus luteus, and Enterococcus faecalis. Staphylococcus epidermidis* is a Gram-positive cocci (0.8-1 µm) that generally appears in single cells or small clusters. *S. epidermidis* is found on the skin of most people but rarely causes disease in health individuals [21]. Although *S. epidermidis* is a commensal organism, it can be considered an opportunistic pathogen causing infections via catheters and medical devices [66]. *S. pyogenes,* a human pathogen, is a Gram-positive cocci (0.6-1 µm) that colonizes the nasopharynx and the skin at portals of entry or as asymptomatic carriers [60]. *Streptococci* species are common on the skin and in the upper respiratory system. Group A *Streptococcus* can be classified by their beta-hemolytic behavior on blood agar. *Streptococci* are transmitted by contact between humans who carry the organism or have an associated disease. When disease occurs, the organism grows on mucous membranes, in skin, or in deep tissues. *M. luteus* is a Gram-positive cocci (0.6-1 µm) that is a normal inhabitant of human skin. This microbe rarely causes infections; however, *M. luteus* has been associated with septic arthritis, meningitis, and endocarditis in patients with immune suppressive conditions [76]. *E. faecalis* is a Gram-positive cocci (0.5-1 µm) that grows in

pairs or chains and ferments carbon sources that produce lactic acid [60]. *Enterococci* are

commensal organisms that survive in intestinal and vaginal tracts and the oral cavity [45].

E. faecalis causes 80-90% of infection of *Enterococci* infections and is the fourth leading

cause of hospital-acquired infections and the third leading cause of bacteremia in the

United States [24,45]. *Enterococci* are frequently isolated with *Staphylococci* in diabetic soft

tissue infections, which is the reason for the use of this species as a negative control [39].

Other potential contaminants that were utilized to recapitulate samples that may

be found in a *Staphylococcal* food-poisoning sample were: *Escherichia coli,*

Enterobacter aerogenes, and *Listeria monocytogenes. Bacillus subtilis* was also utilized

as a potential environmental contaminant. *E. coli* is a flagellated Gram-negative rod (2

μm in length) found living in the intestines of people and animals. Pathogenic *E. coli*

strains can cause urinary tract infections, respiratory illness and pneumonia, and other

gastrointestinal illnesses. *E. coli* is generally transmitted through contaminated water or

food, or via direct contact with animals or humans [13]. *E. aerogenes* is a Gram-negative

(1.2-3.0 μm in length) flagellated rod shaped organism found in soil, water, dairy

products, and in the intestines of humans and animals. *E. faecalis* is a Gram-positive

cocci (0.5-1 μm) that is an opportunistic pathogen that generally infects

immunocompromised individuals and causes urinary tract infections, endocarditis,

abdominal infections, and septic arthritis [81]. *L. monocytogenes* is a Gram-positive rod

shaped (2 μm) bacteria with a temperature regulated flagella [35]. *L. monocytogenes* causes

serious infections by eating food contaminated with this microbe. Symptoms of listeriosis

include fever and muscle aches, sometimes diarrhea and other gastrointestinal symptoms

[12]. *B. subtilis* is an endospore forming Gram-positive rod (3 μm in length) that is typically

found in soil, dust, water, and in the air; however, its primary reservoir is soil [80]. *Bacillus*

species are organisms that are common residents of dirt, soil, and dust and therefore

could be co-isolated with *S. aureus* in samples contaminated with dirt, soil, and/or dust.

Table 1. *Staphylococcus aureus* **Protein A protein BLAST results.** *S. aureus* **Protein A protein sequence was obtained using NCBI a BLAST search was conducted, excluding** *S. aureus* **(taxid: 1280), synthetic constructs (taxid: 32630), plasmids (taxid: 36549), and cloning vectors (taxid: 29278) in order to determine what other organisms had a protein similar in percent identity to** *S. aureus* **Protein A.**

Description	Strain	Max Score	Total Score	Query Cover	E-Value	Percent Identity	Accession
Immunoglobulin G Binding Protein A	*Staphylococcus argenteus*	578	578	99%	0	95%	CDR59589.1
Protein A	*Staphylococcus schweitzeri*	528	1006	99%	0	79%	CDR66424.1
Hypothetical protein	*Staphylococcus hyicus*	333	333	87%	2.00E-108	64%	WP_039643602.1
Peptidoglycan-binding protein LysM	*Staphylococcus pseudintermedius*	333	333	99%	9.00E-108	60%	WP_037542047.1
Immunoglobulin G binding Protein A	*Staphylococcus schleiferi*	328	644	97%	3.00E-104	68%	BAS44959.1
Hypothetical protein	*Staphylococcus intermedius*	252	252	68%	2.00E-77	61%	WP_019167455.1
Immunoglobulin G-binding Protein A	*Staphylococcus* sp. HGB0015	246	403	86%	1.00E-76	62%	EPD49293.1
Immunoglobulin G-binding Protein A spa2	*Staphylococcus pseudintermedius*	243	243	95%	8.00E-74	50%	WP_014612698.1
Hypothetical protein	*Staphylococcus simulans*	100	100	70%	2.00E-20	34%	WP_057510049.1
BBM3XM	*Staphylococcus xylosus*	90.9	320	30%	8.00E-17	90%	AAA26599.1
PPmABPXM precursor	*Staphylococcus carnosus*	79.7	200	17%	4.00E-13	80%	AAA61965.1
Hypothetical protein	*Staphylococcus delphini*	75.9	194	84%	6.00E-12	33%	WP_019166019.1
Hypothetical protein	*Staphylococcus condimenti*	65.9	65.9	38%	2.00E-08	36%	WP_047132808.1
Hypothetical protein	*Staphylococcus chromogenes*	61.6	183	84%	4.00E-07	31%	WP_037577381.1

In addition to the previously mentioned negative controls, another subset of negative controls utilized in this study included organisms that possess Protein A on its surface. A protein BLAST search revealed strains with similar identity to Protein A (Table 1). The bacterial strains found to have a percent identity greater than 30% were included in Table 1. All of the bacterial strains found to have a percent identity greater than 30% were *Staphylococci* species. Of these subspecies, *Staphylococcus argenteus* and *Staphylococcus schweitzer* have been found to be phenotypically similar to *S. aureus*. *S. argenteus* is a non-pigmented human isolated strain that is *nucA* positive; while *S. schweitzer* is a non-human primate strain that is *nucA* negative. *nucA* is a virulence associated gene that codes for thermostable nuclease which plays a role in the evasion of neutrophil extracellular traps [48,82]. Although *S. argenteus* and *S. schweitzer* are the closest known relatives of *S. aureus, S. argenteus* has only rarely been recovered from humans hosts and *S. schweitzer* has only been recovered from a human host once to date [84]. *Staphylococcus intermedius, Staphylococcus pseudointermedius,* and *Stpahylococcus delphini* are zoonotic strains that are typically colonizers of animals including dogs, cats, and pigeons. These strains have typically been associated with infections caused by animal bites but rarely colonize humans [87]. *Staphylococcus simulans* has been isolated from a number of animals and humans and it is also the most frequently isolated species in association with bovine mastitis [1]. Of the strains mentioned in Table 1, *S. intermedius* and *S. simulans* were used in this study.

Rapid bacterial identification by laser-light scattering

Previous studies suggest that particle size, shape, density, and motion can all influence how a particular particle scatters light [6,62]. Bacterial cells can be thought of as

small particles, and different species have specific shapes, sizes, and internal characteristics that cause them to scatter laser-light into distinctive patterns [6,37]. To understand how a specific species of bacteria (like *S. aureus*) scatters laser-light, many thousands of individual cells of a species of interest are measured in a device
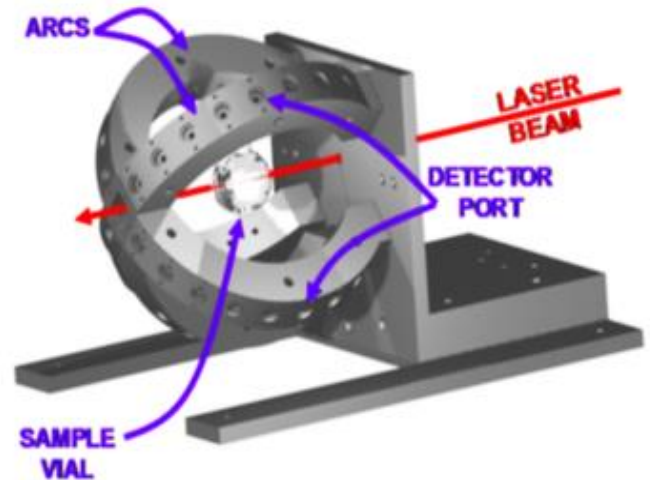
Figure 1. The geometry of the MIT system. A sample vial is placed in the center of the surrounding arcs in order for the laser beam to shine directly through the center of the vial.

developed by Micro Identification Technologies (MIT, located in San Clemente, CA; www.micro-imaging.com). MIT has developed a system for rapid laser based microbial detection and identification. This system measures laser-light scattering intensity as individual microbes pass through a laser beam. The light scattering pattern is influenced by the size, shape, external and internal optical characteristics, and the motility of the microbe. The system can detect and differentiate the size of bacteria by measuring the scattering light at specific angles. The system can detect and differentiate objects within a range of sizes from 0.5 µm to more than 10 µm, which is the typical size range for most bacteria. MIT uses an empirically based "Statistical Classification Algorithm" and a set of pre-measured microbial scattering characteristics called Identifiers. The Identifiers are generated from light scattering measurements of thousands of individual cells of known microbial species or subspecies. The individual cells of specific microbes are analyzed to

identify the measured characteristics that best differentiate that species from another, or subspecies from another.

The MIT system feeds a stream of light scatter measurements into the Statistical Classification Algorithm to determine if the measured cells are members of a specific Identifier within the MIT database. For example, when using the *Staphylococcus* spp. Identifier, the system determines if the sample is or is not *Staphylococcus* spp. The system has the ability to feed the same light scatter measurements into multiple Identifiers simultaneously. Consequently,

Figure 2. Light scattering intensity as a microbe passes though the laser beam. The laser beam is directed toward the reader.

the system can determine if the sample statistically matches any Identifier in the MIT database.

The MIT system consists of five concentric arcs of photodetectors that are located at a variety of positions along the surrounding arcs (Figure 1). Each photodetector is positioned to view through the arcs to the center-of-curvature of the arcs [37]. A solid-state laser (660 nM) passes through the same center-of-curvature where a round bottom flask containing a sample is positioned. As the laser passes through the sample, particles pass through the laser beam resulting in scattered light. Photodetectors record and measure the particles passing through the laser beam. A photon of light striking the photodetector generates a voltage. The MIT system identifies bacteria by analyzing laser-light scatter of

individual bacterial cells suspended in filtered water. When the laser contacts a particle

within a sample, the light both reflects off the outer surface and penetrates the body of the

particle, interacting with structural features that are specific to that particle. As a microbe

passes through the laser beam, the intensity of the scattered light increases as it gets

closer to the center of the beam and decreases as it leaves the beam. The scattered light

measured by the surrounding detectors produces a signal similar to that shown in Figure

2. A single particle that has passed through the laser beam is called an event. Figure 3

displays a typical single particle event measured as a signal (voltage) over time. More

specifically, Figure 3 displays the measured scattered light intensity for two 1.5 µm

diameter polystyrene latex spheres (Figure 3A) and two individual *S. epidermidis* cells
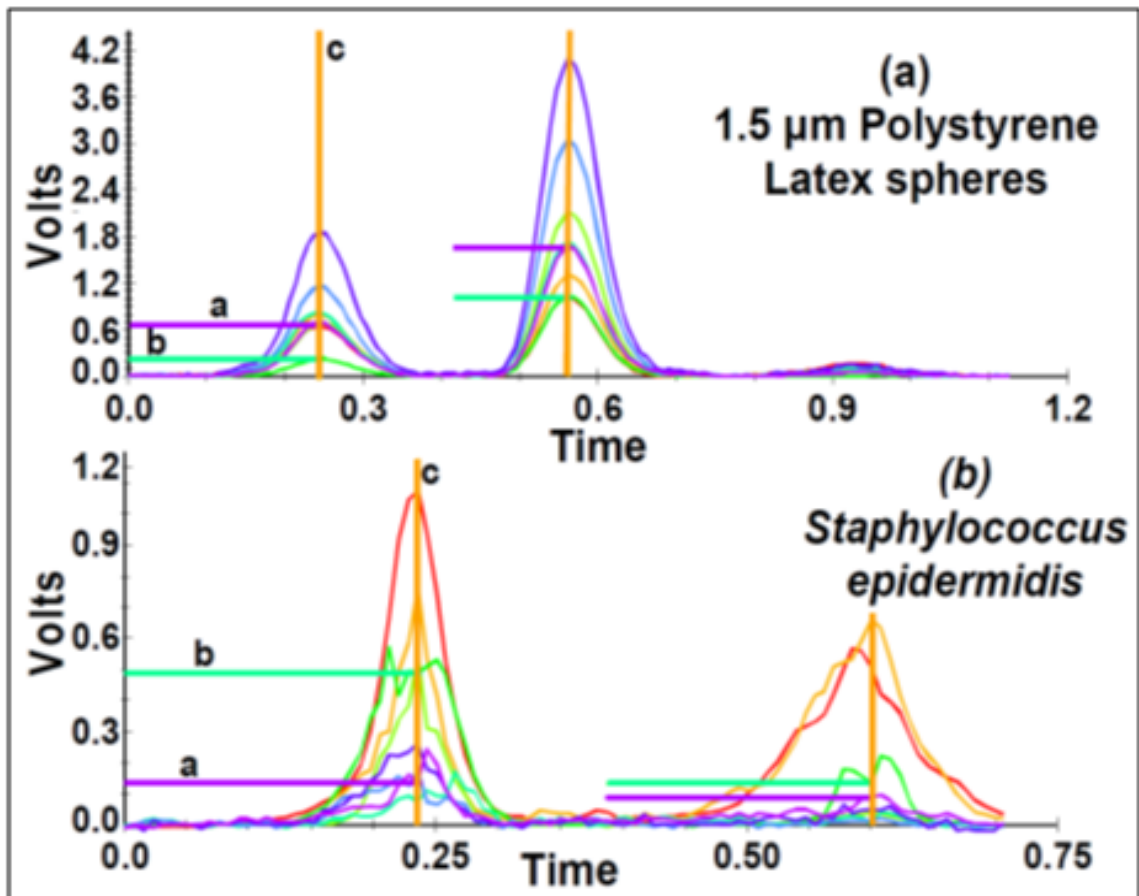
Figure 3. Single particle light scattering measurements: (a) two sequential 1.5 µm diameter polystyrene latex spheres and (b) two sequential *Staphylococcus epidermidis* bacterial cells

(Figure 3B) versus time. The colored curves are the measured signal from all detectors on one arc in the MIT system. The signal measured by the detectors is influenced by size, shape, external and internal optical characteristics and where in the beam the particle is located. In Figure 3, the difference between the polystyrene latex sphere and *S. epidermidis* is apparent; displaying that the individual cells of *S. epidermidis* are not spherically symmetrical thus resulting in variations to its scattering pattern.

By measuring hundreds of thousands of events for a specific bacterial species, the average scattered light pattern for that particular species of interest becomes apparent through statistical analysis. As mentioned previously, MIT has developed a Statistical Classification Algorithm that classifies the sources of events. When bacteria cause the events, the classification is an identification of the bacterial species. The Statistical Classification Algorithm uses a collection of distributions called Judges that are merged Probability Densities constructed from known pre-measured bacterial species and further organized into entities called Identifiers. The Identifier contains Probability Densities derived from measured events. A large number of events for each species are required in
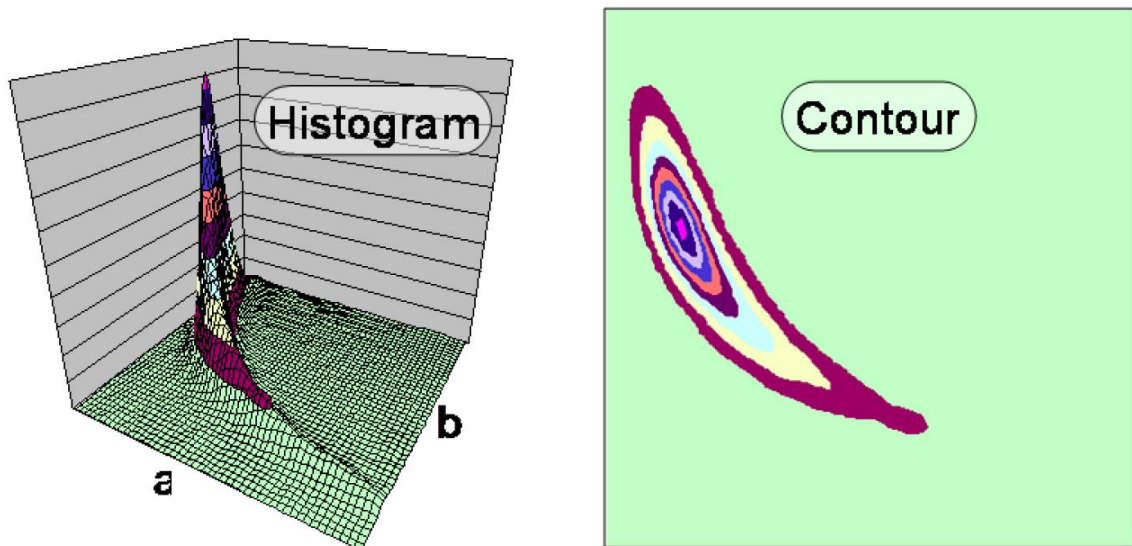
Figure 4. Frequency-of-Occurrence Histogram for 1.5 µm diameter polystyrene spheres. This Frequency-of-Occurrence histogram is an example for 1.5 µm diameter polystyrene spheres.

order to ensure that the derived Probability Densities are representative of the species involved. The same analysis may be applied to any variation in dimensions. This process begins by defining an Observable. An Observable is one or more values calculated from specified measured quantities. Figure 3A displays an instant in time in the left event indicated by the gold colored vertical line labeled **c** This is the instant in time defined as the time that the detector plotted in the same gold color attains a maximum for this event. Looking closely at two detectors in Figure 3A, detector **A** in purple and detector **B** in green at the instant marked by the vertical line **c**. In the left event, the two detectors have a value of **a** and **b** at instant **c**. This results in Observable C(a,b). Values for each Observable can be calculated from any other measurement. Each measurement produces slightly different values for **a** and **b**. For the same sample (polystyrene latex spheres in this case), slightly different Observable values measured by measuring many thousands of different samples.
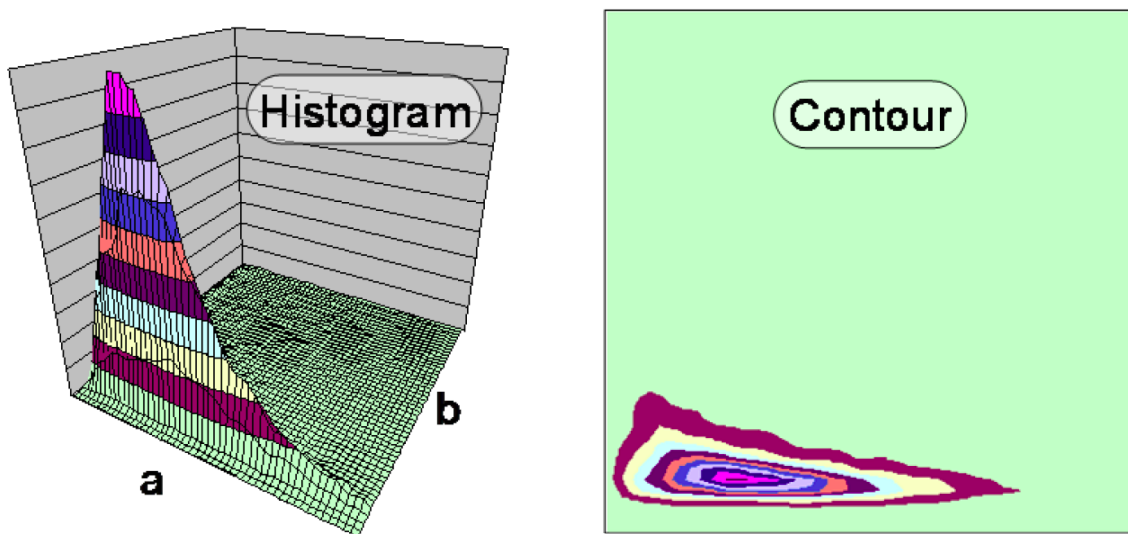
Figure 5. Frequency-of-Occurrence Histogram for *Staphylococcus epidermidis.* This Frequency-of-Occurrence histogram is an example for *S. epidermidis* using the same Observable and plotted on the same scale as figure 4.

Figure 4 shows a Frequency-of-Occurrence histogram and its contour for the Observable C(a,b). This plots the number of times a pair of values, **a** (x-axis) and **b** (y-axis) is measured simultaneously by the detectors **A** and **B** for thousands of individual spheres. When normalized so that the volume of the histogram is 1.0, the histogram can be interpreted as a Probability Distribution, which gives the probability that a range of signals measured by
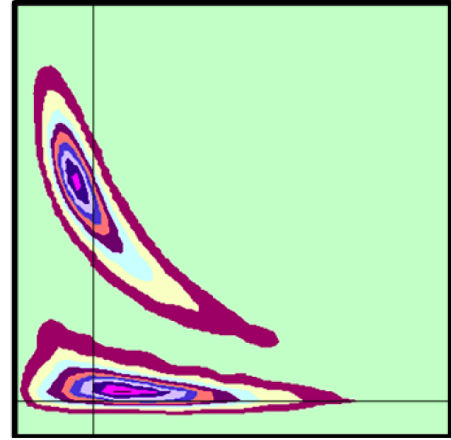
Figure 6. Superposition of the contours of the Frequency-of-Occurrence Histograms in figure 4 and 5.

detectors **A** and **B** are simultaneously measured for the sphere. Figure 3B displays two typical events of individual cells of *S. epidermidis* and the same Observable illustrated in Figure 3A. Figure 5 displays the Frequency-of-Occurrence Histogram and its contour for *S. epidermidis* using the same Observable C(a,b) used to create Figure 4. The scale and the axis limits are the same in both Figure 4 and 5. Since the scales and limits are the same, superposing one of the contours over the other allows direct comparison of the contours which is displayed in Figure 6. There is no overlap between the contours in Figure 6. The Frequency-of-Occurrence histograms therefore display that the size, shape, and location of the contours show a species dependence.

An Observable and the Probability Distribution curves generated from measurements using that Observable for a group of species like in Figure 7 is a Judge. The Judge has one Probability Distribution curve for each species and provides an opinion on the identification of measurements of unknown particles. The Judge's Opinion is the likelihood that the cell is a particular species. The MIT system utilizes a Panel of Judges
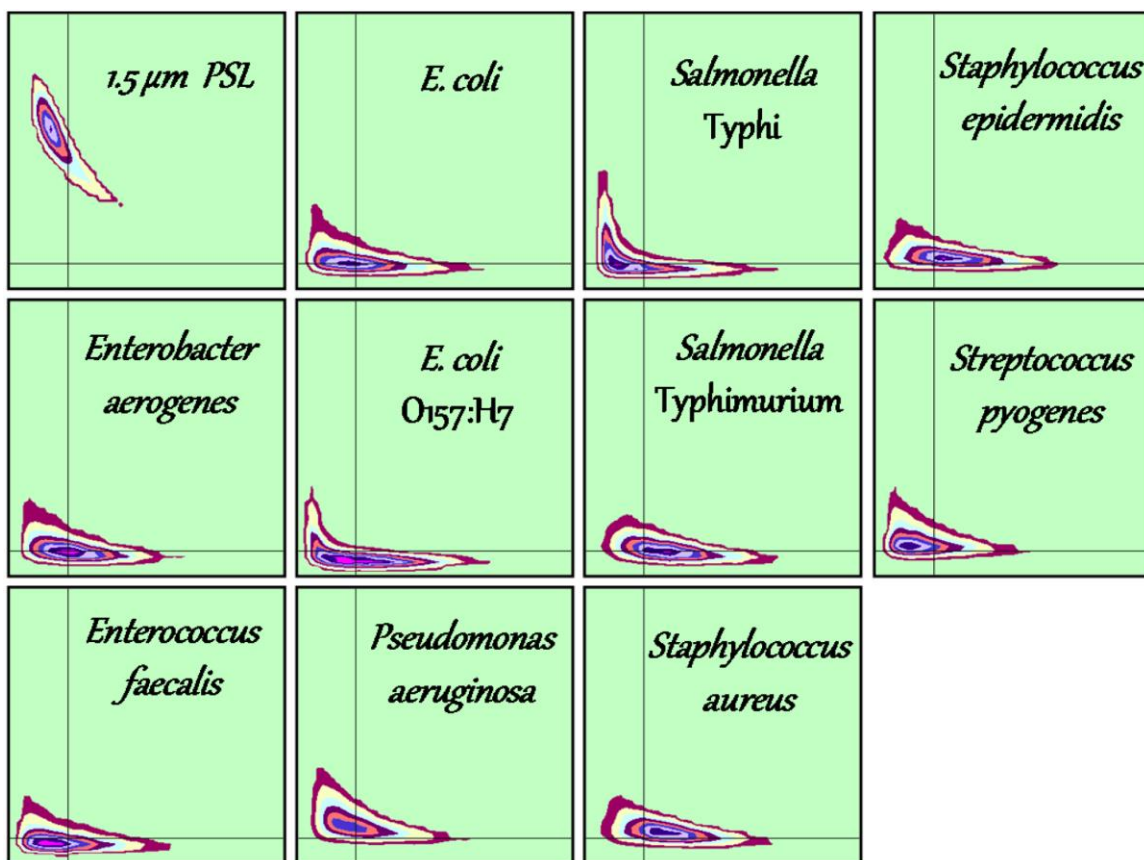
Figure 7. Contours of other bacterial species. These contours are for the same Observable and on the same scale as shown in figures 4 and 5. The crosshair is located at the same coordinate in each plot, this is a Judge.

for a stronger Opinion compared to that of a single Judge's Opinion. Figure 8 displays a series of Judges, each with different Observables and each column is a Judge consisting of Probability Density contours derived from its Observable. Judge 1 is the Observable F(d,e), or the values of detectors D and E when detector F is a maximum. Judge 2 is the Observable I(g,h), or the values of detectors G and H when detector I is a maximum. The axis limits and scale of the contours in each column are the same. This grouping of Judges is referred to as a Panel of Judges.

A Panel of Judges does not always return a definitive identification and additional discrimination information is still needed. The Identification process using MIT

technology is significantly enhanced when multiple cells of the same unknown species are measured in one sample. After measuring the first bacterial cell, the Panel of Judges use event data to produce an identification Opinion of the unknown species.

	Judge 1 F(d,e)	Judge 2 I(g,h)	Judge 3 L(j,k)	Judge ... O(m,n)
1.5 μm PSL				...
Enterobacter aerogenes				...
Enterococcus faecalis				...
Escherichia coli				...
Escherichia coli O157:H7				...
Pseudomonas aeruginosa				...
Salmonella Typhi				...
Salmonella Typhimurium				...
Staphylococcus aureus				...
Staphylococcus epidermidis				...
Streptococcus pyogenes				...
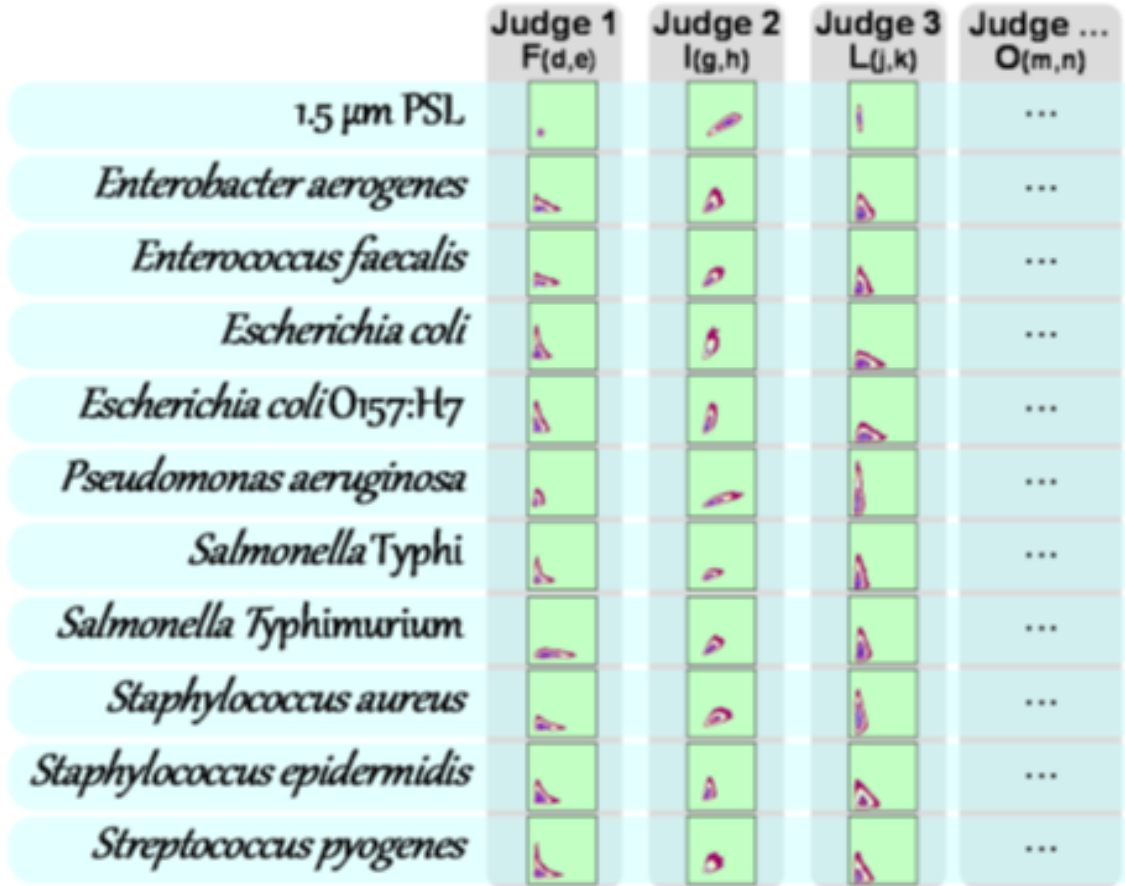
Figure 8. A Panel of Judges. This figure shows a series of Judges each with different Observables. Each column is a Judge consisting of Probability Density contours originating from its Observable.

The Opinion takes the form of a series of identification probabilities, which are combined as the Total Identification Probability. As more cells are measured and processed, the Total Identification Probability for the unknown species then get closer to one. If the probability of one species is greater than 0.999 five times in a row, the species then identifies.
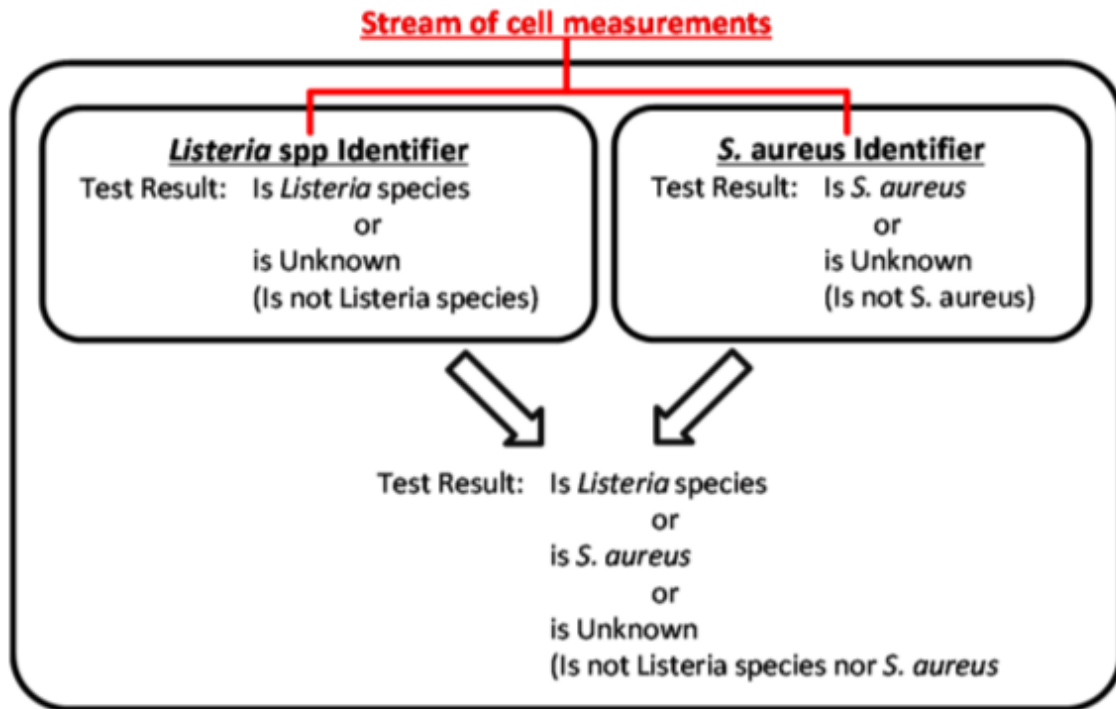
Figure 9. MIT technology cell measurements. MIT technology using *Listeria* species and *S. aureus* Identifiers simultaneously will test the input stream of cell measurements for *Listeria* and *S. aureus* simultaneously.

The Panel of Judges provides quick and accurate identification of any of the species found in the MIT database. This process is closed, meaning, if the unknown sample is not one of the species handled by the Panel of Judges the result is unpredictable. MIT has developed an open equivalent to the Panel of Judges called an Identifier. The Identifier then determines whether the stream of cells is one of the species within its database or if the result is "Unknown." For example, a *Listeria* species Identifier determines whether the stream of cells is *Listeria* species or if it is Unknown. The system can simultaneously run tests for all of its Identifiers within its database, those including *Listeria* species, *Staphylococcus* species, and *S. aureus*. Figure 9 displays the simultaneous identification process utilizing two Identifiers, *Listeria* species and

Staphylococcus species. The identification tests take roughly 30 seconds to 5 minutes with the average test being 2 minutes. In order for this test to be successfully conducted, the system requires 10 to about 40 cells to complete depending on the rate the cells are being measured at.

MIT sample preparation is simple, requires only a few minutes, and is the same for all species. The supplies and apparatus that are required are: 1 inoculating loop, 1 microcentrifuge tube, 1 pipette tip, a vortex mixer, a 0.5-10 µL adjustable pipette, a MIT sample vial, and a 1" x 1" square of parafilm. The sample preparation procedure goes as follows: (1) remove a colony from a culture plate using a sterile loop, (2) dislodge the bacterial colony into a microcentrifuge tube containing filtered water, (3) vigorously agitate the microcentrifuge tube by vortexing the bacterial cells, and (4) inoculate an MIT sample vial with approximately 1 µL of the bacterial suspension. After placing the inoculated vial into the MIT system, click "Identify" on the MIT interface program. The total time until identification using the MIT system totals approximately 5 minutes.

The majority of rapid bacterial identification technologies are designed to detect the presence of genetic material or proteins from a pathogen in a patient specimen. Detection of genomic material can be both highly sensitive and accurate, however these methods may have limitations. Mass spectrometry and antibody-based tests that detect pathogen proteins may require expensive machines, chemical reagents, and/or highly skilled sample processing. Many of these tests have turnaround times as long as 18 to 48 hours. Therefore, a rapid bacterial detection system that is sensitive, accurate, and cost effective would be advantageous. MIT technology is extremely easy to use and reduces time, effort and cost resulting in microbial identification. The system does not rely on

chemical processing, fluorescent tags or DNA analysis. The system eliminates elaborate and lengthy sample preparation required in other identification techniques. The same 1 minute sample preparation is held consistent for every test run in the system. The system requires no other additional chemicals, reagents or processing procedures, other than dilutions. The MIT system identifies all characterized species without reconfiguration using the same procedure. Finally, the amount of supplies and inventory required for identification are reduced drastically compared to other identification methods. MIT has developed their system to detect *Listeria* species (food-borne pathogens) from food samples. Independent laboratories have verified that the MIT system identifies *Listeria* species with greater than 90% accuracy. The MIT system was awarded Association of Analytical Communities Research Institute Performance Tested Methods (AOAC RI PTM) certification for its *Listeria* species Identifier. MIT is working to exapnd the list of Identifiers. *Staphylococcus* species Identifier was utilized for this project. A *S. aureus* Identifier is nearing completion, but was not available at the time of this study.

Rapid bacteria capture by ImmunoMagnetic separation (IMS)

Before this study, the MIT 1000 was being used to identify bacteria from colonies grown on agar plates. The process of growing bacteria colonies can typically take 12-48 hours before the bacteria can be analyzed by laser-light scattering (which takes less than 5 minutes). IMS could potentially capture bacteria cells for laser-light scattering identification within a few hours. IMS is a rapid isolation method that captures proteins or cells using a magnetic field. Magnetic beads (10 mg/ml) are coated with antibodies (2.8 µm, 1 mg/ml) specific for surface antigens of the desired cells [74]. The two components of the IMS method are Streptavidin coated magnetic beads (DynaBeads) and

biotinylated *S. aureus* antibodies (Figure 10). The biotinylated *S. aureus* antibodies can

be linked to Streptavidin on the surface of the DynaBeads due to Streptavidin's extremely

high binding affinity for biotin ($K_d = 10^{-14 \text{ to } -15}$ M) [5,43]. When the conjugated magnetic

beads and antibodies are exposed to a mixed cell population, the magnetic beads attach to

the surface of the desired cells via antibody-antigen interaction. The desired cells can

then be separated by a strong magnetic field [74]. In this study, we are utilizing a protein

found specifically on the surface of *S. aureus,* called Protein A, as the receptor for

antibody binding [17].
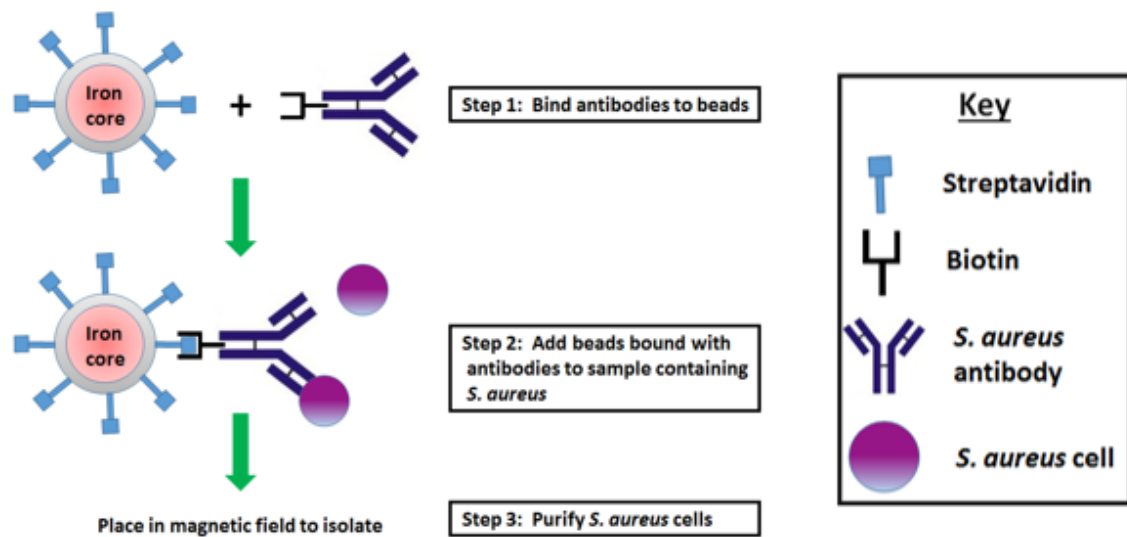
Figure 10. ImmunoMagnetic Separation (IMS) summary. Utilizing magnetic beads to isolate *S. aureus* cells from a sample. Note: multiple antibodies will bind to one bead. Only one antibody is displayed for clarity.

Protein A, a 42-kDa conserved surface protein of *S. aureus* is composed of three

different regions: the S region, which is the signal sequence processed during secretion,

five highly homologous extracellular Ig-binding domains found in tandem and designated

as E, D, A, B, and C, and a cell-wall anchoring region XM [33,42]. Each of the five domains

are arranged in an anti-parallel alpha-helical bundle of approximately 58 amino acids.

This structure is stabilized by a hydrophobic core [42]. Each of these five regions has the ability to independently bind the Fc or Fab region of IgG antibodies [22,33,42,57]. The Fc binding site has shown to involve 11 residues of helix 1 and 2 [22]. The Fab binding region is located separately from the Fc binding region, the Fab binding site involves 11 residues located on helix 2 and 3 [33]. Commercial antibodies against Protein A are available that have biotin conjugated to the Fc region.

Streptavidin (52.8-kDa), isolated from *Streptomyces avidinii,* is a tetrameric protein that can bind up to four d-biotin molecules with extremely high affinity [77]. The ability of streptavidin to bind biotin has led to widespread use in diagnostic assays that require near-irreversible and specific linkage [88]. Streptavidin subunits are organized as eight-stranded, connected, anti-parallel beta sheets. This arrangement produces a cyclically hydrogen-bonded barrel with several extended hairpin loops. One of the extended hairpin loops is located near the carboxyl terminus where it is free to form an extended beta sheet [88]. The pairs of streptavidin barrels hydrogen bonded together form symmetric dimers. Streptavidin forms a tetramer by pairing two dimers, the tetramer is stabilized by Van der Waals interactions between the surfaces of the beta barrels [88]. Biotin then binds in the pockets at the ends of each of the streptavidin beta barrels. Biotin binding involves the removal of bound water molecules that occupy the binding site. After the removal of water, multiple interactions between biotin heteroatoms and the binding site residues of streptavidin allow for the burial of biotin by way of a surface loop of streptavidin [88]. The strength of the streptavidin-biotin bond is the consequence of an expansive number of interactions, which results in a near-irreversible bond that can be exploited for diagnostic assays and other molecular uses.

The use of IMS for bacterial isolation in food samples has been shown to be more efficient than conventional centrifugation and other filtration methods [74]. IMS has the potential to separate *S. aureus* from a sample in 4-8 hours and MIT laser-light scattering technology could complete identification in 5 minutes. It has been shown that live *S. aureus* cells can be isolated using *S. aureus* antibodies bound to magnetic beads, which establishes IMS technology as a method of *S. aureus* bacterial isolation [18].

With this technique in mind, this study aims to (1) develop IMS to magnetically capture cells of *S. aureus* using anti-Protein A antibodies and (2) develop IMS to specifically capture and identify cells of *S. aureus* from swab specimens. The success of this combination could allow for the accurate diagnosis of *S. aureus* infection in 4-8 hours, as opposed to traditional culture based methods which take up to 24-48 hours. Developing these methods for rapid capture and identification of *S. aureus* could lead to improve patient outcomes by resulting in faster treatment administration.

In addition, my thesis seeks to answer the following questions: 1) can *S. aureus* be identified from solid agar plates by laser-light scattering analysis, 2) can *S. aureus* be specifically separated from samples using IMS and 3) in combination with IMS, can laser-light scattering analysis increase the speed and accuracy of *S. aureus* identification? Our goal is to determine if the combination of IMS and laser-light identification can accurately identify *S. aureus* in swab samples while decreasing the total identification time to 4-8 hours. We hypothesized that anti-Protein A conjugated magnetic DynaBeads (also referred to as, anti-Protein A DynaBeads) would bind to surface Protein A on *S. aureus* in a swab sample and facilitate their isolation upon exposure to a magnetic field

within a 4-8 hour procedure. *S. aureus* cells isolated by IMS can then be accurately

identified using MIT technology in less than 5 minutes.

CHAPTER TWO: SINGLE LASER-LIGHT SCATTERING

INTRODUCTION

Laser-light scattering technology developed by MIT measures laser-light scattering intensity as individual microbes pass through a laser beam. The light scattering pattern is influenced by the size, shape, external and internal optical characteristics, and the motility of the microbe. In order to identify microbes via laser-light scattering, the bacteria must first be grown on agar plates. Due to the possibility that different bacteria growth media types may influence bacteria cell size and shape, we needed to determine if the accuracy of bacterial identification was dependent on the growth media that the bacteria was grown on. To test this, four common types of growth media were used to evaluate if the media type influenced the laser-light scattering patterns and identification accuracy of the MIT 1000. To perform this analysis, 10 *Staphylococcus* species and 6 negative control strains were tested a minimum of 10 times in the MIT 1000. Percent identified as *Staphylococcus* species was calculated for each growth media type.

The identification technology developed by MIT was generated by measuring thousands of laser-light scattering measurements of known bacterial strains in order to develop an Identifier specific to that bacteria. Previous studies have shown that laboratory and clinically isolated strains can differ in size and shape despite being the same species [71]. With this in mind, we needed to assess the accuracy of the device from both laboratory and clinical strains. To further assess the accuracy of MIT 1000 bacterial identification technology, a single blind study was conducted using clinical isolates obtained from UP Health System-Marquette. In total, 90 clinical isolates were obtained

31

from UP Health System-Marquette on blood plates. The identity of the bacteria isolates was not revealed to our lab until all identification tests with the MIT 1000 were completed. Each clinical isolate was identified using the MIT instrument and identifications were conducted a minimum of 3 times for each strain. Percent identified as *Staphylococcus* species was calculated and MIT identifications were compared to hospital determined identifications.

METHODS

Bacterial strains and culture conditions

    S. aureus 6538 (ATCC) was used as the positive control in all experiments

conducted during this project. *S. aureus* 6538 has a history of use in quality control

testing, food testing, and other laboratory applications [79]. This strain was initially isolated

from a human lesion. Other strains that were utilized in this study are displayed in Table

2. All bacterial strains were grown in tryptic soy broth (TSB, Hardy Diagnostics) at 37 °C

for 18 hours in aerobic conditions with the exception of *S. pyogenes* which was grown in

reduced oxygen conditions (candle jars). Bacterial cultures were grown in liquid TSB

media shaken at 200 rpm and incubated at 37 °C for 18 hours.

Table 2. Bacterial strains used in this study. Bacterial strains and strain source are listed.	
Bacteria Strain	**Source**
Bacillus subtilis 11774	ATCC
Enterobacter aerogenes 35029	ATCC
Enterococcus faecalis 7080	ATCC
Escherichia coli 29543	ATCC
Listeria monocytogenes 19115	ATCC
Micrococcus luteus SK58	BEI Resources
Serratia marcescens 14756	ATCC
Staphylococcus aureus 25923	ATCC
Staphylococcus aureus 29213	ATCC
Staphylococcus aureus 6538	ATCC
Staphylococcus aureus F003B2N-C	BEI Resources
Staphylococcus aureus HI022	BEI Resources
Staphylococcus epidermidis 35983	ATCC
Staphylococcus epidermidis 49134	ATCC
Staphylococcus epidermidis 49461	ATCC
Staphylococcus epidermidis SK135	BEI Resources
Staphylococcus intermedius 29663	ATCC
Staphylococcus simulans (Clinical isolate)	UP Health System- Marquette
Streptococcus agalactiae MNZ933	BEI Resources
Streptococcus pyogenes MGAS 1882	BEI Resources
Streptococcus salivarius SK126	BEI Resources

Rapid MIT identification from agar colonies
MIT identification procedure from agar plates

Colonies were removed from an agar plate using a sterile loop. The bacteria collected on the loop were dislodged into a microcentrifuge tube that contained about 200 µl of filtered water (3 stage water filter by Watts (North Andover, MA), three stages of filtration (1) sediment, (2) pre-carbon, and (3) VF, 0.6 µm membrane). The microcentrifuge tubes were vigorously agitated by vortexing (20-30 seconds) to dissociate the bacterial cells. The MIT sample vial were inoculated (15 ml round bottom flask, VWR) with about 1 µl of the bacterial suspension. The top of the vial was covered with parafilm (VWR). The MIT sample vial was then secured with parafilm by a rubber band (VWR) and the vial was tipped to resuspend the bacteria. Sample preparation was the same for all species of bacteria. The prepared sample was then placed into the MIT system (MIT 1000). To run the MIT 1000, the User selects the "Identify" button on the MIT Interface Program. The program runs the test and the result was displayed within 2-5 minutes.

Laser-light scattering analysis of bacteria grown on solid media

In order to determine if accuracy of bacteria identification was dependent on the type of growth media used to culture the organisms, the accuracy of the laser-scattering technology was challenged by growing known bacteria strains on 4 different solid media. Various *Staphylococcus* species and negative controls were grown on different medium in order to determine if the type of growth media effected the accuracy of identification. Media utilized in this analysis were common laboratory media: blood agar (Hardy Diagnostics), brain heart infusion agar (BHI, Hardy Diagnostics), lysogeny broth (LB, Hardy Diagnostics), and tryptic soy broth (TSB, Hardy Diagnostics). Strains utilized in

this analysis: *S. aureus* 25923, *S. aureus* HI022, *S. aureus* 29213, *S. aureus* 6538, *S. aureus* F003B2NL, *S. epidermidis* 49461, *S. epidermidis* 49134, *S. epidermidis* 35983, *S. epidermidis* SK135, *S. intermedius* 29663, *S. pyogenes* MGAS 1882, *Streptococcus agalactiae* MNZ 933, *Streptococcus salivarius* SK126, and *M. luteus* SK58. Each strain was tested in the MIT 1000 a minimum of 10 times for each media using MIT identification procedure from agar plates protocol.

Rapid MIT identification from clinical specimens obtained by UP Health System Marquette: A single blind study (Appendix A, approval to use clinical specimens from UP Health System Marquette)

Clinically isolated strains of bacteria can differ significantly from laboratory adapted strains. A single blind study was conducted using bacteria isolated from clinical samples. This single blind study was conducted to evaluate the MIT 1000's ability to identify bacteria from real patient samples rather than laboratory strains. The identities of the bacteria samples were known by UP Health System- Marquette and unknown to our laboratory. In total, 90 clinical isolates were obtained from UP Health System on blood agar plates. Colonies from these 90 clinical isolates were measured a minimum of 3 times in the MIT instrument to determine the accuracy of identification of clinical strains of bacteria. The set of clinical isolates sent to our laboratory included *Staphylococcus* species as well as a subset of unknown negative controls. All samples were tested using the MIT identification procedure from colonies on agar plates mentioned above. After all identification measurements were made, our results were compared to the known identifications made by the UP Health Systems Microbiology Laboratories. The succesful identification of all clinically isolated *Staphylococcus* strains was proof of concept that

light-scattering technology could identify laboratory strans as well as clinical isolates of

Staphylococcus species.

Analysis of MIT identification of bacteria grown on different media

In order to determine if the accuracy of bacteria identification was dependent on the type of growth media used to culture the organisms, organisms were grown on four different commercially available agar plates and tested using the MIT 1000. Ten *Staphylococcus* species were used as positive controls and 6 negative controls strains were also utilized. Each strain was tested in the MIT 1000 a minimum of 10 times and the percent identified as *Staphylococcus* species was calculated.

The average percent identified as *Staphylococcus* species using the MIT 1000 of the 10 *Staphylococcus* strains on blood agar was 97.3% $\pm$ 3.59 ; while the 6 negative controls strains had an average of 3% $\pm$ 2.45 miss-identified as *Staphylococcus* species. The average percent identified as *Staphylococcus* species of the 10 *Staphylococcus* strains on BHI agar was 97.0% $\pm$ 4.22 ; while the 6 negative controls strains had an average of 0.83% $\pm$ 2.04 miss-identified as *Staphylococcus* species. The average percent identified as *Staphylococcus* species of the 10 *Staphylococcus* strains on LB agar was 96.4% $\pm$ 3.89; while the 6 negative controls strains had an average of 0.83% $\pm$ 2.04 miss-identified as *Staphylococcus* species. The average percent identified as *Staphylococcus* species of the 10 *Staphylococcus* strains on TSB agar was 97.1% $\pm$ 3.96 ; while the 6 negative controls strains had an average of 0% $\pm$ 0.00 miss-identified as *Staphylococcus* species. Overall, greater than 95.00% of the positive *Staphylococcus* species identified as *Staphylococcus* species using the MIT 1000 and less than 5.00% of the non-*Staphylococcus* negative controls miss-identified as *Staphylococcus* species. This

displays that bacterial identification by the MIT 1000 is not dependent upon growth

media.

Percent Identified as *Staphylococcus* species: Blood Agar
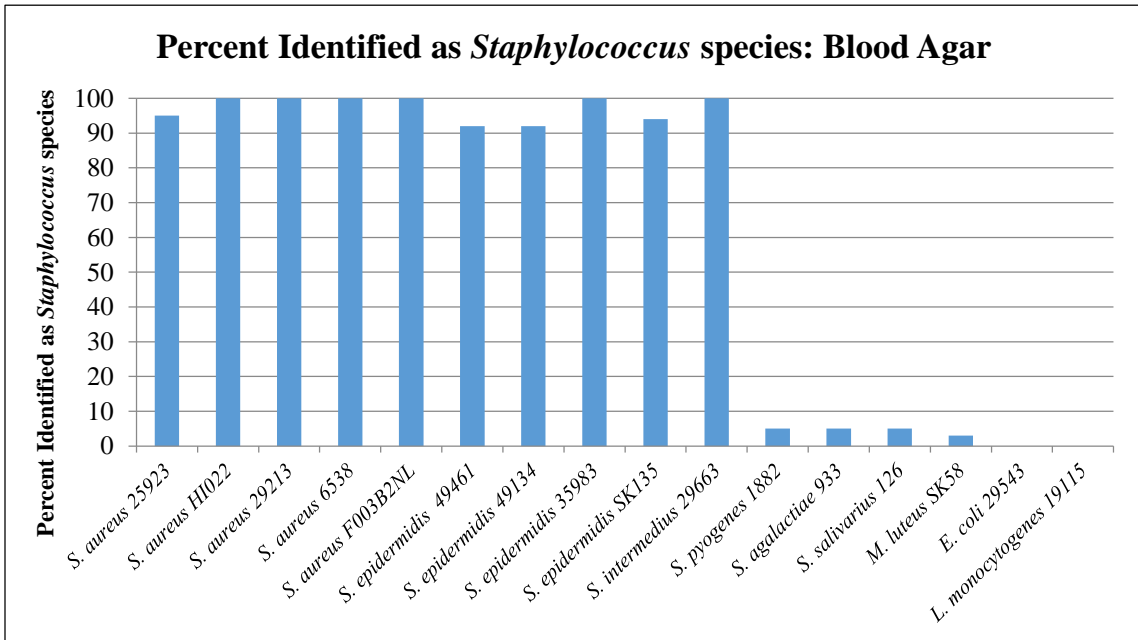
Figure 11. Percent identified as *Staphylococcus* species grown on blood agar. Positive *Staphylococcus* species identified as *Staphylococcus* species using the MIT 1000 an average of 97.3% $\pm$ 3.59. The false positive rate of non-*Staphylococcus* species miss-identified as *Staphylococcus* species using the MIT 1000 an average of 3% $\pm$ 2.45.

Percent Identified as *Staphylococcus* species: BHI Agar
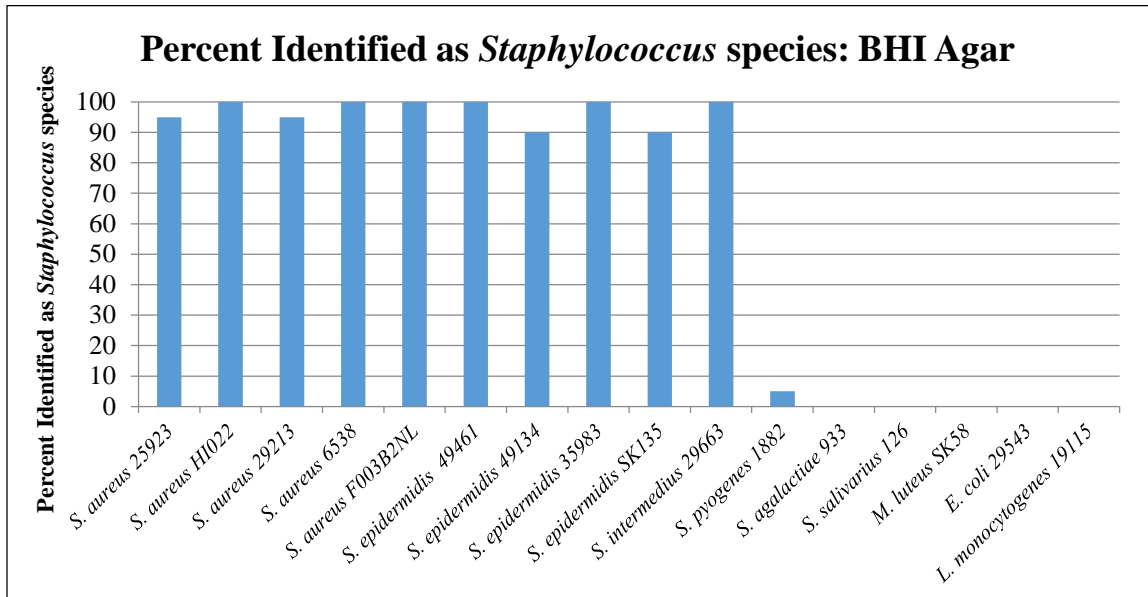
Figure 12. Percent identified as *Staphylococcus* species grown on BHI agar. Positive *Staphylococcus* species identified as *Staphylococcus* species using the MIT 1000 an average of 97.0% $\pm$ 4.22. The false positive rate of non-*Staphylococcus* species miss-identified as *Staphylococcus* species using the MIT 1000 an average of 0.83% $\pm$ 2.04.

Percent Identified as *Staphylococcus* species: LB Agar
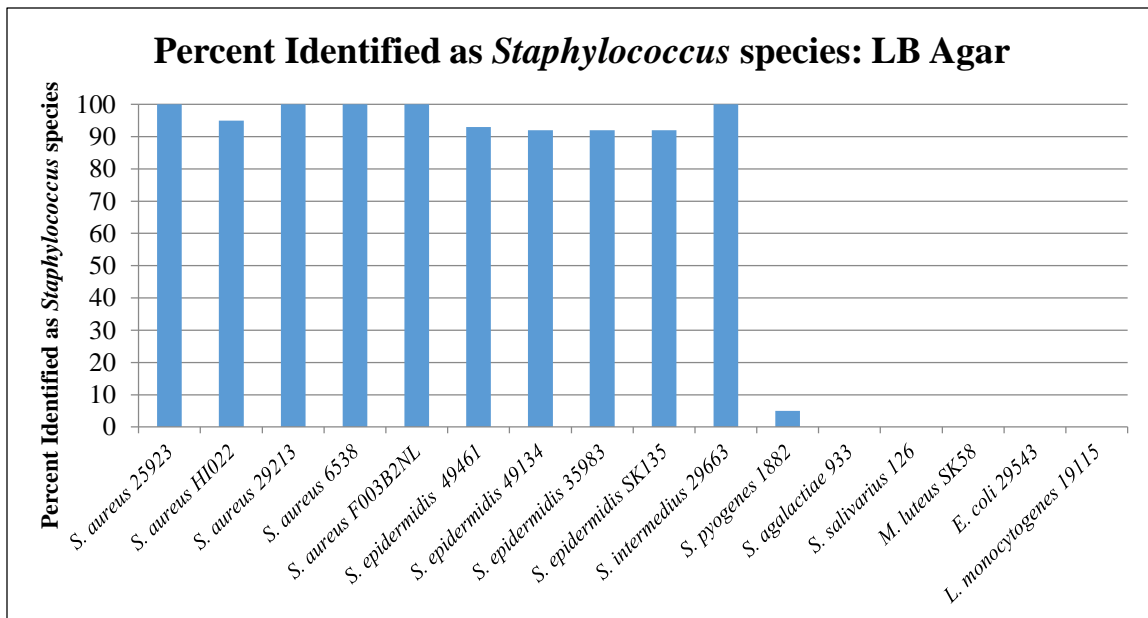
Figure 13. Percent identified as *Staphylococcus* species grown on LB agar. Positive *Staphylococcus* species identified as *Staphylococcus* species using the MIT 1000 an average of 96.4% $\pm$ 3.89. The false positive rate of non-*Staphylococcus* species miss-identified as *Staphylococcus* species using the MIT 1000 an average of 0.83% $\pm$ 2.04.

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Percent Identified as *Staphylococcus* species: TSB Agar

Figure 14. Percent identified as *Staphylococcus* species grown on TSB agar. Positive *Staphylococcus* species identified as *Staphylococcus* species using the MIT 1000 an average of 97.1% $\pm$ 3.96. The false positive rate of non -*Staphylococcus* species miss-identified as *Staphylococcus* species using the MIT 1000 an average of 0.00% $\pm$ 0.00.
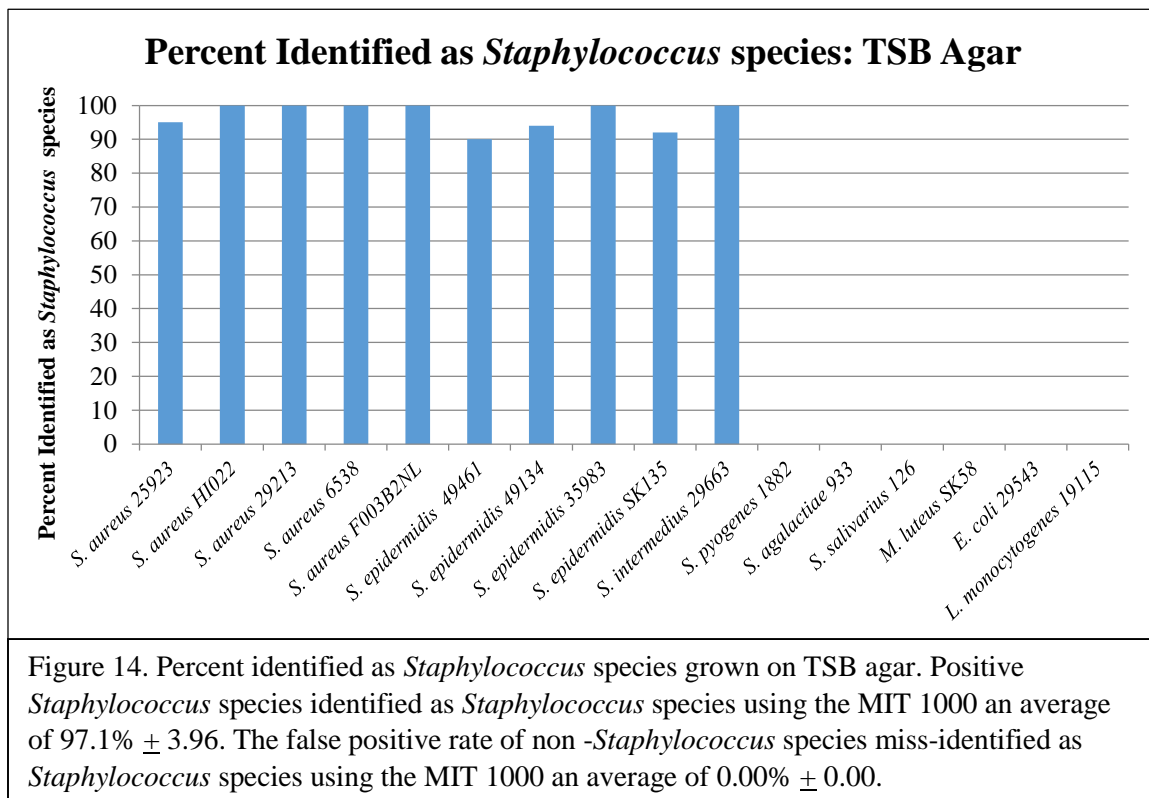
Single blind study- MIT identification from clinical specimens

In total, our lab obtained 90 clinical isolates on blood plates from UP Health System Marquette. Our lab obtained 30 isolates in a month's duration. The isolates were supplied on blood agar, and only a number as an identifier. The hospital-determined identity of the bacteria isolates were not revealed to our laboratory until we finished all of the identification tests with the MIT 1000. Each strain was identified using the MIT instrument and identifications were conducted a minimum of 3 times for each strain. In this single blind study 100% of the *Staphylococcus* species sent to us by the hospital were correctly identified by the MIT instrument (Table 3). In addition, there were no false-positive results. None of the non-*Staphylococcus* bacteria misidentified as

Staphylococcus species, which provides evidence of the MIT instrument's accuracy on

both laboratory and real patient samples.

Table 3. Results of a single blind study. A total of 90 de-identified bacterial isolates were submitted to NMU for identification with the MIT 1000. Each strain isolate was measured at least 3 times in the MIT 1000. *Staphylococcus* species are highlighted in yellow. Other Gram positive cocci are in red. Gram negative cocci are in blue.		
Hospital clinical isolates	**De-identified hospital isolates tested in the MIT 1000**	**MIT 1000 identified as *Staph.* spp.**
Corynebacterium diphtheriae	1	0
Corynebacterium spp.	1	0
Enterobacter cloacae	1	0
Enterococcus faecalis	**13**	**0**
Enterococcus faecium	**1**	**0**
***Enterococcus* spp.**	**2**	**0**
Escherichia coli	14	0
Klebsiella pneumoniae	5	0
Methicillin Resistant *S. aureus*	**8**	**8**
***Neisseria* spp.**	**1**	**0**
Proteus mirabilis	4	0
Providencia rettgeri	1	0
Pseudomonas aeruginosa	2	0
Staphylococcus aureus	**16**	**16**
Staphylococcus epidermidis	**4**	**4**
Staphylococcus simulans	**1**	**1**
***Staphylococcus* spp. (Coagulase negative)**	**1**	**1**
***Streptococcus* group A**	**4**	**0**
***Streptococcus* group B**	**4**	**0**
***Streptococcus* group C**	**1**	**0**
***Streptococcus* group D (not *Enterococcus* spp.)**	**1**	**0**
Streptococcus pneumoniae	**1**	**0**
Viridans streptococci	**3**	**0**
Totals:	90	30

DISCUSSION

Staphylococcus aureus is the most commonly isolated human associated bacterial pathogen. It plays an important role in skin and soft-tissue infections, pneumonia, endocarditis, osteomyelitis, foreign-body infections, and sepsis [21,69]. *S. aureus* is typically identified by coagulase tests or latex agglutination tests; however, identification can also be achieved by detection of Protein A production and heat-stable nuclease [8]. Molecular based tests, such as PCR are conducted by way of *S. aureus*-specific genetic targets. The time until diagnosis of *S. aureus* infections can range from 24-48 hours depending upon the identification method [10]. With this in mind, it has been shown that faster pathogen identification has been linked to improved patient outcome [49]. The impact of faster identification on patient outcome has led us to test alternative methods of *S. aureus* identification. As an alternative to traditional bacteria identification, single cell laser-light scattering technology (MIT 1000 instrument) was utilized as a form of rapid identification of *S. aureus.* In as little as 5 minutes, this technology identifies a pure bacteria sample by comparing laser-light scattering patterns to known scattering patterns (Identifier) in its database. MIT has received AOAC certification for their *Listeria* species Identifier and will soon receive certification for their *Staphylococcus* species Identifier.

In this section, we addressed the following questions: does the growth media that the bacteria is grown with effect the accuracy of identification and can *S. aureus* be identified by single cell laser-light scattering technology and, if so, how accurately? We also asked the question, can laser-light scattering technology identify laboratory strains

and clinical isolates with the same rate of accuracy? To evaluate the effect of growth media on MIT 1000 identification and the accuracy of identification of laboratory strains, 10 *Staphylococcus* species and 6 non-*Staphylococcus* negative control species were tested in 4 different types of common media types (Figures 11-14). Based on the media analysis, we determined that the type of media does not significantly affect the accuracy of MIT 1000 identification on either positive or negative control strains. TSB agar performed the best when considering false positive results, displaying $0.00\% \pm 0.00$ of the negative controls miss-identifying as *Staphylococcus* species. Considering these results, we chose to perform further experiments with TSB media. TSB media also has common overlapping ingredients including: sodium chloride, dextrose, and soybean meal with LB, BHI, and blood agar which further supports our decision to utilize TSB as our main media type.

Previous studies have highlighted significant differences in the genomes of laboratory strains and clinical isolates, which may lead to phenotypic changes to the microbes physical structure [71]. Due to the importance of cell size and shape in laser-light scattering identification, and the knowledge that laboratory and clinical isolates differ, both laboratory and clinical isolates were utilized in the testing of laser-light scattering identification accuracy. A single blind study was conducted in order to address the question, can laser-light scattering technology identify laboratory strains and clinical isolates with the same rate of accuracy. We performed a single blind study in collaboration with UP Health System- Marquette, which included a total of 90 de-identified bacterial isolates. Each of these clinical isolates were identified a minimum of 3 times with the MIT 1000. The provided clinical isolates not only included various

strains of *Staphylococcus* species (MRSA, *S. aureus, S. epidermidis,* and *S. simulans*), but also included Gram positive and negative cocci and rods. Of the 30 *Staphylococcus* clinical strains, all 30 accurately identified as *Staphylococcus* species using laser-light scattering technology. In addition, the remaining non-*Staphylococcus* species did not generate any inaccurate *Staphylococcus* species identifications.

When comparing the results of laboratory versus clinical strains, it was hypothesized that laboratory strains would identify with greater accuracy using laser-light scattering than clinical isolates because laboratory strains were utilized to develop MIT Identifiers. Our results displayed 100% identification accuracy when identifying clinical isolates; while laboratory strains displayed identification accuracy greater than 95.0%. Clinical isolates were tested a minimum of 3 times with the MIT 1000; while laboratory strains were identified a minimum of 10 times. After performing these replication, we did not observe conflicting identifications in the samples tested. Overall, our concern that laser-light scattering identification could not be translated to clinical isolates was addressed. We can conclude that there is no significant difference in the identification accuracy between clinical isolates and laboratory strains. Additionally, none of the growth media tested displayed significant differences in bacterial identification accuracy.

CHAPTER THREE: IMMUNOMAGNETIC SEPARATION VIA ANTI-STAPH DYNABEADS

INTRODUCTION

Prior to the use of anti-Protein A DynaBeads, anti-*Staph* DynaBeads were utilized for *S. aureus* capture via IMS methods. Anti-*Staph* antibodies are antibodies raised against whole *S. aureus* cells as opposed to anti-Protein A antibodies which are raised against a surface protein specific to *S. aureus*. Specificity of anti-*Staph* DynaBeads for *S. aureus* was evaluated by determining the capture ability of the antibodies. We hypothesized that the anti-*Staph* antibodies conjugated to DynaBeads would specifically capture a significant concentration of *S. aureus* cells in both pure and mixed specimen samples.

In order to determine specificity for *S. aureus,* anti-*Staph* DynaBeads were added to pure samples of *S. aureus* and *M. luteus.* The anti-*Staph* DynaBeads were then mixed for 1 hour for binding and washed to remove non-bound cells from the sample. The washed anti-*Staph* DynaBeads were then spot plated on agar plates and incubated at 37 $^{\circ}$C for 18 hours. To further assess anti-*Staph* DynaBead specificity for *S. aureus,* anti-*Staph* DynaBeads were added to separate samples containing *S. aureus* and other negative controls, *B. subtilis, M. luteus,* and *S. marcescens,* mixed for 1 hour for binding and washed to remove non-bound cells from the sample. The washed anti-*Staph* DynaBeads were then spread plated on agar plates to determine the capture efficiencies for the anti-*Staph* DynaBeads when combined with positive and negative control strains. *S. aureus* mixed samples were also utilized to generate a more complex sample and replicate more

realistic sample collections. In order to determine the specificity of anti-*Staph* DynaBeads

for *S. aureus* in mixed samples, *S. aureus* was mixed with each of the negative control

strains: *M. luteus, S. marcescens,* and *B. subtilis.* Anti-*Staph* DynaBeads were added to

each sample, mixed for 1 hour for binding and washed to remove non-bound cells from

the sample. The anti-*Staph* DynaBeads were then quadrant streaked, incubated at 37 °C

for 18 hours and captured colonies were observed. The results of these experiments

allowed us to determine the binding specificity and capture efficiency *anti-Staph*

DynaBeads had for *S. aureus* and other negative control strains.

Preparation of anti-*Staphylococcus* DynaBeads

Anti-*Staph* antibodies (Life Technologies) are antibodies raised against whole *S. aureus* cells as opposed to anti-Protein A antibodies which are raised against surface proteins specific to *S. aureus*. Anti-*Staph* DynaBeads were prepared as follows: 100 µL of magnetic beads (M-280 Streptavidin DynaBeads, Life Technologies) were transferred into a microcentrifuge tube and magnetically separated for 3 minutes in order to remove the storage buffer. The DynaBeads were resuspended in 450 µL of phosphate buffer saline (PBS, NaCl 137mM, KCl 2.7 mM, Na_2HPO_4 10mM, KH_2PO_4 1.8 mM) and mixed by a rotator (VWR, 10136-084) for 1 minute at room temperature. The DynaBeads were magnetically separated for 3 minutes, PBS was removed and the DynaBeads were resuspended in 450 µL of PBS. This process was repeated for a total of 3 washes. The prepared DynaBeads were stored in 100 µL of PBS and held at 4 °C.

Prepared DynaBeads were combined with 20 µL of biotin conjugated chicken anti-*Staph* antibodies (1 mg/ml, Life Technologies) and rotated for 1 hour. The DynaBeads and antibody mixture (now referred to as, anti-*Staph* DynaBeads) was then magnetically separated for 3 minutes. The PBS was removed and the anti-*Staph* DynaBeads were resuspended in 450 µL of PBS and rotated for 1 minute at room temperature. The anti-*Staph* DynaBeads were then magnetically separated for 3 minutes. This washing process was repeated for a total of 4 washes. The washed anti-*Staph* DynaBeads were stored in 100 µL of PBS and held at 4 °C.

Development of IMS methods via anti-Staph DynaBeads and specificity testing

To determine the capture efficiencies of the anti-*Staph* DynaBeads to *S. aureus* in pure culture, bacterial specimens were 10-fold serial diluted from an overnight culture (Undiluted overnight culture to 10^{-7}) in 450 µL of filtered TSB, 20 µL of the anti-*Staph* DynaBeads were added to the specimens and rotated for 1 hour at 22 °C. The samples were then magnetically separated for 1 minute, the TSB was removed and anti-*Staph* DynaBeads were resuspended in 450 µL of wash buffer, PBS plus 0.01% bovine serum albumin (BSA, VWR). The samples were then rotated for 1 minute at room temperature and then magnetically separated for 1 minute. This process was repeated for a total of 4 washes. The anti-*Staph* DynaBeads were then transferred into a sterile microcentrifuge tubes with 100 µL of PBS. The transfer liquid (PBS) was then removed and the DynaBeads were resuspended in 100 µl of PSB. The dilutions were then spot plated (5 µl) to determine the binding specificity of the anti-*Staph* DynaBeads for *S. aureus. M. luteus* was used as a negative control. To further determine the specificity of anti-*Staph* DynaBeads, serial dilutions were created for *S. aureus,* as previously described, and mixed with 20 µl of anti-*Staph* DynaBeads for one hour. The solution from the 10^{-4} dilution was spread plated on LB media and incubated at 37°C for 18 hours. *M. luteus, S. marcescens,* and *B. subtilis* were used as negative controls. Plates were observed after 18 hours and growth was recorded.

Anti-*Staph* DynaBead specificity for *S. aureus* capture when challenged with a mixture of other bacteria was also conducted. Serial dilutions were created as previously mentioned with equal amounts of *S. aureus* and *M. luteus, S. aureus* and *S. marcescens,* and *S. aureus* and *B. subtilis. S. aureus* in combination with *B. subtilis* was plated on LB

agar, *S. aureus* in combination with *S. marcescens* was plated on LB agar, and *S. aureus* in combination with *M. luteus* was plated on MSA agar to differentiate between *S. aureus* and *M. luteus*. This experiment allowed us to determine anti-*Staph* DynaBead specify for *S. aureus* alone, when challenged with other negative control bacteria.

Anti-*Staph* DynaBead specificity testing

To determine the capture efficiencies of the anti-*Staph* DynaBeads to *S. aureus* in pure culture, 20 µl of conjugated anti-*Staph* DynaBeads were mixed with 10-fold serial dilutions of overnight cultures (Undiluted to 10^{-7}) of *S. aureus* for one hour. The dilutions were then spot plated to determine the binding specificity of the anti-*Staph* DynaBeads for *S. aureus. M. luteus* was used as a negative control. Figure 15 displays binding of *S. aureus* to anti-*Staph* DynaBeads and only minimal binding for *M. luteus* the negative control in the undiluted concentration. To further determine the specificity of anti-*Staph* DynaBeads, serial dilutions were created for *S. aureus,* as previously described, and mixed with conjugated anti-*Staph*

Figure 15. Binding specificity for anti-*Staph* DynaBeads for *S. aureus* in pure culture spot plates. 5 µl of bound anti-*Staph* DynaBeads were spot plated on LB media for each dilution to display the bacteria that has bound to the anti-*Staph* DynaBeads. *M. luteus* was used as a negative control.
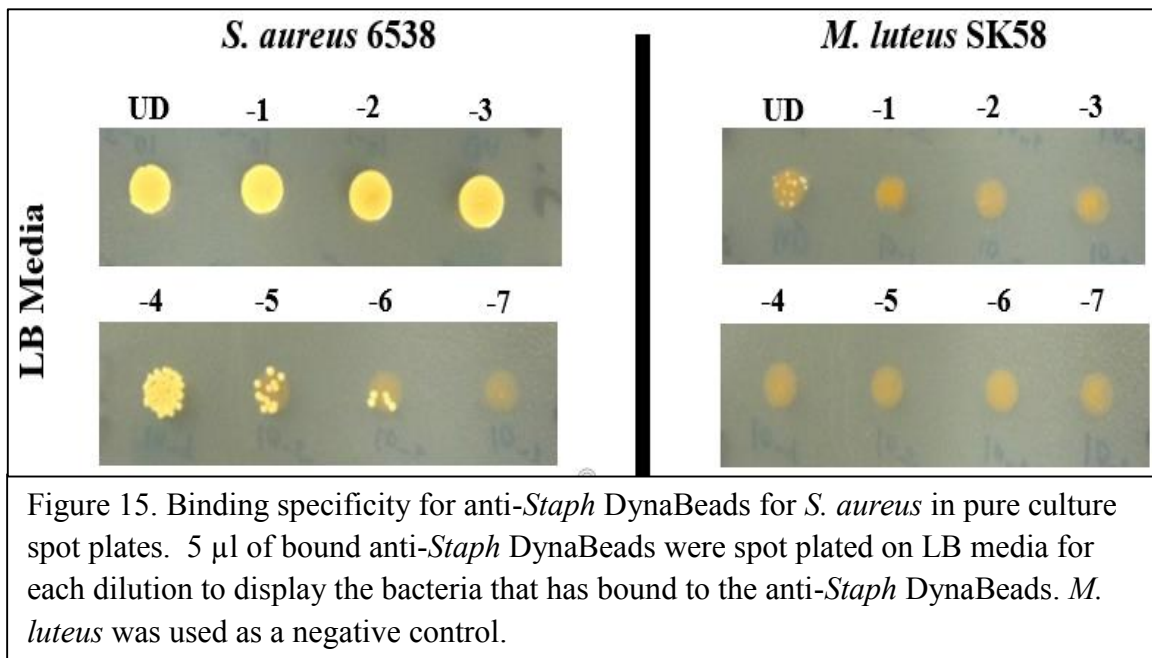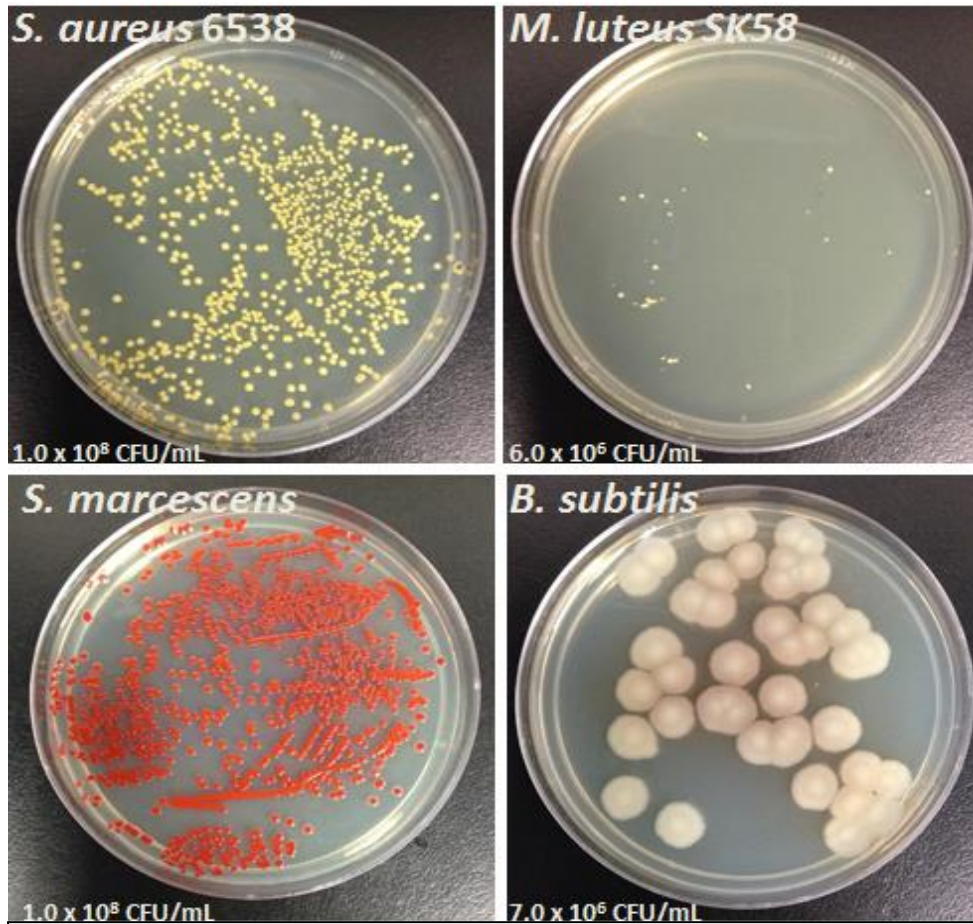
Figure 16. Binding specificity for anti-*Staph* DynaBeads for *S. aureus* in pure culture. 100 µl of bound anti-*Staph* DynaBeads were spread plated on LB media to display anti-*Staph* DynaBead specificity for *S. aureus.* *M. luteus, S. marcescens,* and *B. subtilis* were used as negative controls. Nonspecific binding was observed.

DynaBeads for one hour. The solution from the 10^{-4} dilution was spread plated on LB media and incubated at 37˚C for 18 hours. *M. luteus, S. marcescens,* and *B. subtilis* were used as negative controls. Figure 16 displays anti-*Staph* DynaBeads ability to bind *S. aureus* as well as other negative controls. A final experiment was used to determine anti-*Staph* DynaBead specificity for *S. aureus* when challenged with other bacteria (Figure 17). Serial dilutions were created as previously mentioned with equal amounts of *S. aureus* and *M. luteus, S. aureus* and *S. marcescens,* and *S. aureus* and *B. subtilis.* The

results displayed specificity for *S. aureus* when challenged with *M. luteus* (Figure 17C); however when challenged with *B. subtilis* and *S. marcescens* the anti-*Staph* DynaBeads did not display full specificity for *S. aureus* alone.

The preliminary experiments using anti-*Staphylococcus* antibodies and DynaBeads displayed the ability to capture both *S. aureus* and multiple negative controls but not *S. aureus* specifically. These findings suggest that a more specific antibody was necessary to isolate *S. aureus* alone. Therefore, we proposed to use antibodies against Protein A, a surface protein specific to *S. aureus* but not *S. epidermidis* or other negative controls, to isolate *S. aureus* from mixed samples [18].
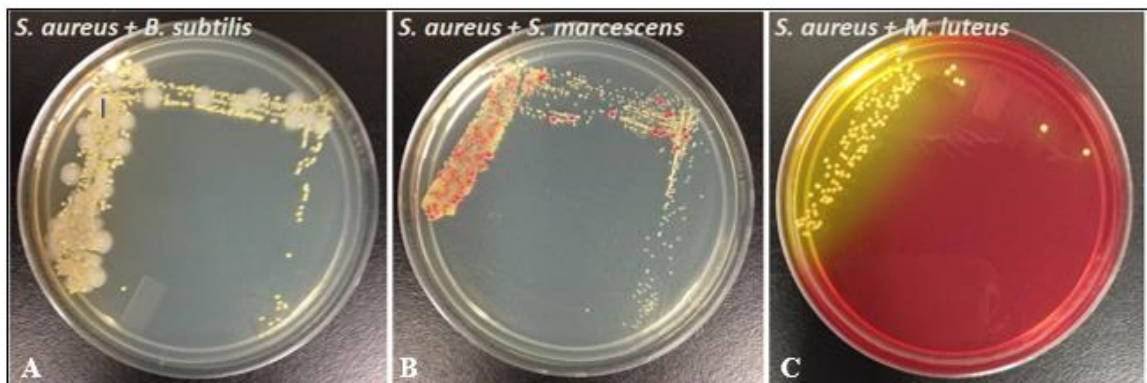
Figure 17. Binding specificity of anti-*Staph* DynaBeads for *S. aureus* when challenged with equal amounts of negative control bacteria. A. *S. aureus* in combination with *B. subtilis* plated on LB agar, showing presence of both bacteria, B. *S. aureus* in combination with *S. marcescens* plated on LB agar, showing presence of both bacteria, and C. *S. aureus* in combination with *M. luteus* plated on Mannitol Salt agar to differentiate between *S. aureus* and *M. luteus,* showing specificity for *S. aureus* alone.

DISCUSSION

Identification methods, such as single laser-light scattering have the ability to rapidly identify pathogens and lead to the quicker administration of treatments. However, rapid identification needs to be paired with a method of rapid capture or isolation of that bacteria prior to identification. In order to bypass the lengthy step of growing bacteria on agar plates, we proposed to utilize IMS as our mode of *S. aureus* isolation prior to rapid laser-light identification. Previous studies have utilized IMS in order to isolate *L. monocytogenes* from pure culture and from contaminated whole milk and ground beef samples.

Utilizing anti-*Staph* antibodies which are antibodies raised against whole *S. aureus* cells, our data initially displayed binding of *S. aureus* to anti-*Staph* DynaBeads and only minimal binding for *M. luteus* the negative control in the undiluted concentration (Figure 15). The deduced that the presence of minimal growth of *M. luteus* on this spot plate could have been due to inefficient washing methods or due to direct binding of *M. luteus* to anti-*Staph* DynaBeads.

To further determine the specificity of anti-*Staph* DynaBeads, 10-fold serial dilutions from overnight cultures were created for *S. aureus,* and mixed with anti-*Staph* DynaBeads for one hour. The solution from the 10^{-4} dilution was spread plated on LB media. *M. luteus, S. marcescens,* and *B. subtilis* were used as negative controls. Figure 16 displayed anti-*Staph* DynaBeads ability to capture *S. aureus* with high efficiency. In addition to a high capture rate for *S. aureus, S. marcescens* and *B. subtilis* were captured at a high rate when combined with anti-*Staph* DynaBeads. We speculate that the high

capture rate of *S. marcescens* and *B. subtilis* could be attributed to non-specific binding of these microbes to the antibodies, DynaBeads, or surfaces of the microcentrifuge tubes. *M. luteus*, much like the results displayed in Figure 15, was captured at a low rate by anti-*Staph* DynaBeads.

A final experiment was used to determine anti-*Staph* DynaBead specificity for *S. aureus* when challenged with other bacteria (Figure 17). The results displayed specificity for *S. aureus* when challenged with *M. luteus* (Figure 17C); however when challenged with *B. subtilis* and *S. marcescens* the anti-*Staph* DynaBeads did not display full specificity for *S. aureus* alone (Figure 17A and 17B). These results suggest that the non-specific binding was occurring during our IMS procedure. The non-specific binding could have been attributed to inadequate washing during the IMS procedure or due to the anti-*Staph* antibody. If non-specific binding was due to the antibody itself, we speculate that the antibody was developed to bind to a cell surface protein that may be commonly found on the surfaces of many microbes, resulting in the binding of not only *S. aureus* but other negative control strains.

The experiments using anti-*Staphylococcus* antibodies and DynaBeads displayed the ability to capture both *S. aureus* and multiple negative controls but not *S. aureus* specifically. The anti-*Staph* DynaBeads are raised against whole *S. aureus* cells, rather than to a specific antigen found on the surface of *S. aureus*. Due to this feature of anti-*Staphylococcus* antibodies, the antibodies have the potential to recognize and bind to the surface of many bacteria species. These findings suggest that a more specific antibody is necessary to isolate *S. aureus* alone. Therefore, we propose to use antibodies against

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Protein A, a surface protein specific to *S. aureus* but not *S. epidermidis* or other negative

controls, to isolate *S. aureus* from samples containing a mixture of bacteria [18].

CHAPTER FOUR: IMMUNOMAGNETIC SEPARATION OF *S. AUREUS* VIA ANTI-PROTEIN A DYANBEADS

INTRODUCTION

Due to anti-*Staph* DynaBeads lack of specificity for *S. aureus,* we proposed to use antibodies against Protein A, which is specific for a surface protein unique to *S. aureus* in order to isolate *S. aureus* [18]. Prior to specificity testing, we first needed to determine the optimal conditions for anti-Protein A DynaBead preparation and capture methods. Wash conditions were first evaluated to determine their effect on non-specific binding and the removal of unbound bacteria that could potentially be stuck to the walls or cap of microcentrifuge tubes. The following wash buffers were evaluated for washing efficiency: PBS, PBS + 0.1% BSA, and PBS + 0.01% Tween 20.

After the establishment of optimal anti-Protein A DynaBead preparation and capture methods, we needed to determine anti-Protein A DynaBeads ability to capture *S. aureus.* To evaluate anti-Protein A DynaBead specificity for *S. aureus,* anti-Protein A DynaBeads were added to pure culture samples of *S. aureus,* mixed for 1 hour, washed with PBS + 0.01% Tween. The anti-Protein A DynaBeads were spread plated on agar plates and then incubated at $37\,^{\circ}$C for 18 hours. Capture efficiencies were then calculated and statistical significance was determined via one-way ANOVA Bonferroni multiple comparison test. This process was repeated with a diverse group of negative control strains. We hypothesized that anti-Protein A DynaBeads would have greater specificity for *S. aureus* than did anti-*Staph* DynaBeads. We further hypothesized that anti-Protein A

DynaBeads would specifically capture a significant concentration of *S. aureus* cells in both pure and mixed specimen samples.

   After determining anti-Protein A DynaBeads ability to capture *S. aureus* in pure cultures, we then sought to determine if our IMS methods could capture *S. aureus* from a more complex biological sample. To evaluate this, we performed our methods in whole milk which contains many competing proteins, enzymes, and other microbial species. We hypothesized that anti-Protein A DynaBeads would have specificity for *S. aureus* in whole milk; however, there would be a reduction in capture efficiency due to the increase in competing cells, fats, proteins, and particulates within the whole milk. In order to determine this, anti-Protein A DynaBeads were added to whole milk containing *S. aureus* and capture efficiencies and statistical significance were calculated. This process was repeated with negative control strains that could be connected to milk contamination (*B. subtilis* and *L. monocytogenes*).

Preparation of anti-Protein A DynaBeads

Anti-Protein A DynaBeads were prepared as follows: 100 µL of magnetic beads (M-280 Streptavidin DynaBeads, Life Technologies) were transferred into a microcentrifuge tube and magnetically separated (Dynamag2, Life Technologies) for 3 minutes in order to remove the storage buffer. The DynaBeads were resuspended in 450 µL of PBS and mixed by a rotator for 1 minute at room temperature. The DynaBeads were magnetically separated for 3 minutes, PBS was removed and the DynaBeads were resuspended in 450 µL of PBS. This process was repeated for a total of 3 washes. The prepared DynaBeads were stored in 100 µL of PBS and held at 4 °C.

Prepared DynaBeads were combined with 20 µL of biotin conjugated chicken anti-Protein A antibodies (1 mg/ml, ICL lab) and rotated for 1 hour. The DynaBead and antibody mixture (now referred to as, anti-Protein A DynaBeads) were then magnetically separated for 3 minutes. The PBS was removed and the anti-Protein A DynaBeads were resuspended in 450 µL of PBS and rotated for 1 minute at room temperature. The anti-

Table 4. Summary table of optimal conditions for enrichment step. This table includes the variations in variables for each experiment performed above. Optimal conditions are highlighted in yellow

Condition	Growth Media	Binding Temperature	Movement	Bead Removal	Growth Media Removal
1	TSB	22 °C	No	No	No
2	TSB	37 °C	No	No	No
3	TSB	37 °C	215 rpm	No	No
4	TSB	37 °C	250 rpm	No	No
5	TSB	37 °C	250 rpm	Yes	No
6	Terrific Broth	37 °C	250 rpm	Yes	No
7	TSB	37 °C	250 rpm	Yes	Yes

Protein A DynaBeads were then magnetically separated for 3 minutes. This washing process was repeated for a total of 4 washes. The washed anti-Protein A DynaBeads were stored in 100 µL of PBS and held at 4 °C. Optimal conditions are shown highlighted in yellow in Table 4.

ImmunoMagnetic Separation of *S. aureus* via anti-Protein A DynaBeads

Development of IMS methods via anti-Protein A DynaBeads

Bacterial strains were grown in TSB at 37 °C for 18-24 hours. Ten-fold serial dilutions from overnight cultures of *S. aureus* were made in TSB media for experiments evaluating ImmunoMagnetic Separation and laser-light scatting identification.

Fifty microliters of overnight bacterial cultures were 10-fold serial diluted in 450 µL of filtered TSB (10^{-2}-10^{-6}), 20 µL of the anti-Protein A DynaBeads were added to the specimens and rotated for 1 hour at either 22 °C or 37 °C. The samples were then magnetically separated for 1-3 minutes, the TSB was removed and anti-Protein A DynaBeads were resuspended in 450 µL of wash buffer, PBS alone, PBS plus 0.1% bovine serum albumin (BSA, VWR), or PBS plus 0.01% Tween 20 (VWR). The samples were then rotated for 1 minute at room temperature and then magnetically separated for 1-3 minutes. This process was repeated for a total of 4 washes. The anti-Protein A DynaBeads were then transferred into a sterile microcentrifuge tubes with 100 µL of PBS. The transfer liquid (PBS) was then removed and the DynaBeads were resuspended in 100 µl of PBS. The experimental conditions (magnetic separation time, wash buffer, tube type, and binding temperatures) were adjusted to determine the most efficient means for ImmunoMagnetic separation of targeted bacterial specimens.

To determine the efficiency of the anti-Protein A DynaBead protocol, the washing buffers were first evaluated. To determine if the wash buffer altered non-specific binding of bacteria to the walls or cap of microcentrifuge tubes, the use of PBS and PBS + 0.1% BSA were evaluated for washing efficiency. In order to evaluate the efficiency of each buffer, 10-fold serial dilutions (10^{-3} to 10^{-7}) of overnight *S. aureus* cultures were created in TSB. To each dilution, 20 µl of anti-Protein A DynaBeads were added, rotated for 1 hour, magnetically separated for 3 minutes and washed with 450 µl of either PBS or PBS + 0.1% BSA. After adding 450 µl of appropriate wash buffer, the tubes were rotated for 1 minute at 22 °C, magnetically separated for 3 minutes and washed with 450 µl of the appropriate wash buffer. This process was repeated for a total number of 4 washed. The washed anti-Protein A DynaBeads were then resuspended in 100 µl of PBS and 5 µl of the solution was spot plated on TBS agar plates and incubated at 37 °C for 18 hours. Another 10-fold serial dilution set (10^{-3} to 10^{-7}) was generated and 20 µl of DynaBeads alone were added and the same protocol was repeated. This dilution set was created to evaluate wash efficiency by media type.

PBS and PBS + 0.01% Tween 20 were then evaluated for their ability to reduce non-specific binding. To evaluate, *S. aureus* was 10-fold serial diluted (10^{-3} to 10^{-6}) from overnight cultures in 450 µl of TSB. To each dilution, 20 µl of anti-Protein A DynaBeads were added and the same IMS protocol as above was repeated. The wash buffer under evaluation were either PBS or PBS + 0.01% Tween 20. Another serial dilution set (10^{-3} to 10^{-6}) was generated and 20 µl of DynaBeads alone were added. After the washes, 100 µl of each dilution was spread plated on TSB agar plates and incubated at 37 °C for 18 hours and colonies were counted in order to calculate and compare capture efficiencies.

Established IMS protocol for S. aureus capture

Bacterial specimens were 10-fold serial diluted of overnight cultures (10^{-2} to 10^{-6}) in 450 µL of TSB, 20 µL of the anti-Protein A DynaBeads were added to the specimens and rotated for 1 hour at 37 °C. The samples were then magnetically separated for 3 minutes, the TSB was removed and anti-Protein A DynaBeads were resuspended in 450 µL of PBS plus 0.01% Tween 20. The samples were then rotated for 1 minute at room temperature and then magnetically separated for 3 minutes. This process was repeated for a total of 4 washes. The anti-Protein A DynaBeads were then transferred into sterile microcentrifuge tubes with 50 µL of PBS. The transfer liquid (PBS) was then removed and the DynaBeads were resuspended in 100 µl of PBS.

Specificity testing in pure culture

To determine anti-Protein A DynaBeads ability to capture *S. aureus*, 50 µl of *S. aureus* were mixed in 450 µl of buffer and 10-fold serial diluted from overnight cultures (10^{-1} to 10^{-8}). Two sets of the same serial dilutions were prepared, 20 µl of anti-Protein A DynaBeads were added to one set of dilutions and mixed for 1 hour. The tube set containing the anti-Protein A DynaBeads were then magnetically separated and washed with 450 µl of PBS plus 0.01% Tween 20 to remove any unbound cells. After 4 washes the bound anti-Protein A DynaBeads were resuspended in 100 µl of PBS. Then 100 µl of DynaBeads were then spread plated on TSB plates and incubated at 37°C for 18 hours. Anti-Protein A DynaBeads were not added to the other set of dilutions. These dilutions were utilized to determine the initial input of bacteria in each sample (input). To determine the input for each dilution, each tube was vortexed for 10 seconds and 100 µl was spread plated on TSB agar plates and incubated at 37°C for 18 hours. After

incubation, colonies on each plate were counted and capture efficiencies were calculated. Capture efficiencies (CE) are defined as the percentage fraction of total bacteria captured on the surface of the anti-Protein A DynaBeads [90]. Capture efficiencies are calculated using the number of unbound cells in the starting dilution (input). The equation for Capture efficiency is calculated using the following equation: Capture efficiency (%) = (1-B/A) x 100% [90]. A is the total number of cells present in the sample (CFU/ml) and B is the number of cells unbound to the anti-Protein A DynaBeads (CFU/ml, input).

In a similar control experiment, *S. epidermidis, S. intermedius, S. simulans, S. pyogenes, M. luteus, L. monocytogenes, E. faecalis, E. coli,* and *B. subtilis* were used as negative controls to determine the anti-Protein A DynaBeads specificity for each. Capture efficiencies were determined using the same protocol mentioned above. These methods allowed us to determine the IMS binding limit (the fewest number of bacteria able to bind in a sample).

Specificity testing in mixed culture

To determine the specificity anti-Protein A DynaBeads have for *S. aureus* when challenged by other bacteria, *S. aureus* was mixed with an equal concentration of *S. epidermidis* which is a normal flora bacteria found on the skin and should not bind to anti-Protein A DynaBeads [15]. To evaluate the specificity, serial dilutions were spread plated using previously established methods and incubated for at 37°C for 18 hours. Capture efficiencies were calculated and analyzed. These experiments were performed to establish the capture limit and specificity for our IMS protocol. (Results not shown)

S. aureus IMS isolation from whole milk by anti-Protein A DynaBeads

To determine anti-Protein A DynaBeads ability to capture *S. aureus* from whole milk, *S. aureus* was 10-fold serial diluted from overnight cultures (10^{-2} to 10^{-6}) in 450 µl of pasteurized whole milk. Two sets of the same serial dilutions (10^{-2} to 10^{-6}) were prepared, 20 µl of anti-Protein A DynaBeads were added to one set of dilutions and mixed for 1 hour. The tube set containing the anti-Protein A DynaBeads were then magnetically separated for 3 minutes and washed with 450 µl of PBS plus 0.01% Tween 20 to remove any unbound cells. After 4 washes the bound anti-Protein A DynaBeads were resuspended in 100 µl of PBS. The 100 µl of DynaBeads are then spread plated on TSB plates and incubated at 37°C for 18 hours. Anti-Protein A DynaBeads were not added to the other set of dilutions. These dilutions were utilized to determine the initial input of bacteria in each sample (input). To determine the input for each dilution, each tube was vortexed for 10 seconds and 100 µl was spread plated on TSB agar plates and incubated at 37°C for 18 hours. After incubation, colonies on each plate were counted and capture efficiencies were calculated.

Analysis of anti-Protein A DynaBead *S. aureus* capture specificity

Development of anti-Protein A DynaBead protocol for S. aureus IMS capture

To determine the efficiency of the anti-Protein A DynaBead protocol, the washing

buffers were first evaluated. To determine if the wash buffer altered non-specific binding

of bacteria to the walls or cap of microcentrifuge tubes, or the DynaBeads themselves, the

use of PBS and PBS + 0.1% BSA were evaluated for washing efficiency. In order to

evaluate the efficiency of each media, 10-fold serial dilutions of overnight cultures (10^{-3}

to 10^{-7}) were generated with *S. aureus.* To each dilution, 20 µl of anti-Protein A

DynaBeads were added, rotated for 1 hour, and washed four times with 450 µl of either

PBS or PBS + 0.1% BSA. Another serial dilution set (10^{-3} to 10^{-7}) was generated and 20

Figure 18. Binding capacity of anti-Protein A DynaBeads for *S. aureus,* PBS versus PBS + 0.1% BSA. After the addition of anti-Protein A DynaBeads and IMS protocol, samples were resuspended in 100 µl of PBS and 5 µl of the solution was spot plated on TSB agar.
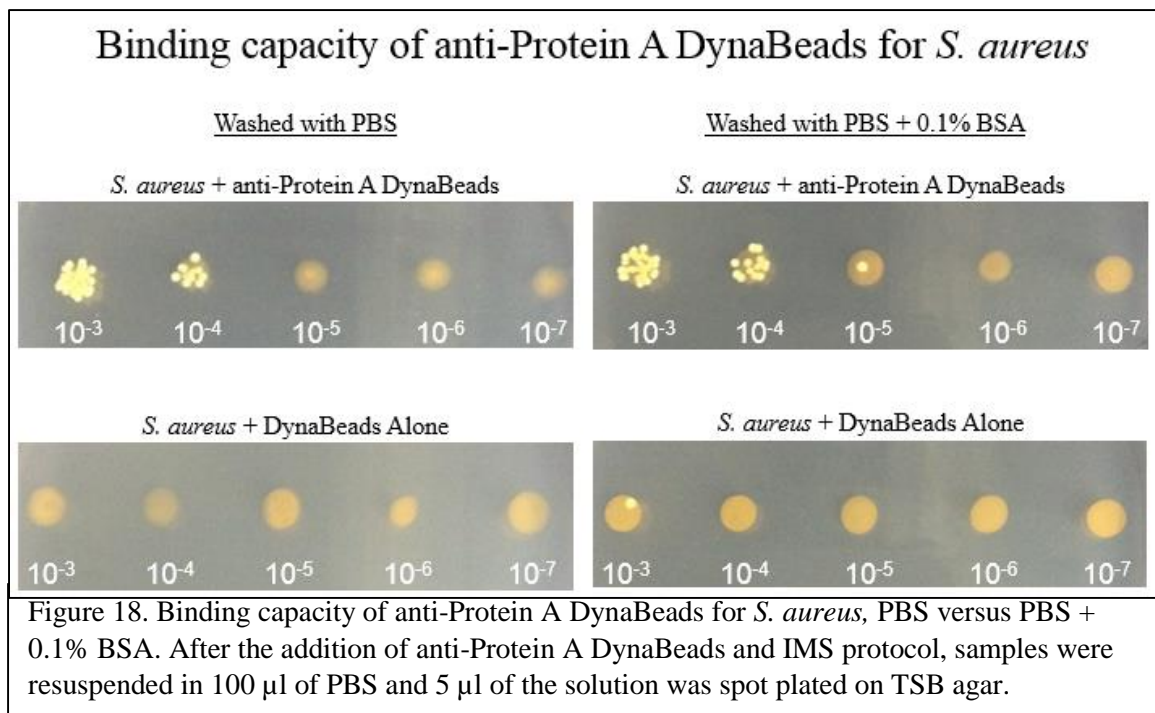
µl of DynaBeads alone were added. This dilution set was created to evaluate wash efficiency by media type. Figure 18 displays similar results in both capture and washing efficiency between PBS and PBS + 0.01% BSA. To prevent further non-specific binding via wash buffer, PBS and PBS + 0.01% Tween 20 were then evaluated for their ability to reduce non-specific binding. To evaluate, *S. aureus* was 10-fold serial diluted from overnight cultures (10^{-3} to
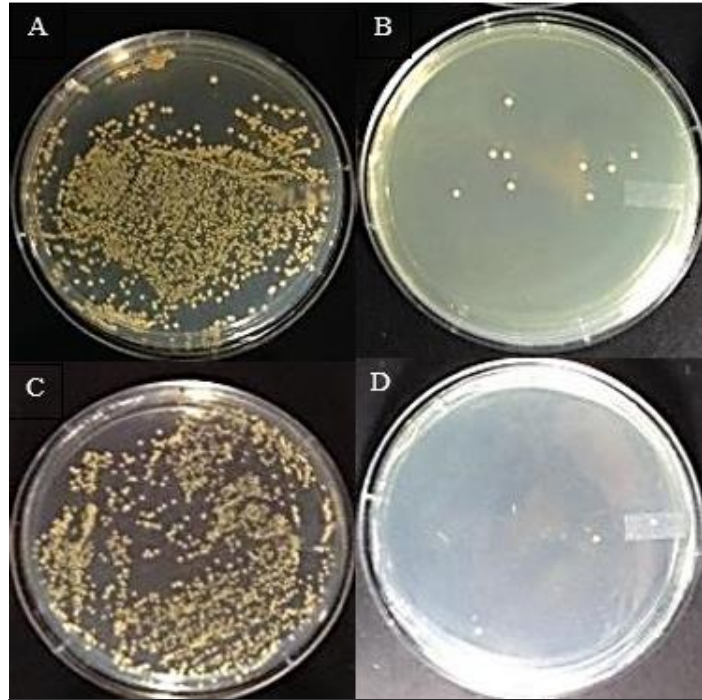
Figure 19. Non-specific binding evaluated with different wash buffer. PBS and PBS + 0.01% Tween 20 were evaluated for their ability to reduce non-specific binding during IMS protocol. (A) *S. aureus* input in 10^{-5} dilution after 4 washes with PBS alone. (B) 10^{-5} *S. aureus* dilution after 4 washes with PBS alone. (C) *S. aureus* input in 10^{-5} dilution after 4 washes with PBS + 0.01% Tween 20. (D) 10^{-5} *S. aureus* dilution after 4 washes with PBS + 0.01% Tween 20.

10^{-6}). To each dilution, 20 µl of anti-Protein A DynaBeads were added, rotated for 1 hour at $22\,^{\circ}$C, and washed four times with 450 µl of either PBS or PBS + 0.01% Tween 20. Another serial dilution set (10^{-3} to 10^{-6}) was generated and 20 µl of DynaBeads alone were added. After the washes with the appropriate media, 100 µl of each dilution was spread plated and colonies were counted in order to calculate and compare capture efficiencies. Figure 19 displays the colonies input into a sample compared to the non-specific binding for the same dilution. When washed with PBS alone, the DynaBeads alone (non-specific binding) displayed an average capture efficiency of 0.83% $\pm$ 0.41.

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When washed with PBS + 0.01% Tween 20, the DynaBeads alone displayed an average capture efficiency of 0.17% $\pm$ 0.13. Although, the media type did not did not display a significant difference in the option between PBS and PBS + 0.01% Tween 20, PBS + Tween did perform better when reducing non-specific binding. This led us to use PBS + Tween as our main wash buffer after the addition of anti-
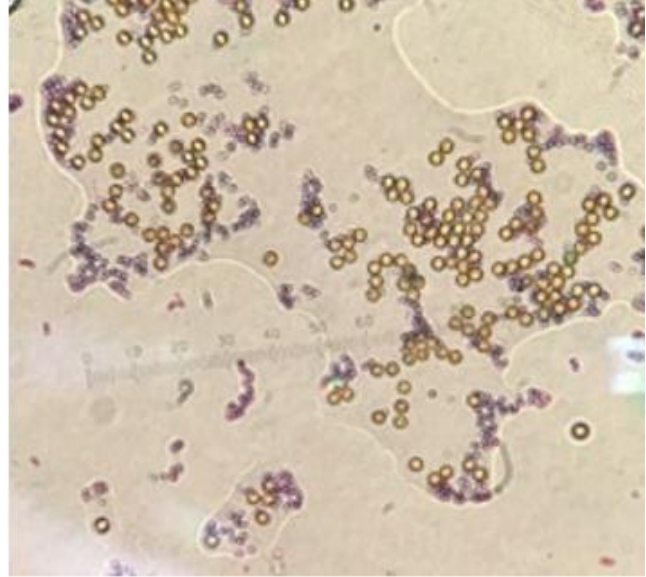
Figure 20. Gram stain of anti-Protein A DynaBeads bound to *S. aureus*. Gram stain was observed under 1000x magnification. *S. aureus* are the purple cells (average diameter is 0.6 μm) and the anti-Protein A DynaBeads are shown as yellow spheres (diameter 2.8 μm).

Protein A DynaBeads to a sample. After using the established protocol, a gram stain of *S. aureus* bound to anti-Protein A DynaBeads was conducted and imaged (Figure 20).

Analysis of capture efficiency and specificity of anti-Protein A DynaBeads for S. aureus in pure culture

In order to determine anti-Protein A DynaBeads specificity for *S. aureus,* pure cultures of *S. aureus* and 8 negative controls were utilized to determine capture efficiencies for each strain when using anti-Protein A DynaBeads. To perform this analysis, overnight cultures of the bacteria strains were 10-fold serial diluted (10^{-3} to 10^{-6}) in duplicate. One set of dilutions were utilized as the initial input of bacteria to the sample and the second set was utilized in order to calculate the number of cells captured by anti-Protein A DynaBeads (Figure 22 and 23). Capture efficiency was calculated as follows: Capture efficiency (%) = (1-B/A) x 100% [90]. A is the total number of cells

present in the sample (CFU/ml) and B is the number of cells unbound to the anti-Protein A DynaBeads (CFU/ml, input) (Table 5).

Capture efficiencies (%) were determined from pure culture samples. The average capture efficiencies were as follows: *S. aureus* 78.21 $\pm$ 2.01, *B. subtilis* 0.00 $\pm$ 0.00, *E. faecalis* 0.55 $\pm$ 0.21, *E. coli* 0.00 $\pm$ 0.00, *M. luteus* 0.00 $\pm$ 0.01, *S. epidermidis* 0.26 $\pm$ 0.34, *S. intermedius* 0.84 $\pm$ 0.95, *S. simulans* 0.07 $\pm$ 0.09, *S. pyogenes* 0.37 $\pm$ 0.69. Statistical analysis using one-way ANOVA with a Bonferroni multiple comparison test displayed all negative controls to have P-values less than 0.001 when compared to *S. aureus* 6538 (Figure 24).
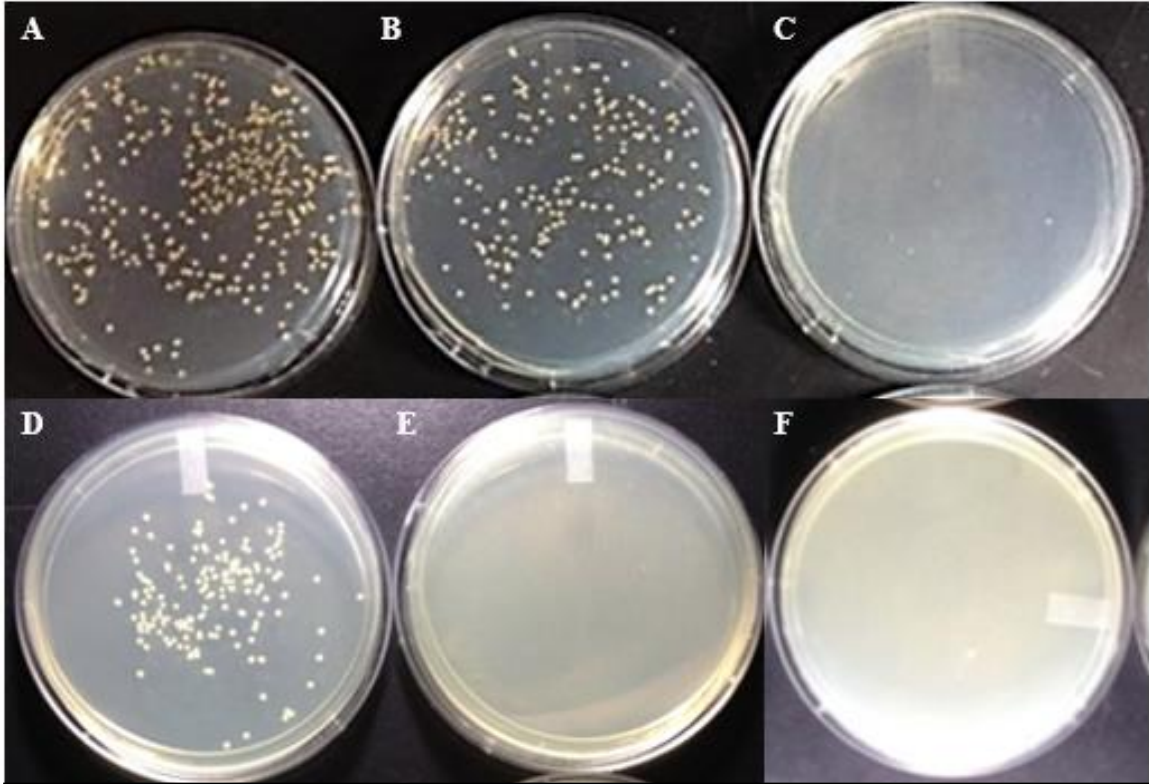
Figure 21. Anti-Protein A IMS capture for strains containing Protein A-like proteins. After 1 hour of anti-Protein A DynaBead exposure and a final wash (to remove unbound bacteria), the anti-Protein A DynaBeads were resuspended in 100 µl of PBS and spread plated on LB agar (10^{-6} dilution is pictured). (A) *S. aureus* input (the initial amount of bacteria added to the sample without anti-Protein A DynaBead exposure) (B) *S. aureus* bacteria captured (C) *S. aureus* combined with DynaBeads alone (D) *S. intermedius* input (the initial amount of bacteria added to the sample without anti-Protein A DynaBead exposure) (E) *S. intermedius* bacteria captured (F) *S. intermedius* combined with DynaBeads alone.
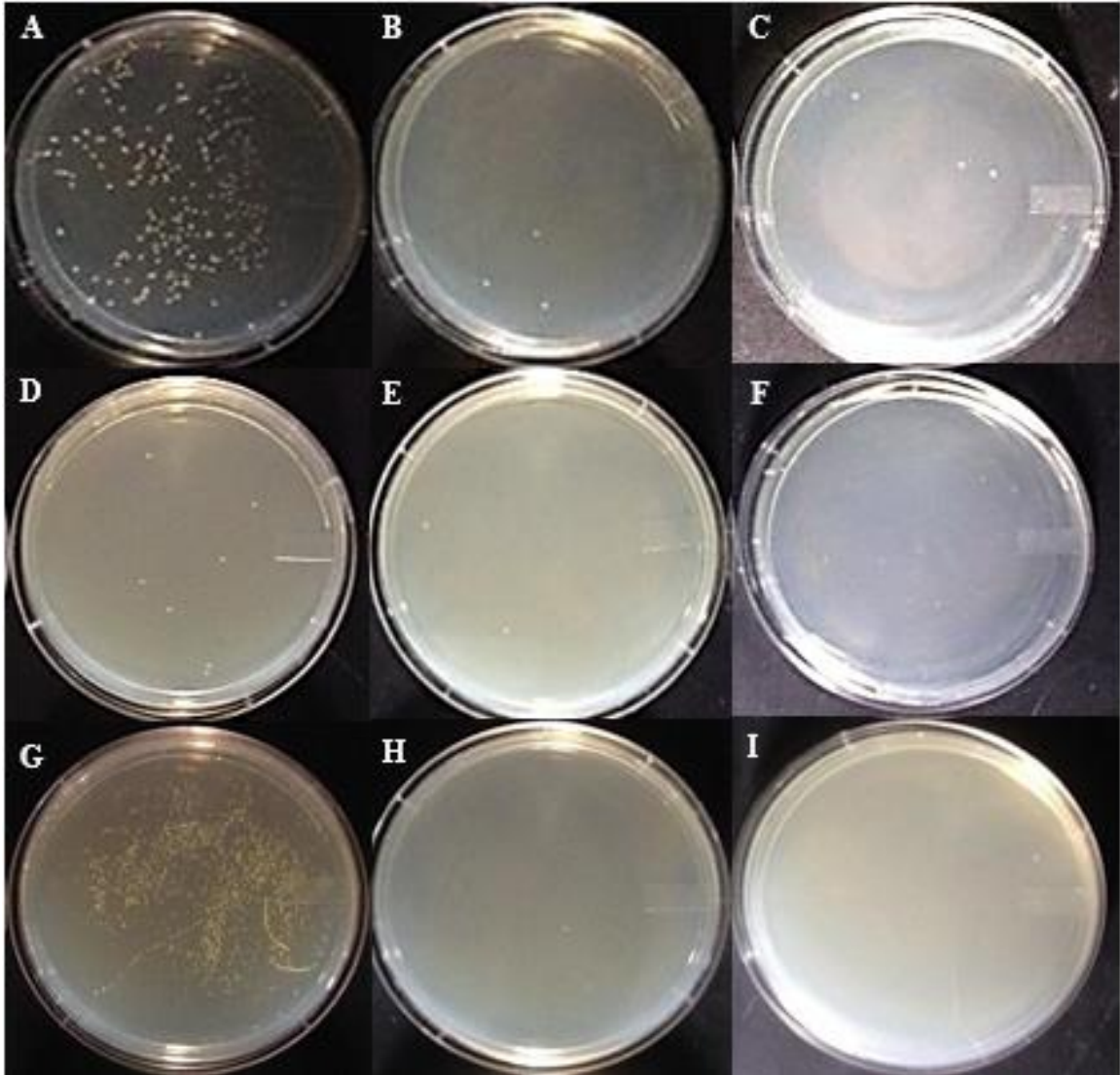
Figure 22. Specificity of conjugated anti-Protein A DynaBeads for negative controls alone. After 1 hour of anti-Protein A DynaBead exposure and a final wash (to remove unbound bacteria), the anti-Protein A DynaBeads were resuspended in 100 µl of PBS and spread plated on LB agar (10^{-6} dilution is pictured). (A) *S. epidermidis* input (the initial amount of bacteria added to the sample without anti-Protein A DynaBead exposure) (B) *S. epidermidis* bacteria captured (C) *S. epidermidis* combined with DynaBeads alone (D) *S. pyogenes* input (E) *S. pyogenes* bacteria capture (F) *S. pyogenes* combined with DynaBeads alone (G) *M. luteus* input (H) *M. luteus* bacteria capture (I) *M. luteus* combined with DynaBeads alone.
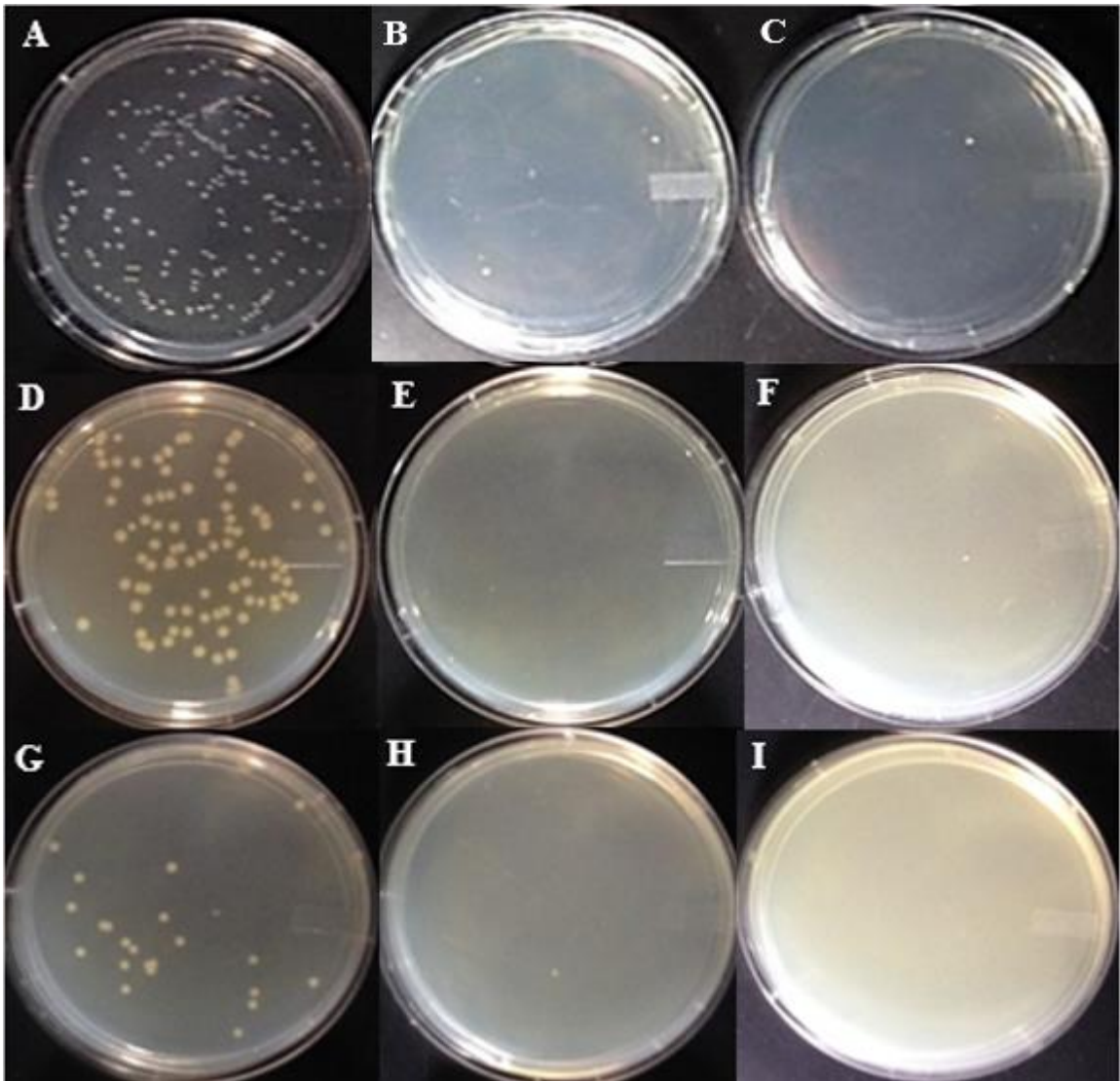
Figure 23. Specificity of conjugated anti-Protein A DynaBeads for negative controls alone. After 1 hour of anti-Protein A DynaBead exposure and a final wash (to remove unbound bacteria), the anti-Protein A DynaBeads were resuspended in 100 µl of PBS and spread plated on LB agar (10^{-6} dilution is pictured). (A) *E. faecalis* input (the initial amount of bacteria added to the sample without anti-Protein A DynaBead exposure) (B) *E. faecalis* bacteria captured (C) *E. faecalis* combined with DynaBeads alone (D) *E. coli* input (E) *E. coli* bacteria capture (F) *E. coli* combined with DynaBeads alone. (G) *B. subtilis* input (H) *B. subtilis* bacteria capture. (I) *B. subtilis* combined with DynaBeads alone.

Table 5. Capture Efficiencies for anti-Protein A DynaBeads from pure culture.		
Organism	**Dilution**	**Capture Efficiency (%)**
S. aureus	10^{-3}	82.08 ± 14.36
	10^{-4}	82.08 ± 14.36
	10^{-5}	78.59 ± 15.75
	10^{-6}	70.10 ± 23.99
S. epidermidis	10^{-3}	0.84 ± 0.20
	10^{-4}	0.84 ± 0.20
	10^{-5}	0.84 ± 0.20
	10^{-6}	0.25 ± 0.50
S. pyogenes	10^{-3}	0.37 ± 0.31
	10^{-4}	1.33 ± 1.18
	10^{-5}	0.00 ± 0.00
	10^{-6}	0.00 ± 0.00
E. faecalis	10^{-3}	0.55 ± 0.03
	10^{-4}	0.66 ± 0.36
	10^{-5}	0.25 ± 0.18
	10^{-6}	1.27 ± 1.87
E. coli	10^{-3}	0.00 ± 0.00
	10^{-4}	0.00 ± 0.00
	10^{-5}	0.00 ± 0.00
	10^{-6}	0.00 ± 0.00
M. luteus	10^{-3}	0.01 ± 0.01
	10^{-4}	0.00 ± 0.00
	10^{-5}	0.00 ± 0.00
	10^{-6}	0.00 ± 0.00
B. subtilis	10^{-3}	0.00 ± 0.00
	10^{-4}	0.00 ± 0.00
	10^{-5}	0.00 ± 0.00
	10^{-6}	0.00 ± 0.00
S. intermedius	10^{-3}	1.87 ± 0.00
	10^{-4}	0.67 ± 0.00
	10^{-5}	0.00 ± 0.00
S. simulans	10^{-3}	0.05 ± 0.03
	10^{-4}	0.05 ± 0.01
	10^{-5}	0.17 ± 0.12
	10^{-6}	0.00 ± 0.00
S. chromogenes	10^{-3}	2.03 ± 0.00
	10^{-5}	0.00 ± 0.00
	10^{-6}	0.00 ± 0.00
S. haemolyticus	10^{-3}	1.48 ± 0.00
	10^{-5}	0.00 ± 0.00
	10^{-6}	0.00 ± 0.00
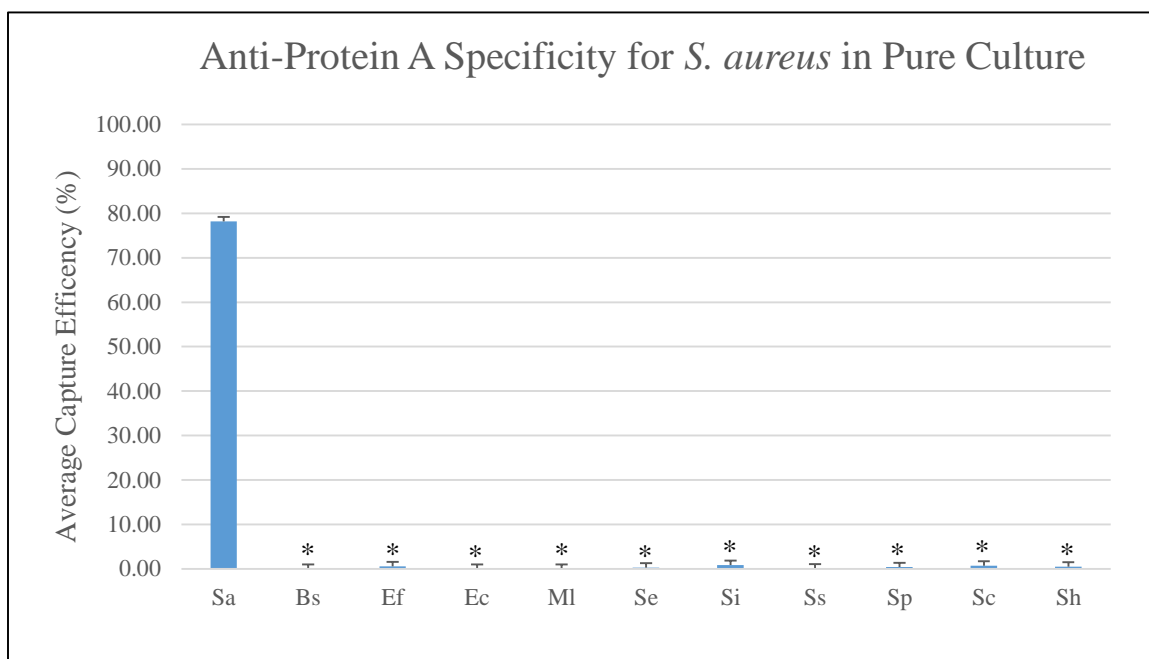
Figure 24. Anti-Protein A specificity for *S. aureus* in pure culture. Sa refers to *S. aureus* 6538, Bs refers to *B. subtilis,* Ef refers to *E. faecalis,* Ec refers to *E. coli,* Ml refers to *M. luteus,* Se refers to *S. epidermidis,* Si refers to *S. intermedius,* Ss refers to *S. simulans,* Sp refers to *S. pyogenes,* Sc refers to *S. chromogenes,* and Sh refers to *S. haemolyticus.* For each bacteria strain, average bacteria inputs were calculated (CFU/ml): *S. aureus* 3.81 x 10^9 CFU/ml, *B. subtilis* 6.04 x 10^9 CFU/ml, *E. faecalis* 1.18 x 10^9 CFU/ml, *E. coli* 1.04 x 10^9 CFU/ml,, *M. luteus* 1.06 x 10^9 CFU/ml, *S. epidermidis* 2.21 x 10^9 CFU/ml, *S. intermedius* 7.47 x 10^8 CFU/ml, *S. simulans* 1.15 x 10^9 CFU/ml, *S. pyogenes* 3.07 x 10^7 CFU/ml. *S. chromogenes* 4.86 x10^8 CFU/ml and *S. haemolyticus* 4.20 x 10^8 CFU/ml. Statistical significance (P-value) was determined by comparing results of each negative control strain to *S. aureus.* One asterisk (*) denotes a P-value less than 0.001.

Analysis of *S. aureus* isolation from whole milk

To determine anti-Protein A DynaBeads ability to capture *S. aureus* from whole milk, capture efficiencies were calculated for *S. aureus, B. subtilis, L. monocytogenes,* and DynaBeads alone. Initial inputs of the bacteria were also determined in order to calculate capture efficiencies. The whole milk alone was also plated out in order to determine the concentration of naturally occurring bacteria within the milk (Figure 25). Whole milk alone typically contains multiple *Bacillus* species, *Lactobacillus* species, and *Streptococcus* species. *S. aureus* is rarely found in Pasteurized milk from healthy

animals; however, animals with mammary glands infected by *S. aureus* can lead to milk

contamination [26]. To determine if *S. aureus* could be captured with anti-Protein A

DynaBeads from a complex biological fluid like whole milk, 50 µl of an overnight

culture of *S. aureus* was initially mixed with 450 µl of whole milk (10^{-1} dilution). This

sample was then used to make further 10-fold dilutions down to 10^{-6}. Twenty microliters

of anti-Protein A DynaBeads were added to the 10^{-3}, 10^{-5}, and 10^{-6} dilutions, then

processed as described above to assess capture efficiencies.

Capture efficiencies within whole milk displayed reduced efficiency for *S. aureus*

compared to capture in TSB media (Table 5). In whole milk, *S. aureus* capture efficiency

was 54.89% $\pm$ 5.78, while negative controls *B. subtilis* and *L. monocytogenes* capture

efficiencies were both 0.00% $\pm$ 0.00. DynaBeads alone (not bound to anti-Protein A

antibodies) were also added to samples containing *S. aureus, B. subtilis,* and *L.*

monocytogenes where they displayed 0.00% $\pm$ 0.00 capture efficiencies in all samples

(Table 5, Figure 26 and 27). Statistical analysis using one-way ANOVA Bonferroni

multiple comparison test displayed significance with a P-value less than 0.001 when

comparing the capture efficiencies of *B. subtilis* to *S. aureus*, *L. monocytogenes* to *S.*

aureus, and DynaBeads alone to *S. aureus.*
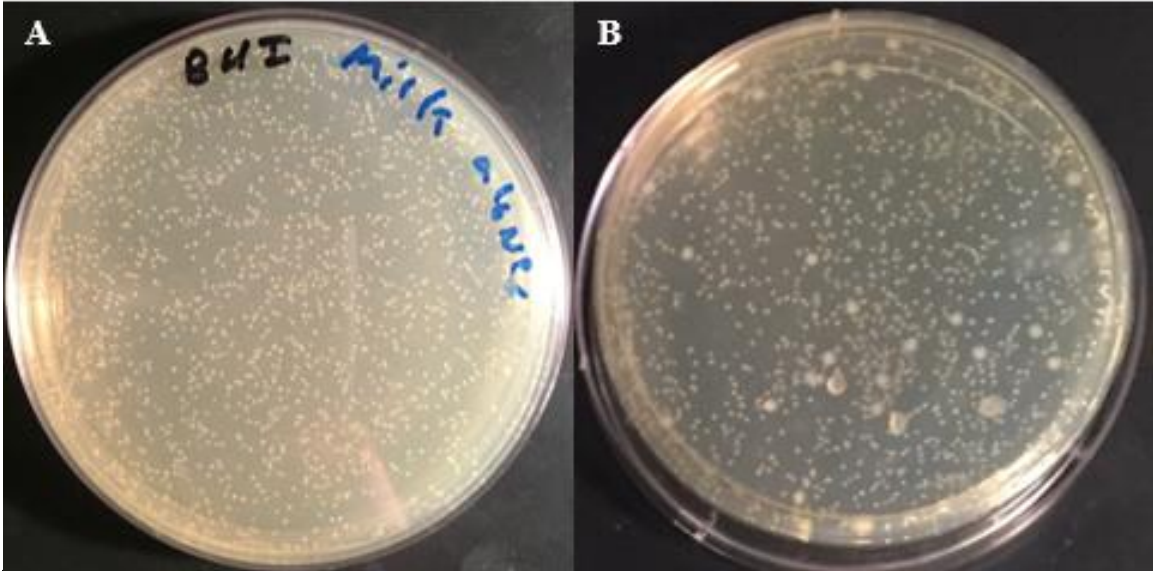
Figure 25. Naturally occurring bacteria from whole milk. Naturally occurring bacteria contaminants found within pasteurized whole milk. Performed in duplicate, 10-fold dilutions of pure whole milk were generated and 100 µl of a 10^{-6} dilution was spread plated on TSB agar and incubated at 37 °C for 18 hours. (A) Naturally occurring bacteria from pasteurized whole milk performed (B) performed in duplicate from separate milk sources. Bacterial strain variation displayed between the two milk sources.
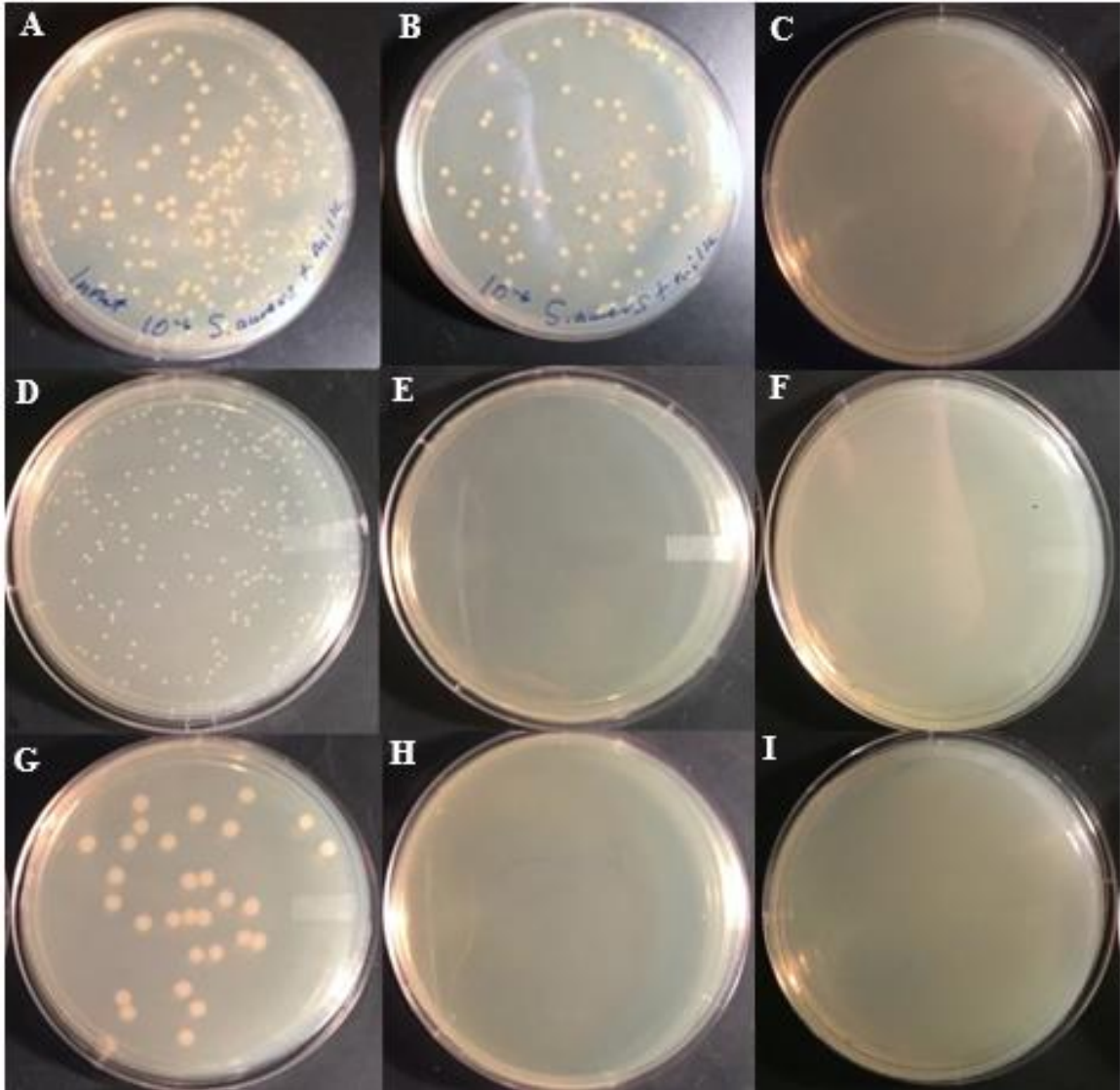
Figure 26. Specificity of anti-Protein A antibodies for *S. aureus* alone in pure culture of whole milk. 100 µl of 10^{-6} dilution was spread plated of bound anti-Protein A DynaBeads. (A) *S. aureus* input without anti-Protein A DynaBeads. (B) *S. aureus* captured from milk by anti-Protein A DynaBeads. (C) *S. aureus* combined with DynaBeads alone in milk (D) *L. monocytogenes* input without anti-Protein A DynaBeads. (E) *L. monocytogenes* captured from milk by anti-Protein A DynaBeads. (F) *L. monocytogenes* combined with DynaBeads alone (G) *B. subtilis* input without anti-Protein A DynaBeads. (H) *B. subtilis* captured from milk by anti-Protein A DynaBeads (I) *B. subtilis* combined with DynaBeads alone.

Table 6. Average capture efficiencies of *S. aureus* from pasteurized whole milk.		
Organism	**Dilution**	**Average Capture Efficiency (%)**
S. aureus	10^{-3}	57.53 ± 3.87
	10^{-5}	57.53 ± 3.87
	10^{-6}	49.62 ± 7.32
B. subtilis	10^{-3}	0.00 ± 0.00
	10^{-5}	0.00 ± 0.00
	10^{-6}	0.00 ± 0.00
L. monocytogenes	10^{-3}	0.00 ± 0.00
	10^{-5}	0.00 ± 0.00
	10^{-6}	0.00 ± 0.00
DynaBeads alone	10^{-3}	0.00 ± 0.00
	10^{-5}	0.00 ± 0.00
	10^{-6}	0.00 ± 0.00
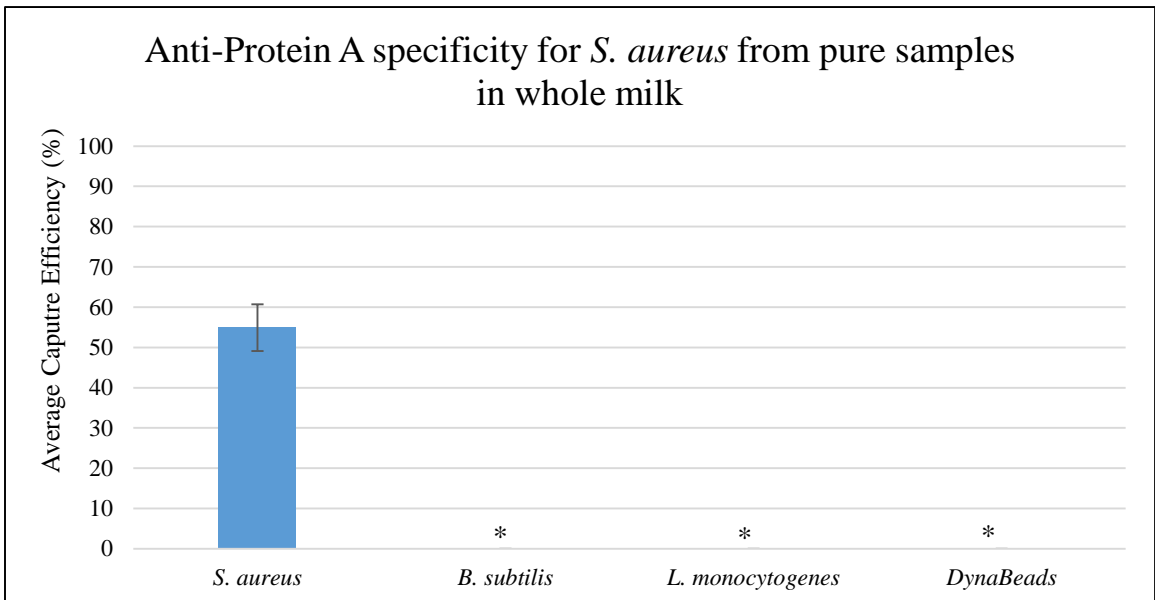
Figure 27. Average capture efficiencies (%) of *S. aureus* 6538 and negative control strains in whole milk and for each bacteria strain, average bacteria inputs were calculated (CFU/ml). *S. aureus* capture efficiency of 54.89% $\pm$ 5.78 with an initial input of 5.54 x 10^9 CFU/ml, *B. subtilis* capture efficiency of 0.00% $\pm$ 0.00 with an initial input of 7.22 x 10^8 CFU/ml, and *L. monocytogenes* with a capture efficiency of 0.00% $\pm$ 0.00 with an initial input of 2.44 x 10^9 CFU/ml. Statistical significance (P-value) was determined by comparing results of each negative control strain to *S. aureus*. One asterisk (*) denotes a P-value less than 0.001.

DISCUSSION

Due to anti-*Staph* DynaBeads lack of capture specificity for *S. aureus,* anti-Protein A antibodies were chosen in order to aid in increasing capture efficiency rate for *S. aureus* and reduce the rate of non-specific binding of negative controls. Protein A is expressed on the surface of nearly all *S. aureus* strains and occupies approximately 7% of the *S. aureus* cell surface [30]. By utilizing an antibody that is specific to a *S. aureus* surface protein rather than against whole *S. aureus* cells (anti-*Staph* DynaBeads), we hoped to increase the specificity of capture and reduce non-specific binding. Prior to the use of anti-Protein A DynaBeads, we needed to develop IMS methods for the greatest recovery of *S. aureus* cells. To develop anti-Protein A IMS methods, we needed to determine if the wash buffer altered non-specific binding of bacteria to the walls or cap of microcentrifuge tubes, or the DynaBeads themselves. The following media types were utilized in the washing process and evaluated for efficiency: PBS, PBS + 0.1% BSA, and PBS + 0.01% Tween 20. PBS is a balanced salt solution used for a variety of cell culture applications including washing cells [70]. BSA is commonly used to reduce non-specific hydrophobic binding and Tween 20 has been used as a blocking agent in immunoassays [73,85]. The addition of a 0.1% of either BSA or 0.01% Tween 20 to PBS was evaluated for its effect on non-specific binding.

The first comparison of media wash efficiency was done by evaluating PBS and PBS + 0.1% BSA. Anti-Protein A DynaBeads were added to pure cultures of *S. aureus,* mixed for one hour to induce capture and then washed 4 times with either PBS or PBS + 0.1% BSA. After the washing process, the samples were spot plated on TSB agar and

incubated at 37 °C for 18 hours. Figure 18 shows that there is not a significant difference between the capture rate when using either PBS or PBS + 0.1% BSA. To determine if non-specific binding was the cause of *S. aureus* capture, rather than actual anti-Protein A capture, DynaBeads alone were added to pure cultures of *S. aureus,* mixed for one hour to induce capture and then washed 4 times with either PBS or PBS + 0.1% BSA.   After the washing process, the samples were spot plated on TSB agar and incubated at 37 °C for 18 hours. Figure 18 showed no non-specific binding when using PBS as a wash buffer, while PBS + BSA displayed minimal non-specific capture.

To further analyze wash buffer efficiency, non-specific binding between PBS was then compared to PBS + 0.01% Tween 20. To evaluate PBS and PBS + Tweens ability to reduce non-specific binding, DynaBeads alone were added to samples containing *S. aureus* and mixed for one hour to induce capture and then washed 4 times with either PBS or PBS + 0.01% Tween. When washed with PBS alone, the DynaBeads alone (non-specific binding) displayed an average capture efficiency of 0.83% $\pm$ 0.41. When washed with PBS + 0.01% Tween, the DynaBeads alone (non-specific binding) displayed an average capture efficiency of 0.17% $\pm$ 0.13. Although there was not a significant difference in non-specific binding, we decided to utilize PBS + 0.01% Tween in order to reduce as much non-specific binding within a sample as possible.

Upon the development of IMS methods for *S. aureus* capture, the specificity of anti-Protein A DynaBeads for *S. aureus* in pure culture was next evaluated. Both capture efficiencies of *S. aureus* and non-specific binding of negative controls were calculated and statistical significance was determined by one-way ANOVA Bonferroni multiple comparison test. By performing this set of experiments we wanted to determine if anti-

Protein A DynaBeads had specificity for *S. aureus* compared to negative controls. We utilized a wide variety of negative controls to represent organisms that may be found in naturally occurring swab specimens including: *M. luteus, S. pyogenes,* and *S. epidermidis* which represent normal skin microbes. *E. faecalis,* which can sometimes be found in skin ulceration with *S. aureus. E. coli*, a Gram-negative gut microbe containing pili on its cell surface, was also utilized as a negative control to challenge our IMS methods [41]. Utilizing IMS with anti-Protein A DynaBeads resulted an average *S. aureus* capture efficiency of 82.00% of the initial input of cells. Comparing the capture efficiency of *S. aureus* to other normal flora negative controls (*M. luteus, S. pyogenes, E. faecalis* and *S. epidermidis)* and *E. coli*, the negative control strains were captured on average less than 1.00% and statistical significance was displayed with P-value less than 0.001 (Table 5, Figure 24).

S. simulans and *S. intermedius* were also utilized to test the specificity of the anti-Protein A DynaBeads specificity for capturing *S. aureus.* As previously mentioned, Protein A is a surface protein specific to *S. aureus.* However, after conducting a BLAST search of *S. aureus* Protein A (Table 1), it was determined that a limited number of other *Staphylococcus* species also contain a protein with some homology to *S. aureus* Protein A. To determine if our antibody was specific to *S. aureus* Protein A, *S. intermedius* (61% Percent Identity to *S. aureus* Protein A) and *S. simulans* (34% Percent Identity to *S. aureus* Protein A) were utilized as negative controls. Comparing the average capture efficiency of *S. aureus* (82.00%) to the capture efficiencies of Protein A containing *Staphylococcus* strains, *S. intermedius* and *S. simulans* both had average capture efficiencies less than 1.00% (Table 5, Figure 24). These results display statistically

significant evidence to suggest that anti-Protein A DynaBeads have specificity for *S. aureus*.

To test anti-Protein A DynaBead specificity for *S. aureus* in a complex biological, IMS methods were performed in pasteurized whole milk and capture efficiencies for *S. aureus* and other negative controls were determined. Milk related pathogens include: *Salmonella, E. coli, Listeria,* and *Staphylococcus* [16]. Non-pathogenic strains such as *Streptococcus*, *Bacillus,* and *Lactobacillus* can also be found in milk samples even after Pasteurization [72]. Figure 25 displays the naturally occurring bacteria that were found specifically in our Pasteurized whole milk which included *Lactobacillus, Streptococcus,* and *Bacillus* species. For our negative control strains we chose *L. monocytogenes* and *B. subtilis. L. monocytogenes* is commonly responsible for dairy related food-poisoning and *B. subtilis* has also been found to be an environmental contaminant in milk [25,72]. As described above, IMS methods were performed in whole milk and capture efficiencies and statistical significance was calculated for *S. aureus* and negative controls. After IMS methods were performed, *S. aureus* capture efficiency was 54.89% $\pm$ 5.78*, B. subtilis* capture efficiency was 0.00% $\pm$ 0.00*,* and *L. monocytogenes* capture efficiency was 0.00% $\pm$ 0.00 (Table 6, Figure 27)*.* When comparing the capture efficiencies of *S. aureus* to both negative controls, we observed statistical significance with a P-value less than 0.001 (Figure 27). DynaBeads alone were also added to samples containing each of the above mentioned bacteria. There was no non-specific binding, further supporting the efficiency of our IMS washing method with PBS containing 0.01% Tween (Table 6, Figure 26).

In conclusion, anti-Protein A DynaBeads displayed significant specificity for *S. aureus* in both pure culture and when isolated from whole milk. In pure culture, IMS capture efficiency was 82% compared to 54% in whole milk. Although *S. aureus* was captured in whole milk, we hypothesized that there would be a reduction in recovery rate due to the complexity of the fluid and the increased rate of competing cells and particles. If IMS methods were to be performed in whole milk in the future, increasing the capture/binding time of anti-Protein A DynaBeads from 1 hour to 2 hours could potentially lead to an increase in capture efficiency for *S. aureus.*

CHAPTER FIVE: COMBINING IMMUNOMAGNETIC SEPARATION AND SINGLE LASER-LIGHT SCATTERING FOR RAPID IDENTIFICATION

INTRODUCTION

In the previous chapter, protocol for IMS of *S. aureus* utilizing anti-Protein A DynaBeads was established. Following *S. aureus* capture, an enrichment step needed to be developed in order to obtain a concentration of bacteria high enough for laser-light scattering identification (which is approximately 1,000 bacteria cells per milliliter) [37]. To determine the optimal conditions for enrichment, variables were altered in order to determine optimal conditions for the IMS of *S. aureus* and identification via laser-light scattering. Variables that were altered included: media volume, media filtration versus non-filtered media, enrichment duration, and sample aeration (200-300 rpm), and incubation.

A protocol was established for anti-Protein A DynaBead *S. aureus* capture, enrichment of bacteria, and subsequent identification via laser-light scattering. Following the development of this protocol, the ability to capture, enrich, and identify *S. aureus* via laser-light scattering was tested on *S. aureus* and the negative control strains (*S. pyogenes, M. luteus, E. faecalis, L. monocytogenes, E. aerogenes,* and *E. coli*). In order to evaluate its specificity for *S. aureus, S. aureus* was measured alone or mixed with negative controls to create a more complex and realistic specimen sample. The capture efficiency and accuracy of the rapid identification methods were then assessed. Percent identified as *Staphylococcus* species was calculated and statistical significance was determined by a one-way ANOVA Bonferroni multiple comparison test.

In typical wound infection and skin/soft tissue infections, specimens are collected from these types of infection sites on sterile swabs. In order to determine if IMS could successfully capture *S. aureus* from a swab sample, we inoculated sterile swabs with a broth culture containing dilutions of *S. aureus*. The swabs were transferred to a test tube containing TSB growth media. The previously established capture, enrichment, and identification protocol was used to isolate the bacteria from swab specimens. Since *S. aureus* is not always isolated in pure culture from the site of infection, we created mixed swab samples to replicate a sample containing normal skin microorganisms. *S. aureus* were combined with *S. pyogenes*, *M. luteus*, and *S. epidermidis* and the previously mentioned methods were repeated. To further assess our developed methods, bacteria taken from healthy human skin and nose swabs were mixed with *S. aureus*. The use of bacteria from human swab samples (likely containing multiple bacterial species) allowed us to increase the complexity of the samples and replicate a mixture of microbes that might be seen from an actual infection site. This set of experiments allowed us to assess if our IMS and laser-light scattering identification methods can be utilized on complex biological samples. Finally, in order to determine the amount of bacteria that grows within a sample during the enrichment stage over time, samples were magnetically separated after each hour for five hours. The average growth (CFU/ml) by enrichment over a 5 hour time frame from swab specimen collection was determined in order to quantify the number of progeny produced during the enrichment step. To calculate this, after IMS capture, each hour, the tubes were magnetically separated for 3 minutes and the supernatant was spread plated on TSB agar and incubated at 37°C for 18 hours. Colony forming units were then calculated.

IMS capture, enrichment, and laser-light scattering identification

Development of IMS capture, enrichment, and laser-light scattering identification methods

Once anti-Protein A DynaBeads specificity was been established for *S. aureus*, an enrichment step needed to take place in order to obtain the proper concentration of bacteria for laser-light scattering identification (approximately 1,000 bacteria cells per milliliter) [37]. To determine the optimal conditions for enrichment 10-fold serial dilutions from overnight cultures (10^{-3} to 10^{-8}) were utilized to represent different concentrations of the bacteria that could be obtained in a clinical sample. Variables that were altered in order to determine optimal conditions for IMS and laser-light scattering identification protocol occurred during each step of the protocol. During IMS protocol the temperature (22°C and 37°C) during IMS binding was tested for effects on binding efficiency. Enrichment step variables altered included: growth media volume, filtered versus non-filtered media, enrichment duration, and sample aeration (200-300 rpm). MIT sample preparation variables altered included: (1) directly testing bacteria resuspended in filtered TSB growth media or (2) testing bacteria resuspended in filtered water after pelleting the capture cells and removing TSB growth media. We also tested if DynaBead removal, after the enrichment step and prior to laser-light scattering identification, would increase the accuracy of identification since DynaBeads are small particles that could interfere with laser-light scattering measurements.

In order to determine optimal conditions, the previously establish IMS protocol was used with the exception of a temperature change during binding from $22\degree$C to $37\degree$C. After washing the 20 µl of conjugated anti-Protein A DynaBeads bound to *S. aureus* in the sample, the DynaBeads were resuspended in 25-50 µl of either non-filtered TSB or filtered TSB liquid media (Thermo Scientific Nalgene vacuum filtration system, 0.2 micron filter*)* and transferred into sterile microcentrifuge tubes. The tube transfer limited the unwanted transfer of unbound cells that could be stuck on the sides/tops of the tubes. After the transfer, the tubes were incubated at $37\degree$C for 1-5 hours. Each hour the tubes were magnetically separated for a minimum of 3 minutes. MIT sample preparation was either conducted by either directly testing about 1.0 µl of the supernatant from the media or by pelleting the cells within the sample (centrifuging for 1 minute at top speed), removing the liquid media and resuspending the cells in filtered water before testing in the MIT 1000. The optimal time for enrichment and the optimal bacterial concentrations were evaluated for accurate identification of *S. aureus* using laser-light scattering analysis. The final variable evaluated during MIT sample preparation was the addition of one 150 µl filtered water wash after pelleting and removing the media. All of the above mentioned variables were adjusted one at a time in order to establish IMS capture, enrichment, and laser-light scattering identification methods for rapid *S. aureus* detection.

IMS capture, enrichment, and laser-light scattering identification (established methods)

Fifty microliters of bacterial specimens from overnight cultures were 10-fold serial diluted (10^{-2} to 10^{-6}) in 450 µL of TSB, 20 µL of the anti-Protein A DynaBeads were added to the specimens and rotated for 1 hour at $37\degree$C. The samples were then magnetically separated for 3 minutes, the TSB was removed and anti-Protein A

DynaBeads were resuspended in 450 µL of PBS plus 0.01% Tween 20. The samples were then rotated for 1 minute at room temperature and then magnetically separated for 3 minutes. This process was repeated for a total of 4 washes. The anti-Protein A DynaBeads were then transferred into a sterile microcentrifuge tubes with 50 µL of PBS. The transfer liquid (PBS) was then removed and the DynaBeads were resuspended in 50 µl of filtered TSB.

The bacteria captured by the anti-Protein A DynaBeads were enriched in 50 µL of filtered TSB. The surface tension of each sample was broken and the tubes were shaken at 250 rpm and incubated at 37 °C for 5 hours. The samples were vortexed for 20-30 seconds and magnetically separated for 3 minutes. The supernatant was removed and transferred to a new microcentrifuge tube. This step was performed in order to reduce DynaBead transfer, which may interfere with laser-light scattering measurements. The supernatant was pelleted (centrifuged for 1 minute at 14000 rpm) and the media was removed. The pellet was then washed with 150 µL of filtered water, vortexed to break up the pellet and centrifuged for 1 minute at top speed. The filter water was then removed and the pelleted bacteria is then ready for laser-light scattering analysis. Filtered water (10-50 µL) was then added to the prepared sample, vortexed for 20-30 seconds and magnetically separated for 3 minutes. A portion of the sample was loaded into a prepared water vial and a laser-light scattering identification analysis is conducted.

Rapid laser-light scattering identification from IMS samples (experiment)

In order to determine the efficiency of the established IMS laser-light scatting rapid identification methods, the procedure was tested on both *S. aureus* and the negative control strains (*S. pyogenes, M. luteus, E. faecalis, L. monocytogenes, E. aerogenes,* and

E. coli) in order to evaluate its specificity for *S. aureus* alone. Using previously established methods, bacterial strains alone were 10-fold serial diluted from overnight cultures in duplicate and anti-Protein A DynaBeads were added to each dilution (10^{-2} - 10^{-6}) of one set of the serial dilutions. The other set of serial dilutions were utilized for counting the initial input of bacteria into each sample. To determine the initial input for each sample, each tube was vortexed for 10 seconds to resuspend the bacteria in the tube and 100 µl was spread plated on TSB agar plates and incubated at 37 °C for 18 hours (performed in triplicate). Colony forming units per milliliter (CFU/ml) were calculated for each tube. The samples containing DynaBeads went through the established IMS and enrichment protocol and were then tested in the MIT 1000 for laser-light scattering identification. The same methods were then utilized in order to perform combination challenges between *S. aureus* and equal concentrations of the negative controls (*S. aureus* and *S. pyogenes, S. aureus* and *M. luteus, S. aureus* and *E. faecalis, S. aureus* and *L. monocytogenes, S. aureus* and *E. aerogenes, S. aureus* and *E. coli).* Combination challenges utilizing *S. aureus* and two negative controls were also utilized (*S. aureus, S. pyogenes,* and *M. luteus AND S. aureus, S. pyogenes,* and *E. coli*).

IMS capture, enrichment, and laser-light scattering identification from swab specimens

Swab specimen collection technique

Swab specimen collection protocol was attained and altered from Panpradist et al. 2014. Ten-fold serial dilutions (10^{-2} to 10^{-6}) of *S. aureus* overnight cultures were generated from overnight cultures in TSB growth media. Swabs (Sterile polyester tipped applicator swabs, VWR) were submerged in initial serial dilutions for 10 seconds. Swabs were then submerged in a transfer microcentrifuge tube containing 450 µl of filtered

TSB. To translate the obtained bacteria to the new microcentrifuge tubes, the submerged swabs were dragged along the edge of the tube in a circular path at a rate of 1 cycle per second (1 HZ) for 10 seconds (10 second 1 HZ side twirl). The swab agitation was done by hand utilizing the timer as a reference for manual control of transfer time [68].

Development of IMS capture, enrichment, and laser-light scattering identification for swab specimens

The most common types of *S. aureus* infections in humans are skin infections, wound infections, and abscesses [55] [14]. Specimens are typically collected from these types of infection sites on sterile swabs. In order to demonstrate that our developed IMS capture methods could successfully capture *S. aureus* from swab samples, we inoculated sterile swabs with a broth culture containing dilutions (10^{-2} to 10^{-6}) of *S. aureus.* The swabs were transferred to a test tube containing growth media and vortexed to dislodge the bacterial cells from the swab to create a suspension. To capture the bacteria in the sample, swabs were removed and 10-20 µl of anti-Protein A DynaBeads were added to the tubes. Previously established IMS protocol was used to isolate the bacteria from swab specimens. The MIT instrument then measured the laser-light scattering pattern of the bacteria in the sample and determine the bacteria matches the known Identifier for *Staphylococcus* species (or *S. aureus* or MRSA when those Identifiers become available). Since *S. aureus* is not always isolated in pure culture from the site of infection, we then created mixed swab samples to replicate normal skin microorganisms. *S. aureus* was then combined with *S. pyogenes*, *M. luteus*, and *S. epidermidis* and the previously mentioned methods were be repeated.

Rapid bacterial identification from IMS swab samples (established protocol)

Bacterial strains were grown in TSB at 37 °C for 18-24 hours. Serial dilutions of cultures in filtered TSB (0.2 micron filter) were produced for IMS and MIT laser-light scattering identification testing. Serial dilutions were made in low-binding polymer microcentrifuge tubes (Life Technologies).

Bacterial specimens were obtained from 10-fold serial dilutions of overnight cultures via swab collection. Swab collections (10 seconds) were transferred into a microcentrifuge tube with 450 µL of filtered TSB. 20 µL of the anti-Protein A DynaBeads were added to the swab collected specimens and rotated for 1 hour at 37 °C. The samples were then magnetically separated for 3 minutes, the filtered TSB was removed and the anti-Protein A DynaBeads were resuspended in 450 µL of PBS plus 0.01% Tween 20. The samples were then rotated for 1 minute at room temperature and then magnetically separated for 3 minutes. This process was repeated for a total of 4 washes. The anti-Protein A DynaBeads were then transferred into natural microcentrifuge tubes with 50 µL of filtered TSB.

Anti-Protein A DynaBeads were enriched in 50 µL of filtered TSB. The surface tension of each sample was broken and the tubes were shaken at 250 rpm and incubated at 37 °C for 5 hours. The samples were vortexed for 20-30 seconds and magnetically separated for 3 minutes. The supernatant was removed and transferred to a new microcentrifuge tube. The supernatant was pelleted (centrifuged for 1 minute at top speed 14000 rpm) and the media was removed. The pellet was then washed with 150 µL of filtered water, vortexed to break up the pellet and centrifuged for 1 minute at top speed. The filter water was then removed and the pelleted bacteria was resuspended in 10-50 µl

of filter water, vortexed for 20-30 seconds and magnetically separated for 3 minutes. A portion of the sample (about 1 µl) was loaded into a prepared water vial and a MIT laser-light scattering identification test was conducted using previously mentioned MIT sample protocol.

Rapid bacterial identification from IMS swab samples (experiment)

In order to determine the accuracy of the established IMS and laser-light scattering identification swab methods, the procedure was tested on both *S. aureus* and the negative control strains (*S. pyogenes, M. luteus, E. faecalis, L. monocytogenes, E. aerogenes,* and *E. coli*) in order to evaluate its specificity for *S. aureus* alone. Using previously established IMS swab methods, bacterial strains alone were 10-fold serial diluted from overnight cultures in duplicate and the specimens were obtained by swabs, and dislodged into microcentrifuge tubes containing 450 µl of growth media, and 20 µl of anti-Protein A DynaBeads were added to each 10-fold dilution (10^{-2} - 10^{-6}) of one set of the serial dilutions. The other set of serial dilutions were utilized for counting the initial input of bacteria into each sample. To determine the initial input for each sample, each tube was vortexed for 10 seconds to resuspend the bacteria in the tube and 100 µl was spread plated on TSB agar plates and incubated at 37 $^{\circ}$C for 18 hours (performed in triplicate). Colony forming units per milliliter (CFU/ml) were calculated for each tube. The samples containing DynaBeads went through the established IMS swab protocol and were tested in the MIT 1000 for laser-light scattering identification.

The same methods were then utilized to capture and identify *S. aureus* in a mixed swab sample containing equal amounts of *S. aureus* and one negative control. The pairings were *S. aureus* and *S. pyogenes, S. aureus* and *M. luteus, S. aureus* and *E.*

faecalis, S. aureus and *L. monocytogenes, S. aureus* and *E. aerogenes, S. aureus* and *E. coli.* Combination challenges containing *S. aureus* and two negative controls were also utilized (*S. aureus, S. pyogenes,* and *M. luteus AND S. aureus, S. pyogenes,* and *E. coli*).

Rapid S. aureus identification following IMS of samples containing mixed human nose and skin microflora

Nose swabs were collected by first soaking the sterile swab in 0.9% saline solution. The swab was then inserted approximately 2 cm (~3/4 inches) into one nostril. The swab was then rotated against the anterior nasal mucosa for 10 seconds. The obtained specimen was then transferred into a microcentrifuge tube containing 450 µl of TSB media. Skin swabs were collected by first soaking the sterile swab in a 0.9% saline solutions. The swab was the rubbed against the inner forearm skin in a 2" x 2" square for 10 seconds. The obtained specimen was then transferred into a microcentrifuge tube containing 450 µl of TSB media. To the obtained swab samples, 50 µl of a *S. aureus* overnight culture was added and the existing swab IMS protocol, enrichment, and MIT laser-light scattering identification was conducted. This procedure was tested on the following samples: (1) skin swab and *S. aureus,* (2) nose swab and *S. aureus,* (3) skin, nose and *S. aureus,* (4) nose swab alone, (5) skin swab alone, and (6) an overnight of a direct nose swab.

Calculation of average bacteria growth by enrichment over time

The average growth (CFU/ml) by enrichment over a 5 hour time frame from swab specimen collection was determined in order to quantify the number of progeny produced during the enrichment step. To calculate this, 10-fold serial dilutions of a *S. aureus* overnight culture were created and 20 µl anti-Protein A DynaBeads were added to each dilution (10^{-3} to 10^{-6}). Using previously established IMS methods, *S. aureus* cells

captured by anti-Protein A DynaBeads were resuspended in filtered TSB agar and incubated at 37°C for 5 hours while shaken at 250 rpm. Each hour, the tubes were vortexed for 10 seconds, magnetically separated for 3 minutes, and the supernatant (50 µl) was spread plated on TSB agar and incubated at 37°C for 18 hours. Colony forming units were then calculated.

Calculation of capture efficiencies and statistical analysis

Capture efficiencies (CE) are defined as the percentage fraction of total bacteria captured on the surface of the anti-Protein A DynaBeads [90]. Capture efficiencies are calculated using the number of unbound cells in the starting dilution (input). The equation for Capture efficiency is calculated using the following equation: Capture efficiency(%) = (1-B/A) x 100% [90]. A is the total number of cells present in the sample (CFU/ml) and B is the number of cells unbound to the anti-Protein A DynaBeads (CFU/ml, input). Statistical analysis of capture efficiencies and MIT laser-light scattering identifications were performed with Prism 3 program (GraphPad Software, Inc.). Statistical significance was measured by one-way ANOVA utilizing Bonferroni multiple comparison test. Statistical significance was defined with a P-value less than 0.05 when comparing negative control strains to positive control *S. aureus* 6538. A P-value greater than 0.05 indicated that there was no statistical significance between capture efficiencies and laser-light scattering results when comparing negative controls to *S. aureus* 6538.

Development of IMS capture, enrichment, and rapid identification by laser-light scattering

In order to determine the optimal conditions for post-IMS rapid identification of *S. aureus* by laser-light scattering, various conditions were evaluated for their effects on successful identification. The following variables were altered: media type for enrichment, filtration of media for enrichment, removal of DynaBeads during enrichment, time of enrichment, removal of growth media after enrichment, and resuspension of captured bacteria in filtered water. Percent identification as *Staphylococcus* species under set conditions were evaluated and a one-way ANOVA Bonferroni multiple comparison test was conducted in order to determine statistical significance.

Figure 28 displays the results of *S. aureus* laser-light identification analysis under varying conditions. The no bead removal section of Figure 28 protocol was performed as indicated in the methods section with the following modifications: samples were bound in filtered TSB media at 22 °C, 1-3 hours of enrichment in filtered TSB at 37 °C, and magnetic separation of anti-Protein A DynaBeads and direct addition of supernatant to MIT vials for laser-light scattering analysis. These variables resulted in *S. aureus* identifying as *Staphylococcus* species in 30% of samples. The shaken at 215 rpm section of Figure 28 protocol was as follows: IMS methods performed in filtered TSB media at 22 °C, 1-3 hours of enrichment in filtered TSB while shaken at 215 rpm at 37 °C, magnetic separation of anti-Protein A DynaBeads and direct addition of supernatant to

MIT vials for laser-light scattering analysis. These conditions resulted in *S. aureus* identifying as *Staphylococcus* species in 45% of samples. The incubated binding section of Figure 28 protocol was as follows: IMS methods performed in filtered TSB media at 37 °C, 1-3 hours of enrichment in filtered TSB while shaken at 215 rpm at 37 °C, magnetic separation of anti-Protein A DynaBeads and direct addition of supernatant to MIT vials for laser-light scattering analysis. Under these conditions, *S. aureus* identified as *Staphylococcus* species in 36% of samples. The shaken at 250 rpm section of Figure 28 protocol was as follows: IMS methods performed in filtered TSB media at 37 °C, 1-3 hours of enrichment in filtered TSB while shaken at 250 rpm at 37 °C, magnetic separation of anti-Protein A DynaBeads and direct addition of supernatant to MIT vials for laser-light scattering analysis. These conditions resulted in *S. aureus* identifying as *Staphylococcus* species in 44% of samples. The bead removal section of Figure 28 protocol was as follows: IMS methods performed in filtered TSB media at 37 °C, 1-2 hours of enrichment in filtered TSB while shaken at 250 rpm at 37 °C, after 1-2 hours of enrichment, anti-Protein A DynaBeads were removed and the enrichment incubation of samples resumed, magnetic separation of anti-Protein A DynaBeads and direct addition of supernatant to MIT vials for laser-light scattering analysis. *S. aureus* identified as *Staphylococcus* species in 64% of samples. The Terrific broth section of Figure 28 protocol was as follows: IMS methods performed in filtered Terrific broth media at 37 °C, 1-3 hours of enrichment in filtered TSB while shaken at 250 rpm at 37 °C, after 1-2 hours of enrichment, anti-Protein A DynaBeads were removed and the enrichment incubation of samples resumed, magnetic separation of anti-Protein A DynaBeads and direct

addition of supernatant to MIT vials for laser-light scattering analysis. Under these conditions, *S. aureus* identified as *Staphylococcus* species in 50% of samples.

The optimal conditions protocol was established as follows: IMS methods were performed in filtered TSB media at 37 °C, 1-5 hours of enrichment in filtered TSB while shaken at 250 rpm at 37 °C, after enrichment anti-Protein A DynaBeads were removed and so was the TSB media (by pelleting the cells in the supernatant), *S. aureus* cells were then resuspended in filtered water, vortexed (to break up the pellet) and samples were then added to MIT vials for laser-light scattering analysis. When these conditions were utilized, *S. aureus* identified as *Staphylococcus* species in 80% of samples (Figure 28). After statistical analysis using a one-way ANOVA Bonferroni multiple comparison test, P-values were greater than 0.05 for all conditions when compared to the established optimal conditions. This displayed no statistical significant difference between any of the conditions; however, when optimal conditions were utilized to identify *S. aureus* as *Staphylococcus* species via laser-light scattering, 80% of the samples identified accurately.
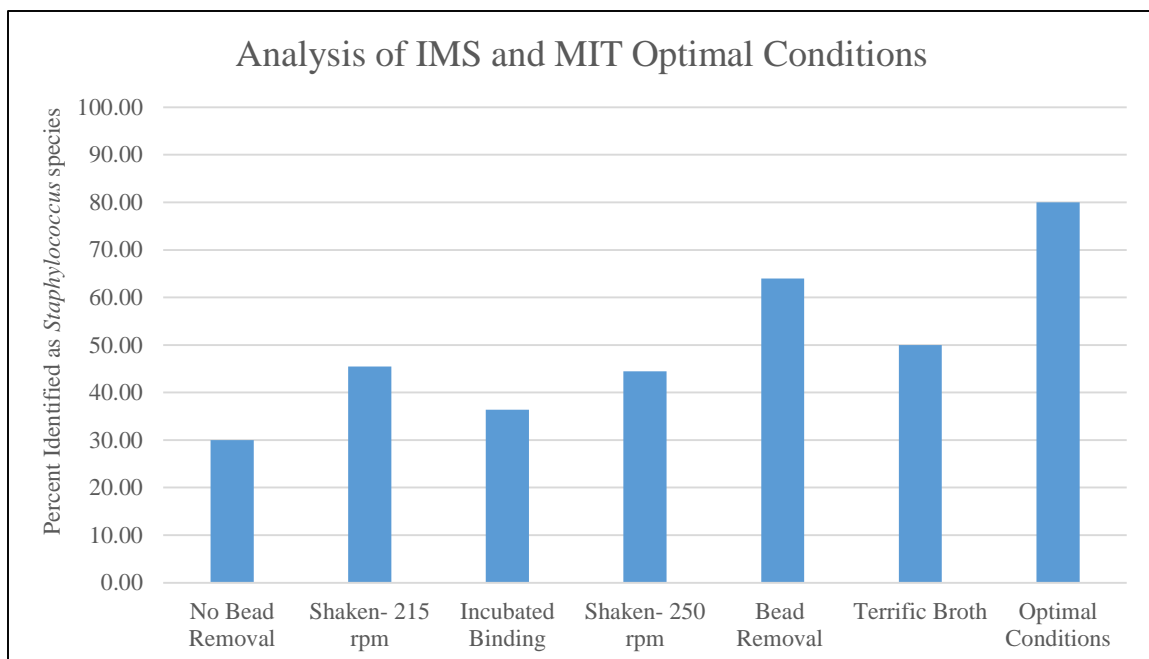
Figure 28. Analysis of IMS and MIT optimal conditions. IMS and MIT conditions were altered in order to determine the optimal conditions for the highest percent identified as *Staphylococcus* species for *S. aureus.* No bead removal resulted in *S. aureus* identifying as *Staphylococcus* species in 30% of samples. When samples were shaken at 215 rpm this resulted in *S. aureus* identifying as *Staphylococcus* species in 45% of samples. When the samples were incubated during anti-Protein A DynaBead binding at 37°C, *S. aureus* identified as *Staphylococcus* species in 36% of samples. When samples were shaken at 250 rpm this resulted in *S. aureus* identifying as *Staphylococcus* species in 44% of samples. When anti-Protein A DynaBeads were removed after 1-2 hours of enrichment, *S. aureus* identified as *Staphylococcus* species in 64% of samples. When IMS and MIT methods were performed in filtered Terrific Broth, rather than filtered TSB, *S. aureus* identified as *Staphylococcus* species in 50% of samples. Optimal conditions included the removal of anti-Protein A DynaBeads and removal of enrichment media after 5 hours of enrichment; when these conditions were utilized, *S. aureus* identified as *Staphylococcus* species in 80% of samples.

Analysis of rapid laser-light scattering detection from pure IMS samples

Upon the establishment of IMS, enrichment, and laser-light scattering identification methods, identification of *S. aureus* was then evaluated in both pure *S. aureus* culture samples and mixed bacteria cultures (Figures 29 and 30). Percent identified as *Staphylococcus* species from pure cultures displayed with an average initial input of 4.86 x 10^9 CFU/ml, *S. aureus* identified as *Staphylococcus* species via laser-light

scattering 80% of the time. With an average initial input of 3.97 x 10^9 CFU/ml, *M. luteus*

falsely identified as *Staphylococcus* species via laser-light scattering 12.5% of the time.

E. aerogenes with an average initial input of 2.15 x 10^8 CFU/ml, *E. coli* with an average

initial input of 4.46 x 10^8 CFU/ml, *E. faecalis* with an average initial input of 1.75 x 10^9

CFU/ml, *L. monocytogenes* with an average initial input of 2.60 x 10^9 CFU/ml, *and S.*

pyogenes with an average initial input of 5.60 x 10^8 CFU/ml all identified as

Staphylococcus species via laser-light scattering 0.00% of the time. Statistical analysis

via one-way ANOVA Bonferroni multiple comparison test displayed P-values of less

than 0.001 for all negative control strains when comparing the results of percent

identified as *Staphylococcus* species of each negative control strain to percent identified

as *Staphylococcus* species for *S. aureus.*

In order to determine percent identified as *Staphylococcus* species from mixed

samples, multi-microbial samples were generated in order to replicate more realistic

clinical samples that are often polymicrobial. When in pure culture, *S. aureus* identified

as *Staphylococcus* species via laser-light scattering 80% of the time with an initial input

of 4.86 x 10^9 CFU/ml. *S. aureus + E. aerogenes* with an initial input, 2.15 x 10^8 CFU/ml

identified as *Staphylococcus* species via laser-light scattering 50% of the time. *S. aureus*

+ E. coli with an initial input of 4.46 x 10^8 CFU/ml identified 0% of the time. *S. aureus +*

E. faecalis with an initial input of 1.75 x 10^9 CFU/ml identified 50% of the time. *S.*

aureus + L. monocytogenes with an initial input, 2.60 x 10^9 CFU/ml identified 60% of the

time. *S. aureus + M. luteus* with an initial input of 3.98 x 10^9 CFU/ml identified 75% of

the time. *S. aureus + S. pyogenes* with an initial input of 5.60 x 10^8 CFU/ml identified

75% of the time. *S. aureus + S. pyogenes + M. luteus* identified 75% of the time. *S.*

aureus + *S. pyogenes* + *E. coli* identified 0% of the time. Statistical analysis via one-way

ANOVA Bonferroni multiple comparison test displayed P-values of less than 0.05 when

comparing the results of percent identified as *Staphylococcus* species of *S. aureus* with

multi-microbial samples containing *E. coli*. Specifically, when *S. aureus* was mixed with

E. coli and when *S. aureus* was mixed with *E. coli* and *S. pyogenes* P-values were less

than 0.05.

Figure 29. Percent identified as *Staphylococcus* species from pure samples. *S. aureus* identified as *Staphylococcus* species via laser-light scattering 80% of the time with an initial input of 4.86 x 10^9 CFU/ml. *M. luteus* identified as *Staphylococcus* species via laser-light scattering 12.5% of the time with an initial input of 3.97 x 10^9 CFU/ml. *E. aerogenes* (initial input, 2.15 x 10^8 CFU/ml), *E. coli* (initial input, 4.46 x 10^8 CFU/ml), *E. faecalis* (initial input, 1.75 x 10^9 CFU/ml), *L. monocytogenes* (initial input, 2.60 x 10^9 CFU/ml), *and S. pyogenes* (initial input, 5.60 x 10^8 CFU/ml) all identified as *Staphylococcus* species via laser-light scattering 0.00% of the time. Statistical significance (P-value) was determined by comparing results of each negative control strain to *S. aureus.* One asterisk (*) denotes a P-value less than 0.001.

Figure 30. Percent identified as *Staphylococcus* species from mixed samples. *S. aureus* identified as *Staphylococcus* species via laser-light scattering 80% of the time with an initial input of 4.86 x 10^9 CFU/ml. *S. aureus* + *E. aerogenes* (initial input, 2.15 x 10^8 CFU/ml) identified as *Staphylococcus* species via laser-light scattering 50% of the time. *S. aureus* + *E. coli* (initial input, 4.46 x 10^8 CFU/ml) identified 0% of the time. *S. aureus* + *E. faecalis* (initial input, 1.75 x 10^9 CFU/ml) identified 50% of the time. *S. aureus* + *L. monocytogenes* (initial input, 2.60 x 10^9 CFU/ml) identified 60% of the time. *S. aureus* + *M. luteus* (initial input 3.98 x 10^9 CFU/ml) identified 75% of the time. *S. aureus* + *S. pyogenes* (initial input, 5.60 x 10^8 CFU/ml) identified 75% of the time. *S. aureus* + *S. pyogenes* + *M. luteus* identified 75% of the time. *S. aureus* + *S. pyogenes* + *E. coli* identified 0% of the time. Statistical significance (P-value) was determined by comparing results of each negative control strain to *S. aureus.* One asterisk (*) denotes a P-value less than 0.05.

Figure 31. Percent identified as *Staphylococcus* species from mixed specimens by dilution. After IMS capture and bacterial enrichment, samples underwent laser-light scattering identification. Percent identified as *Staphylococcus* species was determined for each set of samples. Bar colors represent different concentrations of initial input of *S. aureus* to samples. Estimated equal concentrations of *S. aureus* and negative control strains were added to each sample. Statistical analysis was conducted by one-way ANOVA Bonferroni multiple comparison test, *denotes $P < 0.01$ and **denotes $P < 0.001$. Each dilution by bacteria was compared to each other for statistical analysis, interspecies comparison was not conducted.

Analysis of rapid laser-light scattering detection from IMS swab samples

In clinical settings, specimen collection on swabs is one of the most utilized form of specimen collection for infections of skin and soft tissues. In order to determine if *S. aureus* could be identified from swab specimen collection, pre-established IMS and laser-light scattering identification methods were utilized and evaluated for accuracy of identification. Upon the establishment of IMS, enrichment, and laser-light scattering identification methods from swab samples, identification of *S. aureus* was then evaluated in both swab pure samples and swab mixed samples (Figures 32 and 33). Percent identified as *Staphylococcus* species from pure swab samples displayed that *S. aureus* identified as *Staphylococcus* species via laser-light scattering 82% of the time with an average initial input of 1.12×10^8 CFU/ml. *S. pyogenes* misidentified as *Staphylococcus* species via laser-light scattering 10% of the time with an initial input of 2.22×10^7 CFU/ml. *E. aerogenes* with an average initial input of 2.77×10^8 CFU/ml, *E. coli* with an average initial input of 6.19×10^6 CFU/ml, *E. faecalis* with an average initial input of 3.49×10^8 CFU/ml, *L. monocytogenes* with an average initial input of 1.75×10^8 CFU/ml, *M. luteus* with an average initial input of 7.35×10^7 CFU/ml, human nose swab with an average initial input of 3.00×10^8 CFU/ml, direct human nose swab with an average initial input of 1.82×10^5 CFU/ml, human skin swab with an average initial input of 4.05×10^8 CFU/ml all identified as *Staphylococcus* species via laser-light scattering 0.00% of the time. Statistical significance (P-value) was determined by comparing results of each negative control strain to *S. aureus* via one-way ANOVA Bonferroni multiple comparison test. Statistical significance, P-value less than 0.001 was present for all negative controls when compared to *S. aureus* (Figure 32). Figure 31 displays how the

percent identified as *Staphylococcus* species differed by each dilution for the averages displayed in Figure 30.

By generating mixed, multi-microbial samples, we were able to evaluate the accuracy of our methods for *S. aureus* capture and identification when challenged with multiple species of bacteria in a sample. Evaluation of *S. aureus* capture and identification from multi-microbial samples gave a more realistic analysis of *S. aureus* capture from clinical swab samples. In order to evaluate mixed swab specimens, percent identified as *Staphylococcus* species from mixed swab samples was recorded and statistical significance was determined by one-way ANOVA Bonferroni multiple comparison test. *S. aureus* identified as *Staphylococcus* species via laser-light scattering 83% of the time with an initial input of 4.86 x 10^9 CFU/ml. *S. aureus + E. aerogenes* with an initial input of 2.77 x 10^8 CFU/ml identified as *Staphylococcus* species via laser-light scattering 78% of the time. *S. aureus + E. coli* with an initial input of 6.19 x 10^6 CFU/ml identified 29% of the time. *S. aureus + E. faecalis* with an initial input of 3.49 x 10^8 CFU/ml identified 29% of the time. *S. aureus + L. monocytogenes* with an initial input of 1.75 x 10^8 CFU/ml identified 57% of the time. *S. aureus + M. luteus* with an initial input of 7.35 x 10^7 CFU/ml identified 71% of the time. *S. aureus + S. pyogenes* with an initial input of 2.22 x 10^7 CFU/ml identified 71% of the time. *S. aureus* + a human nose swab with an initial input of 3.00 x 10^8 CFU/ml identified 75% of the time. *S. aureus* + a human skin swab with an initial input of 4.05 x 10^8 CFU/ml identified 75% of the time. *S. aureus* + a human nose swab + and a human skin swab identified 70% of the time. *S. aureus + S. pyogenes + M. luteus* identified 43% of the time. *S. aureus + S. pyogenes + E. coli* identified 0% of the time. Statistical significance (P-value) was

determined by comparing results of each negative control strain to *S. aureus.* Statistical significance was found with a P-value less than 0.01 when *S. aureus* + *S. pyogenes* + *E. coli* was compared to *S. aureus* alone (Figure 33). Figure 34 displays how the percent identified as *Staphylococcus* species differed by each dilution for the averages displayed in Figure 33.

The average growth (CFU/ml) by enrichment over a 5 hour time frame from swab specimen collection was determined in order to quantify the number of progeny produced during the enrichment step. To calculate this, after IMS capture, the *S. aureus* cells captured by anti-Protein A DynaBeads were resuspended in filtered TSB agar and incubated at 37°C for 5 hours while shaken at 250 rpm. Each hour, the tubes were magnetically separated for 3 minutes and the supernatant was spread plated on TSB agar and incubated at 37°C for 18 hours. Colony forming units were then calculated. At hour 0 of enrichment, average growth was 4.73×10^7 CFU/ml, after 1 hour of enrichment average growth was 2.19×10^8 CFU/ml, after 2 hours of enrichment average growth was 4.57×10^8 CFU/ml, after 3 hours of enrichment average growth was 2.66×10^9 CFU/ml, after 4 hours of enrichment average growth was 4.36×10^9 CFU/ml, and after 5 hours of enrichment average growth was 1.27×10^{10} CFU/ml (Figure 36).

Figure 32. Percent identified as *Staphylococcus* species from pure swab samples. *S. aureus* identified as *Staphylococcus* species via laser-light scattering 82% of the time with an initial input of 1.12 x 10^8 CFU/ml. *S. pyogenes* identified as *Staphylococcus* species via laser-light scattering 10% of the time with an initial input of 2.22 x 10^7 CFU/ml. *E. aerogenes* (initial input, 2.77 x 10^8 CFU/ml)*, E. coli* (initial input, 6.19 x 10^6 CFU/ml)*, E. faecalis* (initial input, 3.49 x 10^8 CFU/ml)*, L. monocytogenes* (initial input, 1.75 x 10^8 CFU/ml)*, M. luteus* (initial input, 7.35 x 10^7 CFU/ml)*,* human nose swab (initial input, 3.00 x 10^8 CFU/ml), direct human nose swab (initial input, 1.82 x 10^5 CFU/ml), human skin swab (initial input, 4.05 x 10^8 CFU/ml) all identified as *Staphylococcus* species via laser-light scattering 0.00% of the time. Statistical significance (P-value) was determined by comparing results of each negative control strain to *S. aureus.* One asterisk (*) denotes a P-value less than 0.001.

Figure 33. Percent identified as *Staphylococcus* species from mixed swab samples. *S. aureus* identified as *Staphylococcus* species via laser-light scattering 83% of the time with an initial input of 4.86 x 10⁹ CFU/ml. *S. aureus + E. aerogenes* (initial input, 2.77 x 10⁸ CFU/ml) identified as *Staphylococcus* species via laser-light scattering 78% of the time. *S. aureus + E. coli* (initial input, 6.19 x 10⁶ CFU/ml) identified 29% of the time. *S. aureus + E. faecalis* (initial input, 3.49 x 10⁸ CFU/ml) identified 29% of the time. *S. aureus + L. monocytogenes* (initial input, 1.75 x 10⁸ CFU/ml) identified 57% of the time. *S. aureus + M. luteus* (initial input 7.35 x 10⁷ CFU/ml) identified 71% of the time. *S. aureus + S. pyogenes* (initial input, 2.22 x 10⁷ CFU/ml) identified 71% of the time. *S. aureus + Nose* (initial input, 3.00 x 10⁸ CFU/ml) identified 75% of the time. *S. aureus + Skin* swab (initial input, 4.05 x 10⁸ CFU/ml) identified 75% of the time. *S. aureus + Nose + Skin* identified 70% of the time. *S. aureus + S. pyogenes + M. luteus* identified 43% of the time. *S. aureus + S. pyogenes + E. coli* identified 0% of the time. Statistical significance (P-value) was determined by comparing results of each negative control strain to *S. aureus.* One asterisk (*) denotes a P-value less than 0.01.
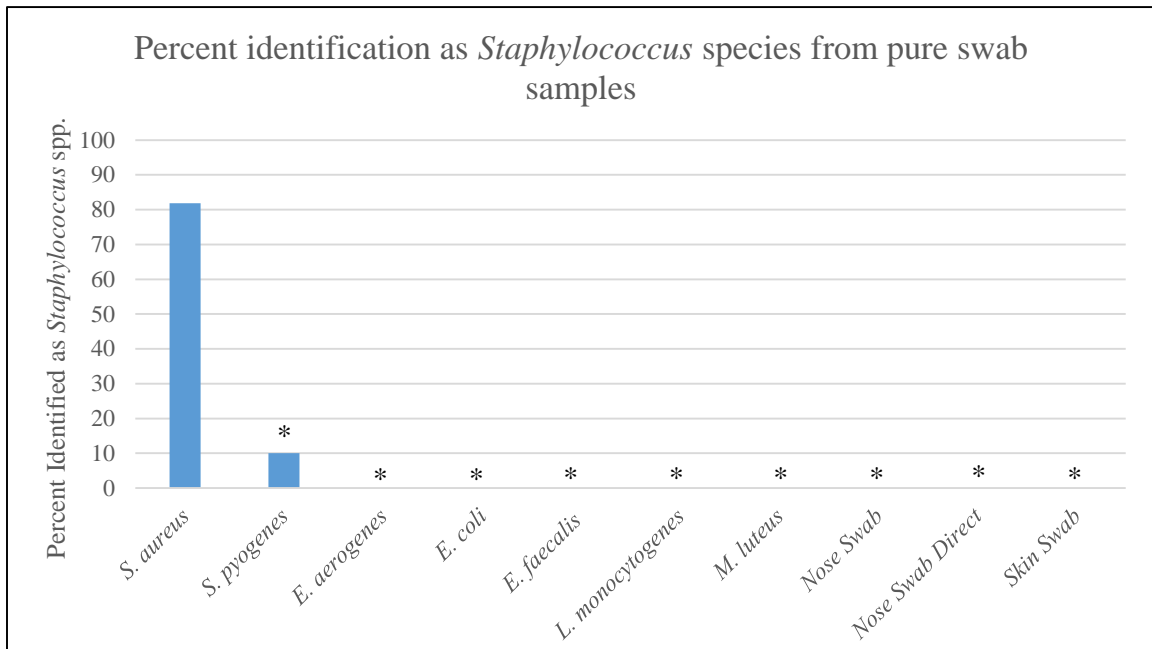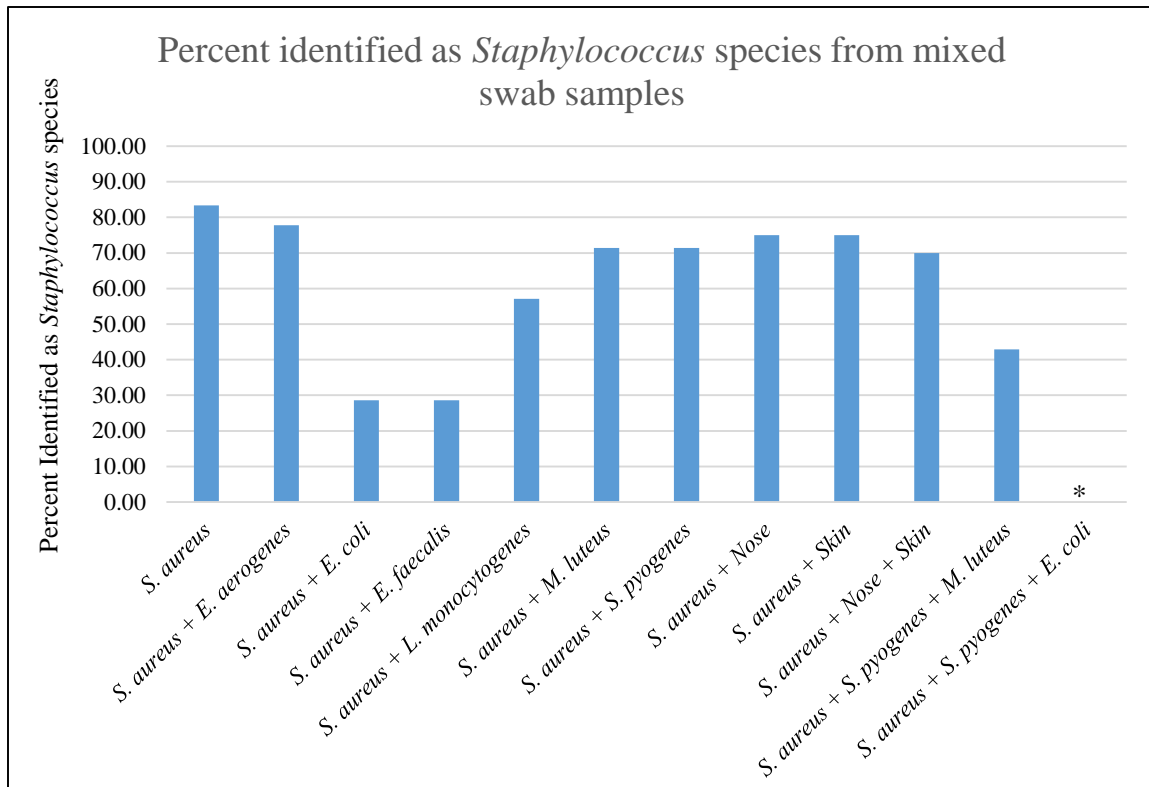
Figure 33. Percent identified as *Staphylococcus* species from mixed swab samples. *S. aureus* identified as *Staphylococcus* species via laser-light scattering 83% of the time with an initial input of 4.86×10^9 CFU/ml. *S. aureus + E. aerogenes* (initial input, 2.77×10^8 CFU/ml) identified as *Staphylococcus* species via laser-light scattering 78% of the time. *S. aureus + E. coli* (initial input, 6.19×10^6 CFU/ml) identified 29% of the time. *S. aureus + E. faecalis* (initial input, 3.49×10^8 CFU/ml) identified 29% of the time. *S. aureus + L. monocytogenes* (initial input, 1.75×10^8 CFU/ml) identified 57% of the time. *S. aureus + M. luteus* (initial input 7.35×10^7 CFU/ml) identified 71% of the time. *S. aureus + S. pyogenes* (initial input, 2.22×10^7 CFU/ml) identified 71% of the time. *S. aureus + Nose* (initial input, 3.00×10^8 CFU/ml) identified 75% of the time. *S. aureus + Skin* swab (initial input, 4.05×10^8 CFU/ml) identified 75% of the time. *S. aureus + Nose + Skin* identified 70% of the time. *S. aureus + S. pyogenes + M. luteus* identified 43% of the time. *S. aureus + S. pyogenes + E. coli* identified 0% of the time. Statistical significance (P-value) was determined by comparing results of each negative control strain to *S. aureus.* One asterisk (*) denotes a P-value less than 0.01.
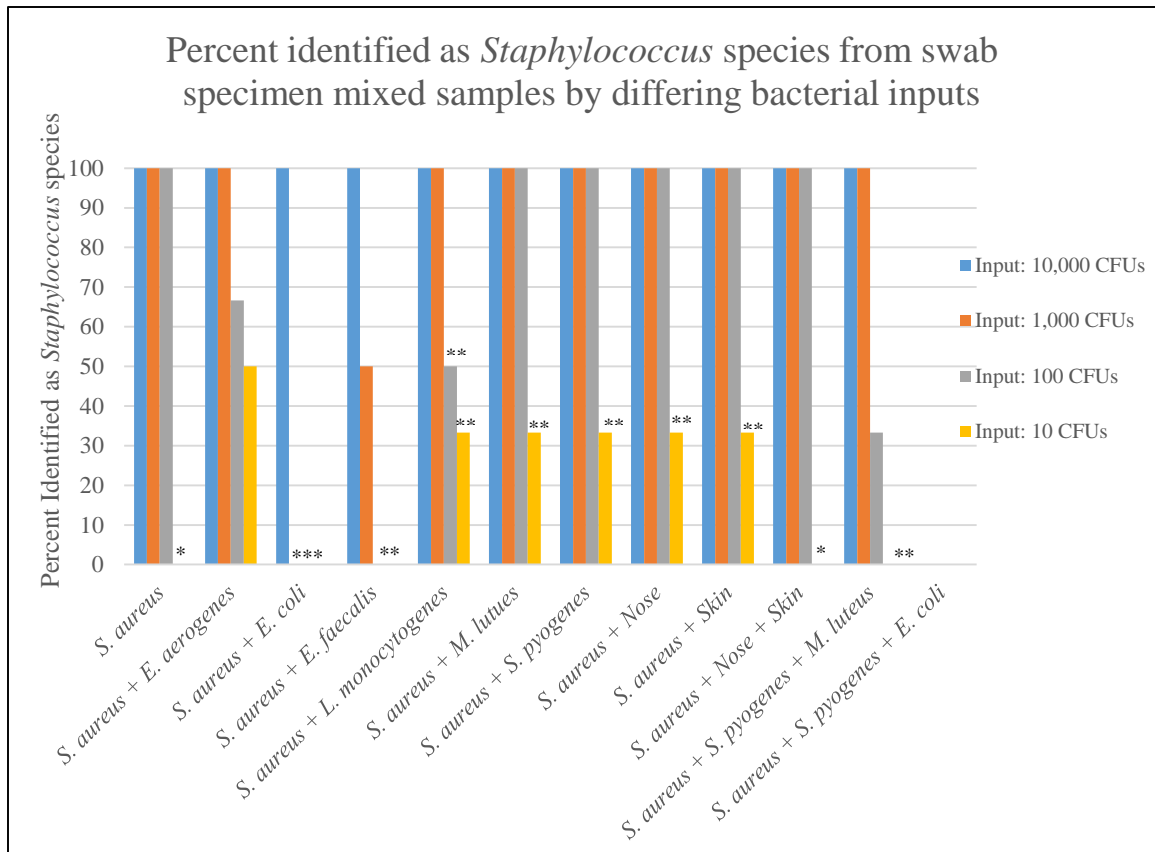
Figure 34. Percent identified as *Staphylococcus* species from mixed swab specimens by dilution. Percent identified as *Staphylococcus* species was determined for each set of swab samples. Bar colors represent different concentrations of initial input of *S. aureus* to samples. Estimated equal concentrations of *S. aureus* and negative control strains were added to each sample. Statistical analysis was conducted by one-way ANOVA Bonferroni multiple comparison test, **denotes $P < 0.01$ and *denotes $P < 0.001$. Each dilution by bacteria was compared to each other for statistical analysis, interspecies comparison was not conducted.
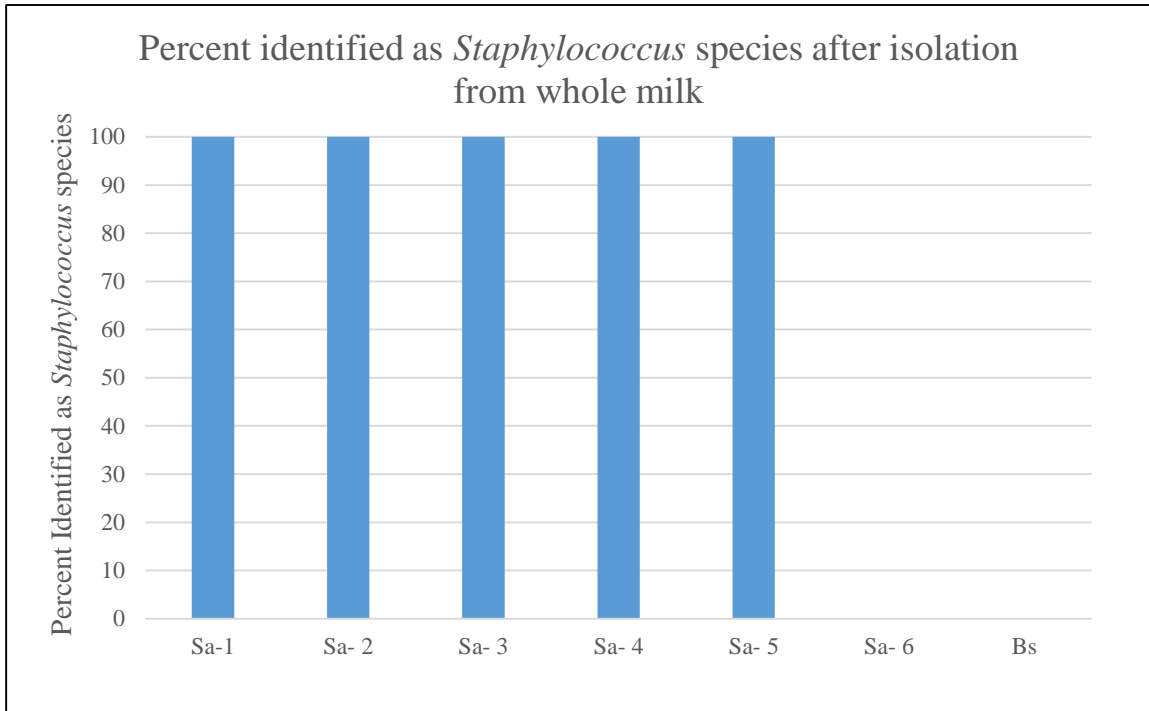
Figure 35. Percent identified as *Staphylococcus* species from whole milk samples. After IMS capture and bacterial enrichment, samples underwent laser-light scattering identification. Percent identified as *Staphylococcus* species was determined for each set of samples. Samples were 10-fold serial diluted from an overnight of *S. aureus* or *B. subtilis,* Sa-1 represents the 10^{-2} *S. aureus* serial dilution (initial input 2282000 CFUs), Sa-2 represents the 10^{-3} *S. aureus* serial dilution (initial input 228200 CFUs), Sa-3 represents the 10^{-4} *S. aureus* serial dilution (initial input 22820 CFUs), Sa-4 represents the 10^{-5} *S. aureus* serial dilution (initial input 2282 CFUs), Sa-5 represents the 10^{-6} *S. aureus* serial dilution (initial input 275 CFUs), Sa-6 represents the 10^{-7} *S. aureus* serial dilution (initial input 27 CFUs), and Bs represents the 10^{-2} *B. subtilis* serial dilution (initial input 1800000 CFUs). *S. aureus* identified as *Staphylococcus* species in 100% of samples when the initial input of bacteria exceeded 275 CFUs. When the initial input of *S. aureus* was less than 275 (Sa-6), the sample identified as *Staphylococcus* species 0% of the time. Negative control, *B. subtilis* identified as *Staphylococcus* species at a rate of 0% indicating no non-specific binding during IMS methods. Statistical analysis was not conducted due to low sample size.
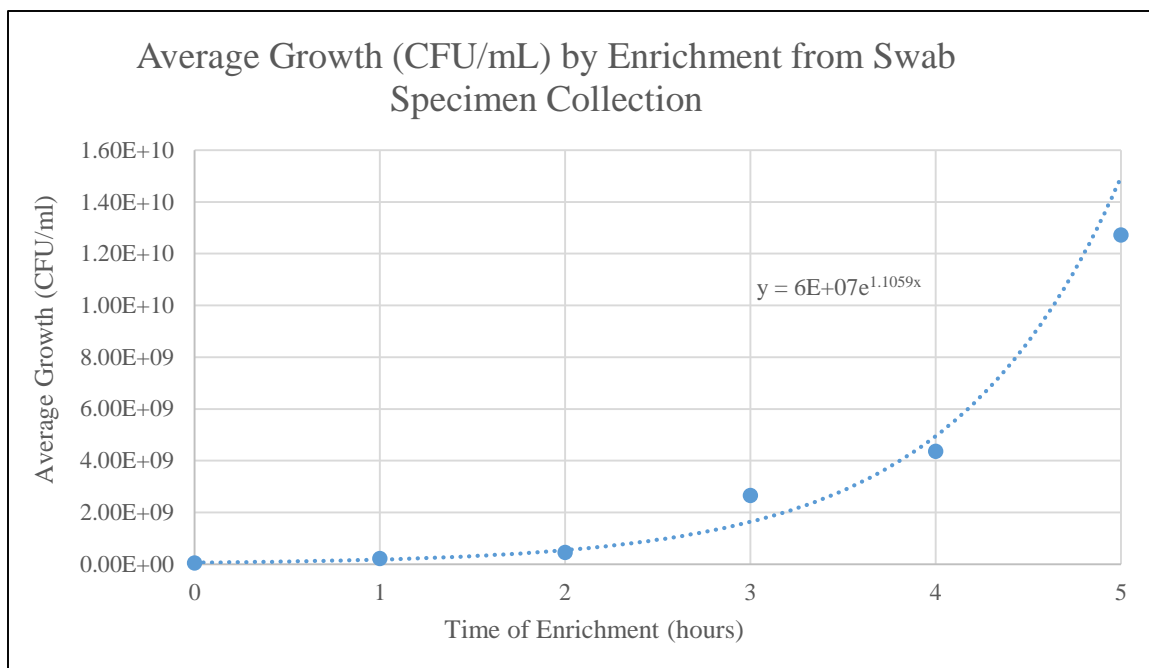
Figure 36. Average growth (CFU/ml) of *S. aureus* by enrichment from swab specimen collection over 5 hours. After IMS capture, *S. aureus* captured by anti-Protein A DynaBeads were resuspended in filtered TSB agar and incubated at 37°C for 5 hours while shaken at 250 rpm. Each hour, the tubes were magnetically separated for 3 minutes and the supernatant was spread plated on TSB agar and incubated at 37°C for 18 hours. Colony forming units were then calculated. At hour 0 of enrichment, average growth was 4.73×10^7 CFU/ml, after 1 hour of enrichment average growth was 2.19×10^8 CFU/ml, after 2 hours of enrichment average growth was 4.57×10^8 CFU/ml, after 3 hours of enrichment average growth was 2.66×10^9 CFU/ml, after 4 hours of enrichment average growth was 4.36×10^9 CFU/ml, and after 5 hours of enrichment average growth was 1.27×10^{10} CFU/ml.

DISCUSSION

IMS capture, enrichment, and laser-light scattering identification method development

After establishing the methods for rapid *S. aureus* capture via IMS, we needed to combine IMS and laser-light scattering technology in order to rapidly identify *S. aureus.* Previous studies suggest that faster pathogen identification leads to faster administration of appropriate treatments, and this has been linked to improved patient outcomes. Improved patient outcomes include a reduction in hospitalization time, decreased risk of nosocomial infections, and decreased in medical costs [44]. After establishing anti-Protein A DynaBead IMS capture methods for *S. aureus*, we developed an enrichment step which was performed in order to obtain the proper concentration of bacteria for laser-light scattering identification (1,000 bacteria cells per milliliter) [37]. The following conditions were altered in order to determine optimal conditions for the highest percent identified as *Staphylococcus* species for *S. aureus*: media type for enrichment, filtration of media for enrichment, removal of beads during enrichment, time of enrichment, removal of media after enrichment, and resuspension of captured bacteria in filtered water.

The first attempt at combining IMS, enrichment, and laser-light scattering technology, the following methods were utilized: samples were bound by anti-Protein A DynaBeads in filtered TSB media at 22 $^\circ$C, 1-3 hours of enrichment in filtered TSB at 37 $^\circ$C, and magnetic separation of anti-Protein A DynaBeads and direct addition of supernatant to MIT vials for laser-light scattering analysis. These variables resulted in *S. aureus* identifying as *Staphylococcus* species in 30% of samples. In the search to further

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enhance the rate of *S. aureus* capture, we found that previous studies had established a link between Protein A expression and growth dependence. Previous studies have determined that Protein A is expressed on the surface of nearly all *S. aureus* strains and occupies approximately 7% of the *S. aureus* cell surface and that it is upregulated during the exponential growth phase [30,59]. We then sought to determine if increasing the temperature from 22 °C to 37 °C during the binding procedure between anti-Protein A DynaBeads and *S. aureus* cells would affect the overall capture rate of *S. aureus*. We hypothesized that increasing the binding temperature from 22 °C to 37 °C would lead to an upregulation in Protein A on the surface of *S. aureus* cells present in the sample. The upregulation of Protein A would then lead to an increased capture rate of *S. aureus* by anti-Protein A antibodies, ultimately leading to a higher concentration of *S. aureus* for laser-light scattering identification. After subsequent testing, we determined that incubation during anti-Protein A DynaBead binding at 37 °C resulted in *S. aureus* identifying as *Staphylococcus* species in 36% of samples compared to the 30% without incubation.

To further develop our methods, it has been suggested from previous studies that shaking liquid cultures of bacteria helps increase the rate of growth within that sample [38]. Shaking allows the culture to remain aerated and this provides adequate oxygen for the bacteria within the sample, thus increasing the rate of bacterial growth [38]. Knowing this, we used previously mentioned methods and combined them with agitation. We used two different agitation speeds, 215 rpm and 250 rpm, to determine if the addition of agitation would enhance the rate of *S. aureus* growth during the enrichment step. When samples were shaken at 215 rpm this resulted in *S. aureus* identifying as *Staphylococcus* species

in 45% of samples. When samples were shaken at 250 rpm this resulted in *S. aureus* identifying as *Staphylococcus* species in 44% of samples.

In order to increase the percentage of *S. aureus* identifying as *Staphylococcus* species using laser-light scatter, we hypothesized that removing the anti-Protein A DynaBeads after hours 1 or 2 of enrichment would aid in accurate laser-light scattering identification. Removing the anti-Protein A DynaBeads would potentially result in an increase in accurate identification by preventing anti-Protein A DynaBeads from entering the MIT sample. If both *S. aureus* cells and anti-Protein A DynaBeads were added to a MIT sample, the laser-light scattering pattern could be altered and identification accuracy would decrease. These methods included: IMS methods performed in filtered TSB media at 37 °C, 1-2 hours of enrichment in filtered TSB while shaken at 250 rpm at 37 °C, after 1-2 hours of enrichment, anti-Protein A DynaBeads were removed and the enrichment incubation of samples resumed, magnetic separation of anti-Protein A DynaBeads and direct addition of supernatant to MIT vials for laser-light scattering analysis. Although the DynaBeads were removed, each sample was still magnetically separated for 3 minutes after enrichment in order to reduce the risk of including DynaBeads into a sample. When anti-Protein A DynaBeads were removed after 1-2 hours of enrichment, *S. aureus* identified as *Staphylococcus* species in 64% of samples.

In order to determine if we could increase the rate of *S. aureus* growth during the enrichment step, filtered terrific broth was utilized instead of filtered TSB media. Terrific broth is a highly enrichment media that is generally used to increase the yield of plasmid DNA from transformed microbial strains [19]. The following protocol was utilized to compare the efficiency of Terrific broth with TSB: IMS methods performed in filtered

Terrific broth media at 37 °C, 1-3 hours of enrichment in filtered TSB while shaken at 250 rpm at 37 °C, after 1-2 hours of enrichment, anti-Protein A DynaBeads were removed and the enrichment incubation of samples resumed, magnetic separation of anti-Protein A DynaBeads and direct addition of supernatant to MIT vials for laser-light scattering analysis. When IMS and laser-light scattering identification methods were performed in filtered Terrific Broth, rather than filtered TSB, *S. aureus* identified as *Staphylococcus* species in 50% of samples. Due to the reduced percent identification of *S. aureus* when enriched in Terrific Both, we decided to utilized TSB media in future experiments.

After altering variables to increase the accuracy of laser-light scattering identifications of *S. aureus,* we wanted to determine if removing the anti-Protein A DynaBeads and TSB enrichment media would increase the accuracy of identification. Knowing the importance of particulates within a MIT sample in laser-light scattering patterns, we hypothesized that removing potential laser-light scattering pattern particulates could help to increase the rate of *S. aureus* identification. After altering the above variables for IMS capture, enrichment, and laser-light scattering identification, the following methods were established as the optimal conditions: IMS methods performed in filtered TSB media at 37 °C, 1-5 hours of enrichment in filtered TSB while shaken at 250 rpm at 37 °C, after enrichment anti-Protein A DynaBeads were removed and so was the TSB media (by pelleting the cells in the supernatant), *S. aureus* cells were then resuspended in 10-50 µl of filtered water, vortexed (to break up the bacteria pellet and resuspend the bacterial cells) and samples were then added to MIT vials for laser-light scattering analysis. When these conditions were utilized, *S. aureus* identified as *Staphylococcus* species in 80% of samples (Figure 28). It is worth noting that all sample

concentrations (10^{-2} to 10^{-6}) were included in the average percent identified as *Staphylococcus* species. Samples that were more highly concentrated with *S. aureus* had higher percent identifications as *Staphylococcus* species.

IMS capture, enrichment, and laser-light scattering identification

After establishing anti-Protein A DynaBead capture, enrichment, and identification, the efficiency of the established rapid identification methods were assessed. The ability to capture, enrich, and identify *S. aureus* was tested on both *S. aureus* and the negative control strains (*S. pyogenes, M. luteus, E. faecalis, L. monocytogenes, E. aerogenes,* and *E. coli*) in order to evaluate its specificity for *S. aureus* alone. *S. aureus* was then mixed with negative controls to create a more complex and realistic specimen sample, the efficiency of the rapid identification methods was then assessed. For each sample that was tested, the average initial input (CFU/ml) of bacteria were determined.

When IMS capture, enrichment, and single laser-light scattering identification methods were performed *S. aureus* identified as *Staphylococcus* species via laser-light scattering 80% (Figure 29). *M. luteus* miss-identified as *Staphylococcus* species via laser-light scattering 12.5% of the time. *E. aerogenes, E. coli, E. faecalis, L. monocytogenes, and S. pyogenes* all identified as *Staphylococcus* species via laser-light scattering 0.00% of the time. When comparing *S. aureus* percent identified as *Staphylococcus* species individually to all negative controls via one-way ANOVA Bonferroni multiple comparison tests, statistical significance was found for each negative control, P-value less than 0.001. The only negative control that miss-identified as *Staphylococcus* species was *M. luteus.* Reasoning for miss-identification could be due to the fact that *M. luteus* cell characteristics closely resemble *S. aureus* cells. *M. luteus* cells are Gram-positive cocci

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that are approximately 0.5-3.5 µm in diameter and usually arrange in tetrads or irregular clusters and *S. aureus* cells are gram positive cocci that are approximately 0.6 µm in diameter and usually arrange in clusters [4,83]. If the *M. luteus* cells had grown closer to 0.6 µm in diameter, this could explain why *M. luteus* identified as *Staphylococcus* species using laser-light scattering. MIT is currently working on developing a *Staphylococcus aureus* identifier, which could help increase the accuracy for *S. aureus* identification and reduce miss-identifications of samples that may closely resemble *S. aureus* cell characteristics, like *M. luteus*. Although *M. luteus* miss-identified as *Staphylococcus* species 12.5% of the time, we hope that our IMS capture and washing methods could help remove these unbound or non-specifically bound cells in future experiments.

In order to increase the complexity of our laboratory made samples, *S. aureus* was combined with negative control strains and IMS capture, enrichment, and laser-light scattering methods were utilized in order to assess the efficiency of our methods for *S. aureus* isolation from mixed samples. All of the polymicrobial results (Figure 30) were then compared to the results of *S. aureus* alone (Figure 29). *S. aureus* identified as *Staphylococcus* species via laser-light scattering 80% of the time. When *S. aureus* was combined with *E. aerogenes,* it was identified as *Staphylococcus* species via laser-light scattering 50% of the time. When *S. aureus* was combined with *E. coli, S. aureus* was identified as *Staphylococcus* species 0% of the time. When *S. aureus* was combined with *E. faecalis, S. aureus* identified as *Staphylococcus* species 50% of the time. When *S. aureus* was combined with *L. monocytogenes, S. aureus* was identified as *Staphylococcus* species 60% of the time. When *S. aureus* was combined with *M. luteus, S. aureus* was identified as *Staphylococcus* species 75% of the time. When *S. aureus* was combined

with *S. pyogenes, S. aureus* was identified as *Staphylococcus* species identified 75% of the time. In order to generate further complex samples, *S. aureus* was combined with two negative control strains. When *S. aureus* was combined with *S. pyogenes* and *M. luteus, S. aureus* identified as *Staphylococcus* species 75% of the time. When *S. aureus* was combined with *S. pyogenes* and *E.coli, S. aureus* identified as *Staphylococcus* species 0% of the time. Statistical significance (P-value < 0.05) was shown when samples containing *E. coli* were compared to *S. aureus.*

After analyzing IMS capture, enrichment, and laser-light scattering methods in polymicrobial cultures, we determined that when *S. aureus* was combined with skin associated microbes, *M. luteus* and *S. pyogenes, S. aureus* was identified as *Staphylococcus* species 75% of the time, compared to 80% when samples contained *S. aureus* alone. However, when *S. aureus* was combined with *E. aerogenes, E. faecalis, L. monocytogenes* displayed a reduction in percent identified as *Staphylococcus* species, ranging from 50-60% identified as *Staphylococcus* species. Although there was a reduction in percent identified as *Staphylococcus* species when *S. aureus* was combined with these strains, there was no statistical significance to the reduction (P > 0.05). When *S. aureus* was combined with *E. coli,* this combination resulted, with statistical significance (P < 0.05), in a 0% identification rate for *Staphylococcus* species.

In typical wound infections, specimens are collected from these types of infection sites on sterile swabs. In order to determine if IMS could successfully capture *S. aureus* from a swab sample, sterile swabs were inoculated with a broth culture containing dilutions of *S. aureus*. The swabs were transferred to a test tube containing and previously established capture, enrichment, and identification protocol were used to

isolate the bacteria from swab specimens. Since *S. aureus* is not always isolated in pure culture from the site of infection, we created pure and mixed swab samples to replicate normal skin microorganisms and IMS capture, enrichment, and laser-light scattering identification methods were repeated. In addition, we also combined *S. aureus* with real human skin and nose swabs in order to increase the complexity of the samples and assess if our methods could be utilized in complex biological samples. We hypothesize that our swab methods would produce results similar to those in pure and mixed culture methods (Figures 29-30); however, the average bacterial input would be reduced.

Our results of pure swab IMS capture, enrichment, and laser-light scattering identification displayed that *S. aureus* identified as *Staphylococcus* species via laser-light scattering 82% of the time (Figure 32). *S. pyogenes* identified as *Staphylococcus* species via laser-light scattering 10% of the time. *E. coli, E. faecalis, L. monocytogenes, M. luteus,* human nose swab, direct human nose swab, human skin swab all identified as *Staphylococcus* species via laser-light scattering, with statistical significant ($P < 0.001$), 0.00% of the time (Figure 32).

In order to increase the complexity of our laboratory made swab samples, *S. aureus* was combined with negative control strains and IMS capture, enrichment, and laser-light scattering methods were utilized in order to assess the efficiency of our methods for *S. aureus* isolation from mixed samples. All of the polymicrobial results (Figure 32) were then compared to the results of *S. aureus* alone (Figure 30). Overall, these results displayed similar results to pure and mixed samples (Figures 29 and 30) and *E. coli* once again displayed a significant reduction in percentage identified as *Staphylococcus* species.

In both the pure and swab specimen samples, *E. coli* contributed to a reduction in *S. aureus* identification when mixed with *S. aureus*. *E. coli* have multiple virulence factors that contribute to its pathogenicity, one of those virulence factors is the fimbriae [41]. The fimbriae, or pili, are involved in adhesion/adherence of *E. coli* to epithelial cells within its host [29,64]. Adherence fimbriae are also involved in biofilm formation within the epithelial intestinal cells [41]. Previous studies have suggested that temperature regulation may play a role in the upregulation of fimbriae related genes, thus increasing the presence of this adhesion protein on the surface of *E. coli* [78].

When conducting initial anti-Protein A DynaBead specificity for *S. aureus, E. coli* was utilized as one of the negative control strains. It was determined that *E. coli,* much like the other controls strains, had a capture efficiency of less than 1.00% (Figures 29-33). However, when developing IMS capture, enrichment, and laser-light scattering methods we adjusted the binding temperature from 22 °C to 37 °C. This temperature change was conducted in order to increase the rate of *S. aureus* capture by upregulating Protein A on the surface of *S. aureus* and increase the replication rate of *S. aureus.* The known temperature regulation of both Protein A and *E. coli* fimbriae may result in enhanced binding competition. Although we determined that anti-Protein A DynaBeads have specificity for *S. aureus* when bound at 22 °C in pure culture, we speculate that increasing the binding temperature could potentially lead to an upregulation in fimbriae. The primary function of fimbriae is adhesion and its binding has been found to be non-specific host tissues, via electrostatic hydrophobic/hydrophilic interactions [20]. In addition to non-specific binding to host tissues, fimbriae can interact with inanimate objects and can often bind fimbriae to fimbriae creating biofilms [20,41,67]. We hypothesize that *E. coli*

could have been incorporated in the MIT samples even after the washing step. If *E. coli* was incorporated into the samples undergoing the enrichment step, both *S. aureus* and *E. coli* growth would occur, resulting in a highly concentrated mixed sample that would not identify as *S. aureus*.

The final goal of this section was to quantify the average amount of bacterial growth each hour (1-5 hours) during the enrichment step. Figure 36 displays the results recorded from this experiment. On average, after 1 hour of enrichment average growth was 2.19×10^8 CFU/ml, after 2 hours of enrichment average growth was 4.57×10^8 CFU/ml, after 3 hours of enrichment average growth was 2.66×10^9 CFU/ml, after 4 hours of enrichment average growth was 4.36×10^9 CFU/ml, and after 5 hours of enrichment average growth was 1.27×10^{10} CFU/ml. By using the average *S. aureus* bacterial input (CFU/ml), we were able to determine the necessary time of enrichment needed to produce the appropriate concentration of bacteria for a positive identification using laser-light scattering technology. Using the trend line produced in Figure 36 ($y = 6E+07e^{1.1059x}$) we determined that with the average *S. aureus* input (4.86×10^9 CFU/ml) via swab specimen collection, 3.36 hours of enrichment was needed to produce a positive identification using laser-light scattering technology.

Future directions

In the future, we hope to increase the rate of *S. aureus* identification above 95% for both pure and mixed cultures and adjust the IMS washing methods to limit non-specific binding of pileated bacteria (*E. coli* and *E. aerogenes*). In order to increase the rate of *S. aureus* identification above 95%, we need to reduce non-specific binding. Non-specific binding could occur on the walls or caps of the tubes as well as, binding to the

anti-Protein A DynaBeads, which could be addressed by modifying the washing procedure. By increasing the percentage of Tween included in our PBS wash buffer or testing other detergents may aid in reducing non-specific binding.

In the future we also plan to perform IMS capture, enrichment, and laser-light scattering identification on patient swab samples obtained directly from infected wounds or soft tissue infections. We hope to perform a single blind experiment, similar to the single blind experiment conducted in chapter two, with the addition of IMS capture and enrichment directly from patient swab specimens.

Future directions in combination with our collaborator, MIT, include improving the *Staphylococcus* species Identifier to specifically identify *Staphylococcus aureus.* Currently MIT reports that the *S. aureus* Identifier is 95% completed. Once the *S. aureus* Identifier is complete the *S. aureus* Identifier will be validated by testing in the Sharp lab. We have over two dozen different *S. aureus* isolates in our strain collection to test the strength of this Identifier. MIT is also making initial light scattering measurement on a MRSA strains. We purchased two MRSA panels from the American Type Collection that contains 17 different MRSA strains with different *sccMec* types. It will be interesting to determine if MRSA strains have any consistent light scattering pattern differences compared to methicillin-sensitive *S. aureus* (MSSA) strains. The *mecA* gene genomic insertions that confer methicillin resistance are relatively small, between 2.820-2.903 Mb nucleotides and the protein content of the MRSA cell is likely very similar to MSSA strains. It may push the limits of the technology to differentiate MRSA from MSSA. MIT also plans to automate the methods we have generated. Although this system generates an identification for *S. aureus* faster than traditional methods it is still fairly labor intensive.

Automating this system could decrease sample preparation time and labor efforts. Overall, this study has generated a novel and rapid method for *S. aureus* capture and identification from pure and mixed swab specimens. Further development of these methods could lead to applications in both clinical and dairy industries for faster *S. aureus* identification. By changing the target of the antibody used in our IMS methods, there is the potential to apply this technology to any bacteria of interest.

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LETTER OF SUPPORT FROM UP HEALTH SYSTEMS- MARQUETTE
(FORMERLY MARQUETTE GENERAL) TO USE CLINICAL PATIENT ISOLATES

MARQUETTE GENERAL

A Duke LifePoint Hospital

LABORATORY
1-888-818-3879 • 1-906-225-3050

July 29, 2014

Dear Grant Reviewers,

It is my pleasure to be writing this letter of support. The Microbiology Laboratory at Marquette General Hospital (MGH) is extremely enthusiastic about helping Dr. Sharp and Micro Identification Technologies (MIT) with their proposed research project to further develop a rapid means to identify Staphylococcus *aureus* and Methicillin Resistant Staphylococcus *aureus* (MRSA). *S. aureus* is one of the most commonly identified organisms in the clinical microbiology laboratory and drug resistant MRSA is also an important concern. Tests that can accurately identify and differentiate these two organisms quickly and in a cost effective manner is of critical importance.
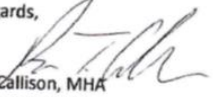
We recently visited the Sharp laboratory at Northern Michigan University (NMU) and observed a demonstration of a rapid identification technology developed by MIT. This technology utilized laser-light scattering measurements of individual bacteria cells to identify *Staphylococcus* species. During the demonstration two laboratory strains of *S. aureus* strains and *S. epidermidis* identified as *Staphylococcus* species while *Micrococcus luteus* and *Streptococcus pyogenes* returned an Unknown identification. These are encouraging preliminary results. Based on our visit we believe the technology is fast, and simple to use, and with further funding and development would have promise to aid in rapid identification of *Staphylococcus aureus* and MRSA.

To aid in this research project we have discussed with Dr. Sharp about the possibility of installing a MIT 1000 laser system in our laboratory at MGH, and MIT is supportive of this idea. We have also discussed the possibility of supplying the Sharp laboratory at NMU with bacteria on agar plates isolated from patient specimens for analysis in the MIT 1000 system. Experiments could be designed to assess how rapidly and accurately the MIT 1000 system is able to identify *S. aureus* and MRSA compared to our currently used methods. Such experiments could also determine if it is feasible to implement the MIT 1000 in a clinical setting.

It is very important to note that the proposal of supplying bacteria from the hospital to the Sharp laboratory at NMU, or for testing bacteria in a MIT 1000 system installed at MGH, is currently undergoing review by the Grants and Contracts Office at NMU and the Administrative offices at MGH. While this process is not currently completed by these offices at the time of grant submission, we do not anticipate any significant difficulties with achieving approval within the next eight weeks.

In closing, we are excited to offer our help and support as outlined above for this extremely important research project. Upon approval by the appropriate boards and offices at MGH and NMU we look forward to working with Dr. Sharp and MIT. Marquette, Michigan is a small community, and there has been a history of successful partnerships and interactions between MGH and NMU in the past. We sincerely hope that funding of this project will not only help in continuing successful research interactions between NMU and MGH, but also facilitate the creation of unique learning opportunities for NMU students and people in our community.

Best Regards,

Preston Callison, MHA
Assistant Administrator

CC. Dr. J. Weiss
Dr. J. Gephart
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