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The Effect of Media Selection on the Membrane Characteristics and Fatty Acid Composition of Staphylococcus Aureus

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The cellular membrane is a vital structure to a microorganism and impacts important aspects of environmental regulation and growth. The physical structure of cellular membranes plays a key role in bacterial growth at low temperatures, membrane permeability and susceptibility to membrane active molecules as well as broader aspects of bacterial physiology and pathogenesis. Mueller Hinton medium is the required medium for antimicrobial susceptibility testing in the US, but there are many other types of media used in staphylococcal research. Different media vary in their nutrient compositions, and the effect of media composition on bacterial physiology is incompletely understood. The aim of this study is to elucidate possible mechanisms underlying alterations to important membrane characteristics of *Staphylococcus aureus* when grown in different nutrient media. The characteristics of interest in this study are fatty acid composition, membrane fluidity and carotenoid content of the cellular membrane. Each of these characteristics plays an important role in *S. aureus* growth, virulence and resistance to antibiotics. *S. aureus* cells of different strains were grown on a number of media types and their membrane characteristics were investigated. Growth in Mueller Hinton medium yielded cells with a much higher content of branched-chain fatty acids in their cellular membranes

compared to cells grown in Tryptic Soy Broth or Brain Heart Infusion broth. However, the membrane of Mueller-Hinton Broth grown cells was less fluid than cells grown in other the other media types, which appeared to be due to high carotenoid content in Mueller Hinton grown cells. Growth in Serum yielded cells with unusual unsaturated fatty acids. Changes in bacterial physiology attributed to media selection could cause a rethinking of which types of media are used for antimicrobial susceptibility testing as well as other important experimental procedures.

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S.J

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CHAPTER I

INTRODUCTION

Staphylococcus aureus is a facultative anaerobic Gram-positive coccal bacterium, and is a common component of the flora on the skin and nasal passages (Klutymans et al., 1997). *S. aureus* infections are a global health concern due to the increasing rates of antibiotic resistance observed for many of the currently available antibiotics on the market. Due to the threat of multiply antibiotic resistant *S. aureus*, various features of staphylococcal biology are being investigated to expand our understanding of the organism's physiology, antibiotic resistance and pathogenesis.

S. aureus is one of the most prevalent pathogens of humans; causing pneumonia, food poisoning, and toxic shock syndrome in addition to various suppurative diseases. Frequently appearing as grape-like clusters of golden pigmented colonies, *S. aureus* contains a host of virulence factors that enable the bacteria to effectively colonize its host. Enzymes such as coagulase, hyaluronidase, lipase, beta-lactamase and staphylokinase work in conjuction to prevent phagocytosis by host immune cells and alter the surrounding environment to increase the spread of the bacteria. Other virulence factors such as the golden pigment staphyoxanthin are important antioxidants that protect *S. aureus* from host reactive oxygen species. Every year close to half a million Americans

contract a *S. aureus* infection during their stay in a hospital (Boxersox, 1999). The cost of hospital acquired *S. aureus* infections runs into the billions globally and threatens to continue to grow as *S. aureus* continues to evolve mechanisms to combat the antibiotic arsenal. Scientists across the globe are investigating many aspects of *S. aureus* biology in order to uncover novel chemicals or targets to bring infections back under control.

Cellular Membrane

The composition of a microorganism's cellular membrane is an interesting site for investigation as membrane physical structure impacts many aspects of cellular growth and affects an organism's ability to respond to environmental stress (Annous et al., 1997; Suutari and Laasko, 1992). The physical state of a cell membrane is manipulated by the incorporation of a mixture of fatty acids with different melting temperatures (T_m) into phospholipids. Membrane phospholipid composition is crucial to temperature and osmotic regulation, and changes in normal membrane composition may prevent growth under stressful conditions (Kikuchi et al., 2000). A decrease in temperature increases membrane rigidity and many bacteria respond by increasing the proportion of unsaturated fatty acid incorporated into the phospholipids. When membrane fluidity increases due to rising temperature in the environment, the proportion of unsaturated fatty acids incorporated into the membrane decreases (Zhang and Rock, 2008). However, the role that nutrient media may play in membrane composition is poorly understood. Nutrient media come in a variety of mixtures to encourage the growth of microorganisms, but what effect if any the makeup of the media may have on membrane physical structure has not been thoroughly and systematically investigated.

An interesting observation by a PhD student Yang Song sparked the interest in this project. While working on an alternate project, Yang sent off samples of *S. aureus* grown in different media types (Mueller Hinton, MHB; Tryptic Soy Broth, TSB; and Brain Heart Infusion, BHIB) for fatty acid analysis. She was surprised to note that the *S. aureus* cells grown in MH medium had membrane compositions of over 80% branched chain fatty acids (BCFAS) compared to TSB and BHI, which had compositions of ~60% BCFAS.

Unsaturated fatty acids increase membrane fluidity when incorporated into phospholipids, reducing the need for further desaturation of existing lipids (Aguilar et al., 2001). De novo fatty acid synthesis is an energy intensive process; certain bacteria such as *S. aureus* are able to uptake exogenous unsaturated fatty acids through a pathway which may act as an energy saving mechanism (Parsons et al., 2011). It has been suggested that the bacterial cell membrane is always kept at the limit of stability by the introduction of non-bilayer lipids into the membrane to allow a flexible response to extracellular stimuli that disturb the membrane biophysical properties (Jorach et al., 1998, Morein et al., 1996). Membrane lipids are fluid in the liquid-crystalline state but become rigidified when the temperature drops below the T_m of the membrane. The fluidity of the membrane is required for movement and activity of membrane proteins; therefore it is paramount to bacterial survival that T_m is adjusted during growth at lower temperatures (Seltmann and Host, 2002). Gram-positive organisms typically increase the BCFA content of their membranes to aid in their growth at low temperatures (Annous et al., 1997; Suutari and Laakso, 1992).

S. aureus colonies are distinct for their golden color caused by the carotenoid pigment staphyloxanthin. Staphyloxanthin promotes the bacterium's resistance to reactive oxygen species and host neutrophil-based killing (Liu et al., 2005; Clauditz et al., 2006). In addition to the antioxidant properties of carotenoids, staphyloxanthin can also alter membrane fluidity, which is important in protecting against host defenses mediated by cationic peptides (Mishra et al., 2011a). Based on the contributions of carotenoid pigments toward staphylococcal fitness, inhibition of carotenoid biosynthesis is viewed as a potential therapeutic target in treating *S. aureus* infections (Liu et al., 2008).

Media Effects on Staphylococcal Membranes

MHB medium is the required media for antibiotic susceptibility testing in clinical microbiology laboratories in the United States (National Committee for Clinical Laboratory Standards, 2006). In a study by Mishra et al. (2011b), *S. aureus* with increased membrane fluidity was associated with decreased susceptibility to daptomycin and other microbial peptides. If it is determined that nutrient media selection impacts important aspects of membrane physiology such as fatty acid composition, carotenoid content, or membrane fluidity it may cause a rethinking of which media types are used for a wide variety of experimental protocols.

Analysis of *S. aureus* wound cultures in clinical laboratories is performed solely using MH media, but academic research on *S. aureus* utilizes a variety of media of varying composition. With such a wide variety of carbon and amino acid sources used in *S. aureus* growth there has been little directed research into how the membrane physiology of *S. aureus* may be affected by such diversity in growth conditions. Previous

research has noted that alterations in characteristics of the cellular membrane such as fatty acid composition, carotenoid content, and membrane fluidity have effects on *S. aureus* virulence, resistance and growth.

In order to elucidate any connection between nutrient media selection and alterations in cellular membrane characteristics in *S. aureus* we undertook this study to answer our primary objective by documenting that when grown in different nutrient media, the fatty acid composition, membrane fluidity and carotenoid content of *S. aureus* cells is altered. Our study hypothesized that these characteristics will be significantly altered in *S. aureus* cells between different media types due to the variety of carbon and amino acid sources present in different media. In addition, we sought to document membrane property differences in fatty acid composition, membrane fluidity and carotenoid content between various strains of *S. aureus* grown in multiple media types. We hypothesized that antibiotic resistant bacteria will respond differently to different media types and exhibit altered membrane characteristics compared to the susceptible parent strains.

The outcome of this project could prove vital for future clinical *S. aureus* testing because variation of membrane characteristics due to growth media may impact testing results. Accurate clinical testing is an essential tool for medical providers to tailor their treatment regimens to each patient's specific needs. With the results of this study, we expect to gain a greater understanding of how media composition alters *S. aureus* membrane physiology and spark a debate as to whether MH media should continue to be the standard for clinical lab testing of *S. aureus*.

CHAPTER II

MATERIALS AND METHODS

Nine stains of *S. aureus* were chosen to compare the effects of growth in MHB vs TSB media on membrane characteristics. COL-R (a well-studied MRSA strain), ATCC 29213 (extensively used in antibiotic susceptibility studies), VISA strains 13136 p^-m^+ V5, 13136 p^-m^+ V20 and their parent 13136 p^-m^+, daptomycin-resistant strain CB1540 and its parent strain CB1118, SH1000 and SH1000-BKD. These strains were chosen because they were available in house and provided an appropriate range of antibiotic resistance as well as comparative value between parent and mutant strains. Strains were grown in MHB and TSB to mid-exponential phase and analyzed in triplicate for each experiment.

Methanol Extraction of Carotenoids

Cultures of *S. aureus* were grown overnight in 50 ml flasks containing 10 ml of TSB at 37°C with shaking at 200 rpm. Cultures were then inoculated in 250 ml flasks containing 50 ml of media and grown to mid exponential phase (mid exponential phase was determined through periodic spectrophotometric measurements of media cultures until OD_{600nm} reached ~0.6) in (MHB, TSB). Samples were subjected to methanol extract following the protocol of (Morikawa et al 2001) as described in (Davis et al 2005) and the total carotenoid content analyzed by analysis of the OD_{460}.

Sample Collection for Fatty Acid Analysis

Cultures of *S. aureus* were grown overnight in 50 ml flasks containing 10 ml of media at 37°C with shaking at 200rpm. The samples were then inoculated in 250 ml flasks containing 50 ml of media and grown to mid exponential phase in at 37°C with shaking at 200 rpm. The cells were then harvested in mid-exponential phase (OD600 0.6) by centrifugation at 3000 x g at 4°C for 15 minutes and the pellet were washed three times in cold distilled water. The samples were then sent for Fatty Acid Methyl Ester (FAME) analysis whereby the fatty acids in the bacterial cells (30 to 40 mg wet weight) are saponified, methylated, and extracted. The resulting methyl ester mixtures were then separated using an Agilent 5890 dual-tower gas chromatograph and identified by Midi microbial identification system (Sherlock 4.5 microbial identification system) at Microbial ID, Inc. (Newark, DE).

Serum Growth

Bovine growth serum (Fisher Scientific) was aliquoted into 10 ml and 50 ml samples in 50 ml and 250 ml flasks respectively. The aliquoted serum was incubated in a water bath at 56°C for 30 min to heat inactivate any complement proteins. SH1000 and SH1000 BKD were grown for 24 hours in 50 ml of serum in a 250 ml flask at 37°C with shaking at 200 rpm.

Membrane Fluidity

The fluidity of the cell membranes of two *S. aureus* strains (SH1000, SH1000 BKD) were measured by fluorescence polarization measurements using diphenylhexatriene (DPH). Following the protocol described in Mishra et al.(2011a),

overnight cultures of each strain were grown in TSB at 37°C with shaking at 200 rpm. Samples were inoculated and mid exponential phase cultures were grown in MHB, TSB, LB and BHI then harvested and washed twice in 0.85% (wt/vol) NaCl. The pellets were then resuspended in 0.85% (wt/vol) NaCl containing 3 mM DPH to an OD_{600} of about 0.3. Fluorescence polarization measurements were measured using a PTI Model QM-4 Scanning Spectrofluorometer. The experiments were performed on three fresh batches for each strain with SH1000 grown in all four media (TSB, MHB, BHI and LB) and SH1000 Bkd grown in two media, BHI and TSB.

Comparison of the Effects of Different Media on Lipid Composition

The preliminary results of the effect of media selection on membrane fluidity, carotenoid content and fatty acid composition required further experimental support. In an effort to further document the effect of media selection on membrane characteristics, two strains of *S. aureus* (SH1000 and SH1000 BKD) were grown in an even wider array of nutrient media (MHB, TSB, BHI, LB, Tryptone Broth (TB), Mueller Hinton Oxoid (OX), Cy Broth (Cy), Cation supplemented MHB, defined media (Towsend and Wilkinson,1992)) and subjected to the same experimental approach as previously described. The BKD mutant contains an inactivated branched-chain α-keto acid dehydrogenase (BKD) enzyme complex that is used by the wild type strain (SH1000) to catalyze the early stages of BCFA production. By comparing the BKD mutant to the wild type SH1000 strain a better idea of how *S. aureus* membrane characteristics are affected by media choice can be examined. The SH1000 strain is a derivative of NCTC 8325 and has the *rsbU* mutation repaired making the strain more robust in its response to

environmental stress thus making it a good control strain that has been used by many authors in their studies of staphylococcal biology.

CHAPTER III

RESULTS

Methanol Extraction of Carotenoids

To test the hypothesis that carotenoid composition changes in different nutritional conditions, nine clinical *S. aureus* isolates were grown overnight in either MHB or TSB. Carotenoids were methanol extracted and concentrations measured by OD_{465nm} as described in methods and materials. The initial data from the methanol extract of carotenoids from different strains of *S. aureus* grown in MHB vs TSB indicate that in all but one strain (SH1000 BKD) TSB increases the content of carotenoids in the cell membrane (Figure 1). Next, we focused on the prototypical strain SH1000 and its BKD mutant to further expand and compare the types of media tested. In the media comparison between SH1000 and its BKD mutant the media with the largest content of carotenoids for both strains during each phase of growth was MHB (Figure 2, Figure 3). It is important to note that the growth rates of both strains of *S. aureus* varied greatly between media. In media such as TSB and BHI has reached log phase within 3 hours of inoculation, while in media such as MHB and TB it took as long as 7 hours to reach log phase and up to 13 hours to reach stationary phase. The data from the media comparison of SH1000 and its BKD mutant also indicates that for all media types except for Serum

and MH Oxoid the amount of carotenoid in the cellular membrane is greater in BKD compared to SH1000 in the log phase of growth (Figure 2). The trend is similar in the stationary phase as the carotenoid content of BKD cells surpasses SH1000 in all medias except for MH Oxoid and Cy Broth (Figure 3).

Figure 1. Mean Carotenoid Content of *S. aureus* Strains Grown in MHB vs TSB

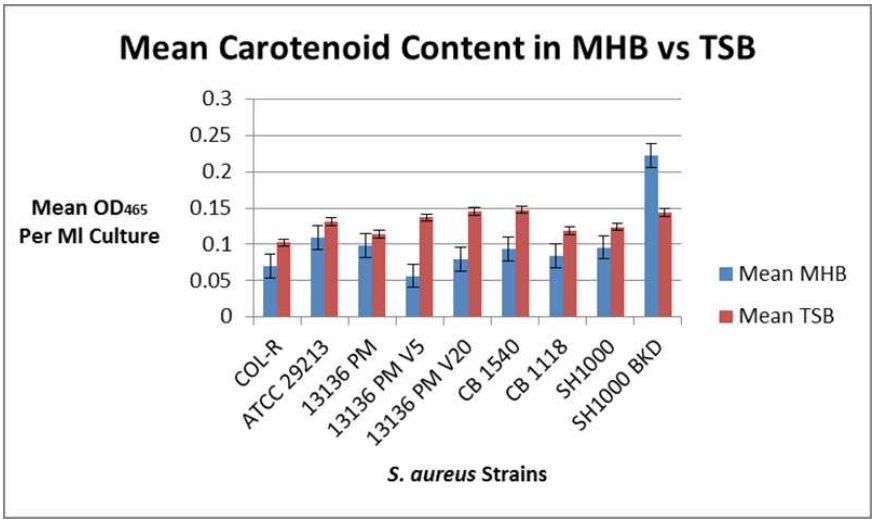


Figure 2. Carotenoid Content of SH1000 vs SH1000 BKD at Log Phase of Growth

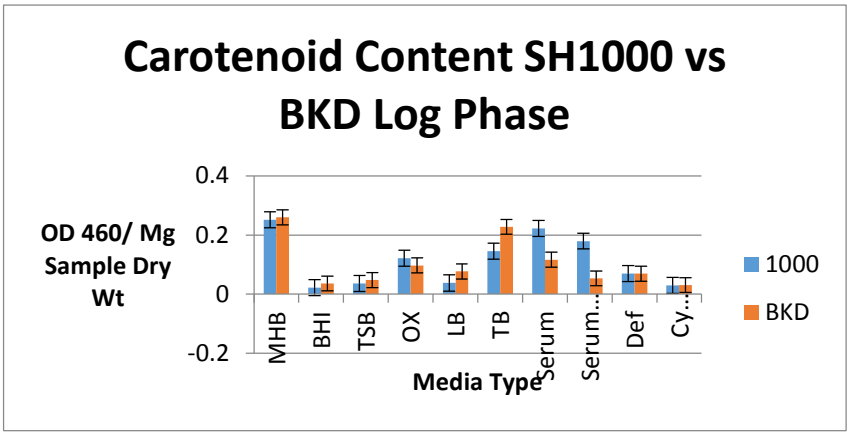
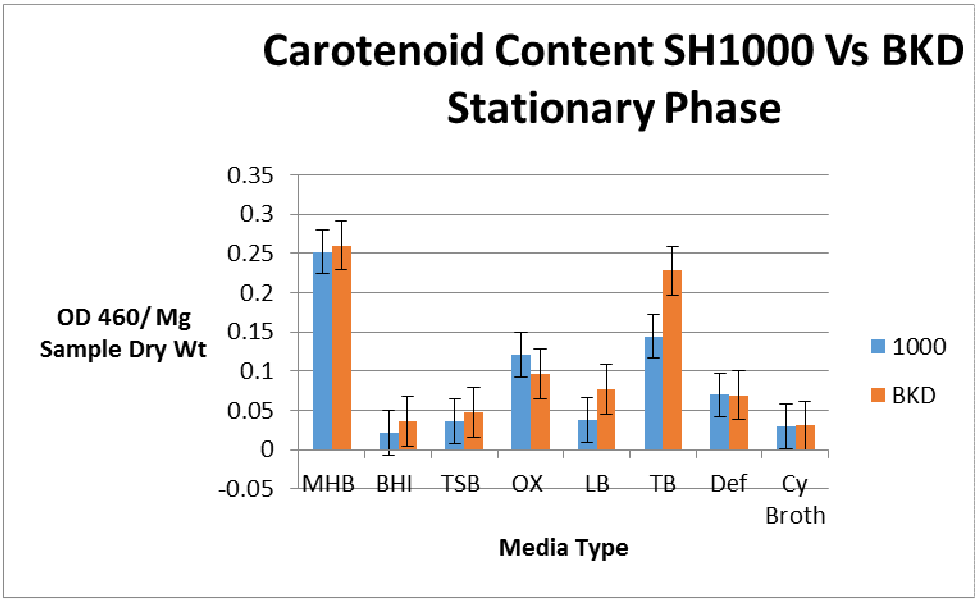


Figure 3. Carotenoid Content of SH1000 vs BKD Cells at Stationary Phase of Growth



FAME Analysis

As noted earlier, unsaturated fatty acids increase membrane fluidity when incorporated into phospholipids of the cellular membrane. In order to determine the composition of the cellular membranes of *S. aureus* grown in different media, samples were collected and subjected to FAME analysis. If different nutrient media have varying effects on the biosynthesis of fatty acids by the cell we would expect to see differences in the ratios of BCFA and SCFA between cells grown in different media. Analysis of the initial FAME data indicates that *S. aureus* cells grown in MHB exhibit a greater ratio of anteiso/iso fatty acids incorporated into the cellular membrane compared to cells grown in TSB (Figure 4). The data also indicate that *S. aureus* cells grown in MHB have an increased percentage of BCFAs incorporated into the cellular membrane compared to TSB in all but two strains COL-R and 13136 $p^{-}m^{+}$ V5 (Figure 5). The initial data indicates that *S. aureus* cells grown in TSB have an increased percentage of SCFAs

incorporated into the cellular membrane in all but two strain COL-R and 13136 $p^{-}m^{+}$ V5 (Figure 5). The initial data are supported by the data from the comparison of SH1000 and the Bkd mutant in various media types. The comparison data indicates that when grown in almost any media SH1000 will have a greater percentage of BCFA than the BKD mutant (Figure 6). The comparison data substantiates the initial data in that cells grown in MHB have a greater percentage of BCFAs compared to cells grown in TSB for both strains (Figure 6). The comparison data also substantiates the initial FAME data that cells grown in MHB will have a greater anteiso/iso ratio compared to cells grown in TSB (Figure 7). Of particular interest are the FAME profiles of SH1000 and BKD cells grown in bovine serum (Figures 8 and 9). The profiles of serum grown cells contrast with those of *S. aureus* grown in other media types as both SH1000 and the BKD mutant exhibit high concentrations of fatty acids containing odd numbered carbon chains, compared to cells grown in other medias where even number carbon containing fatty acids predominate. The membranes of serum grown cells also contain greater amounts of iso BCFAs than anteiso BCFAs, as opposed to cells grown in other medias (MHB, TSB, LB) where anteiso BCFAs comprise a greater percentage of the membrane than iso BCFAs (Figure 7).

Figure 4. Anteiso/Iso ratio of *S.aureus* Strains Grown in MHB vs TSB

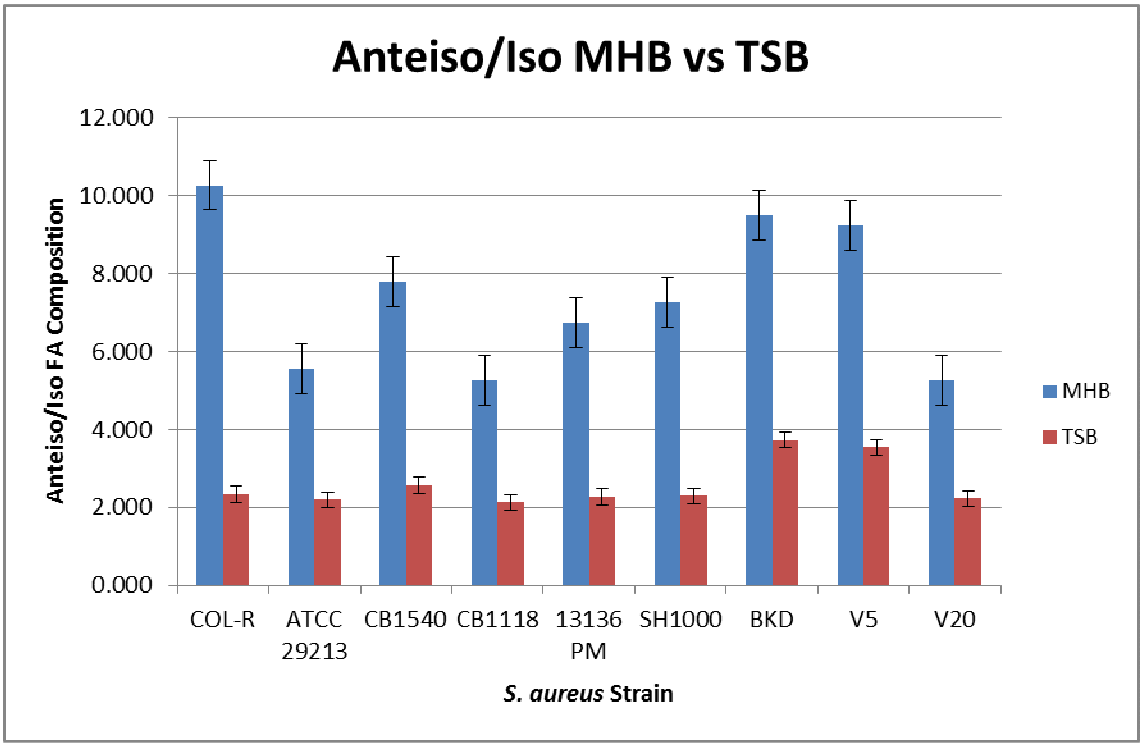


Figure 5. BCFA vs SCFA Content in *S. aureus* Strains Grown MHB Compared to TSB

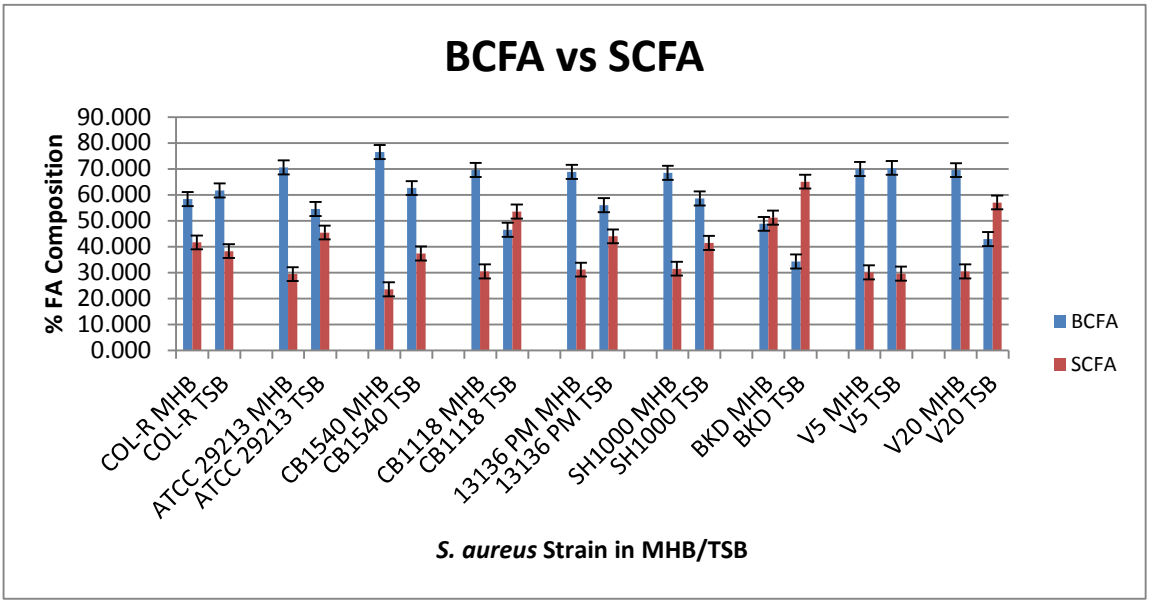


Figure 6. SH1000 vs BKD BCFA and SCFA Content when Grown in Different Media Types

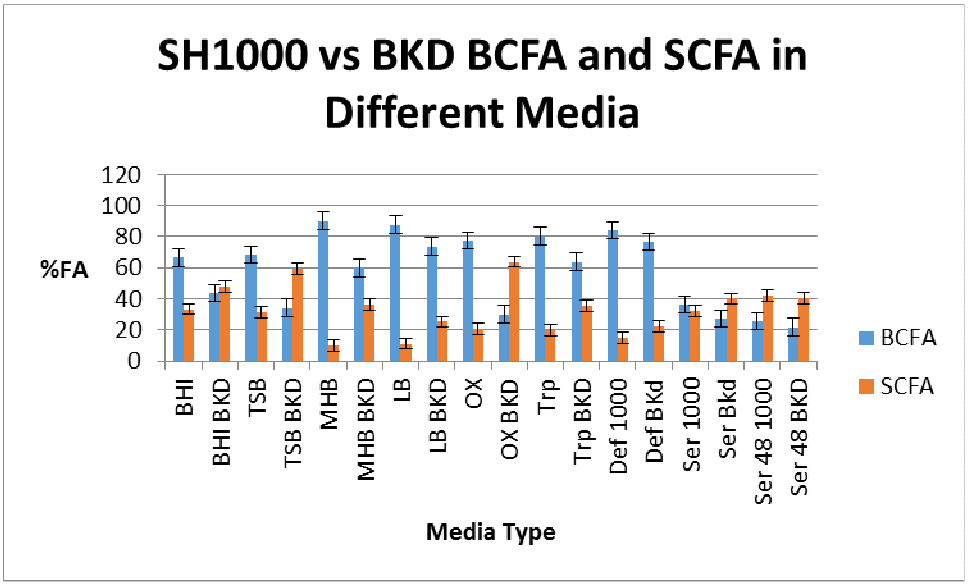


Figure 7. Anteiso/Iso Ratios of SH1000 vs BKD Cells Grown in Different Media

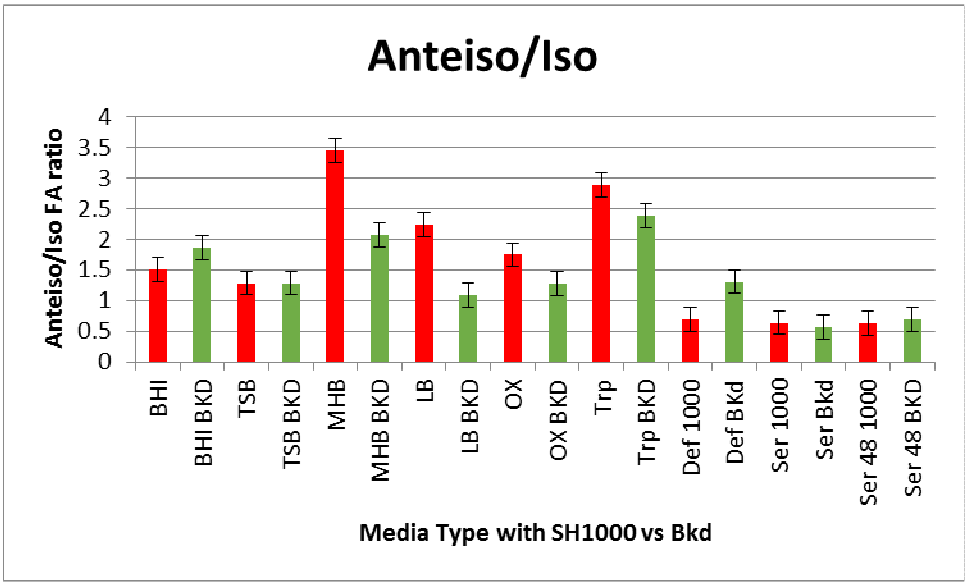


Figure 8. Comparison of the FA Profiles of SH1000 Cells Grown in Different Media

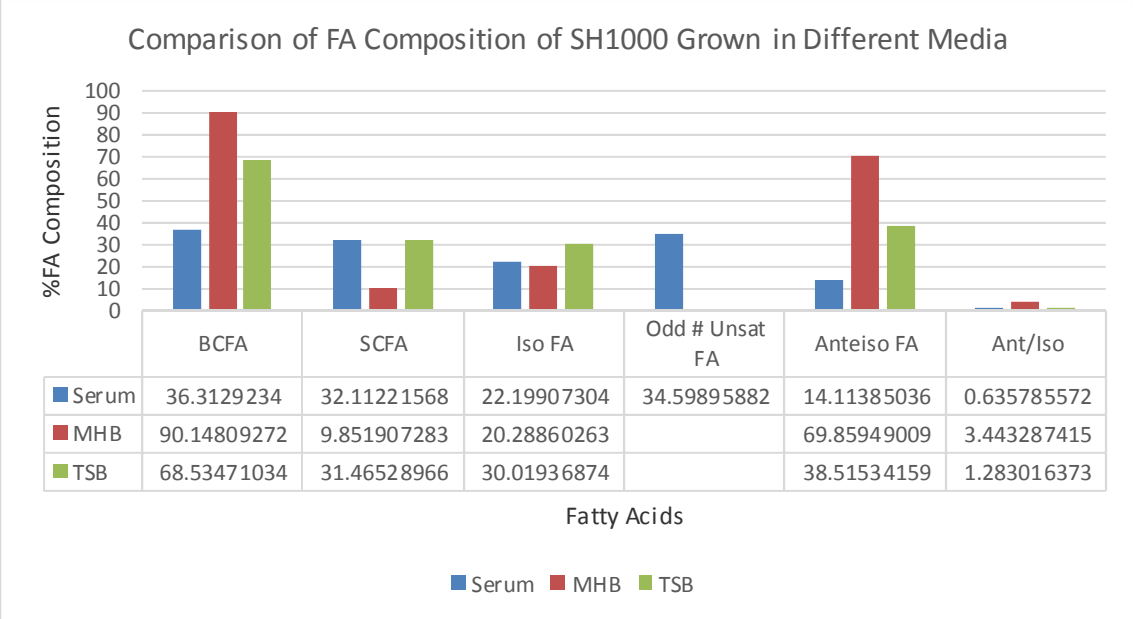
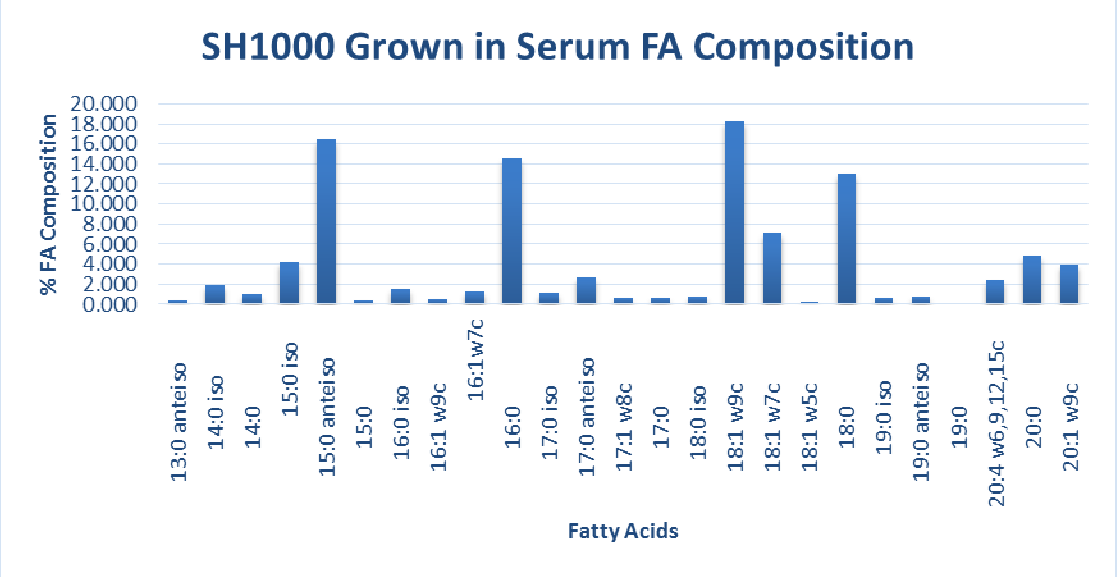


Figure 9. Fatty Acid Composition of SH1000 Grown in Serum



Membrane Fluidity

In an effort to determine if any variation in membrane fluidity could be observed between *S. aureus* cells grown in different media types, samples were collected and subjected to fluorescence anisotropy. Using a fluorescent dye DPH that can permeate the cellular membrane and be excited by laser, a determination of membrane fluidity can be assessed. The lower the value of the fluorescence reading, the greater the fluidity of the cellular membrane. The initial fluorescence anisotropy measurements indicate the membrane of SH1000 cells grown in TSB media are more fluid than cells grown in MHB (Figure 11). These results are indicated by the lower polarization values seen in TSB-grown cells compared to MHB-grown cells. The initial measurements indicated that MHB-grown SH1000 cells had the least fluid membrane compared to cells grown in TSB, BHI and LB (Figure 11). The initial measurements indicate that SH1000 Bkd cells grown in TSB have greater membrane fluidity than cells grown in BHI (Figure 12). These results are substantiated by the comparison results (Figure 10) which indicate that cells grown in TSB have membranes more fluid than cells grown in MHB.

Figure 10. Membrane Fluidity of SH1000 vs BKD Cells Grown in Different Media

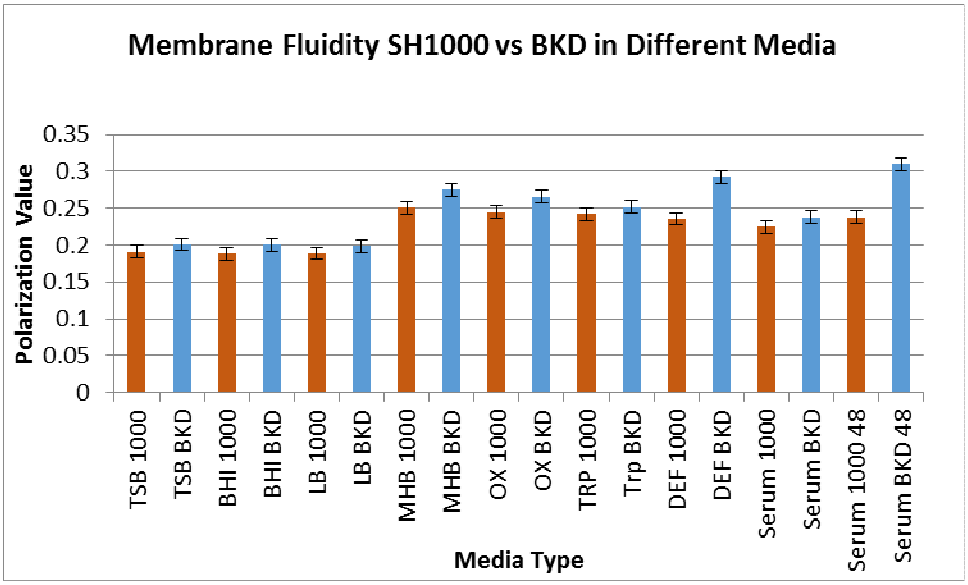


Figure 11. Membrane Fluidity of SH1000 Cells Grown in Different Media

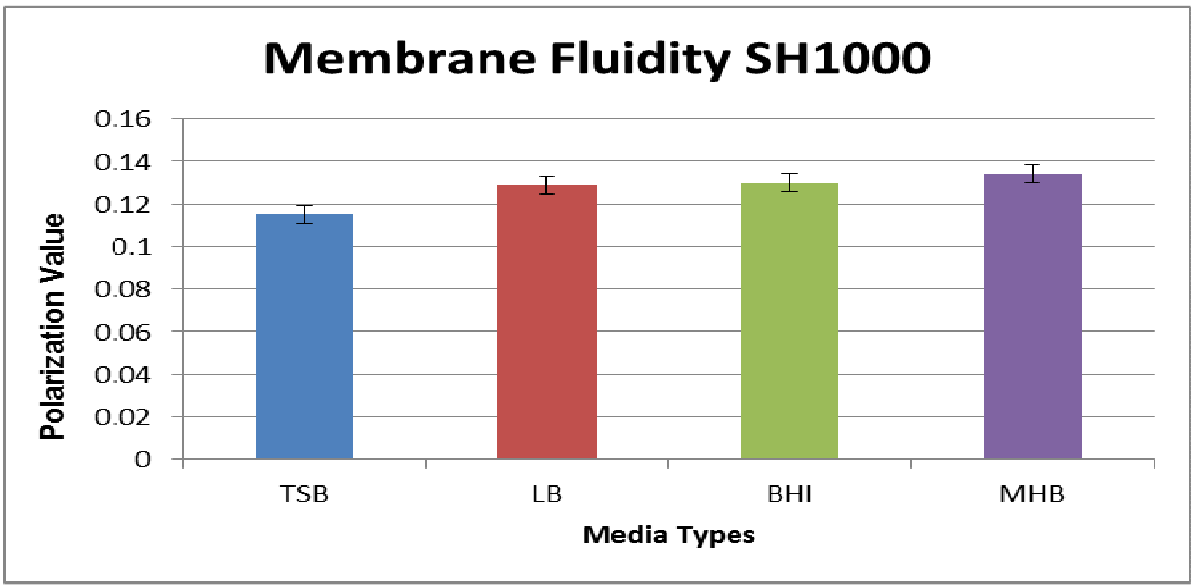
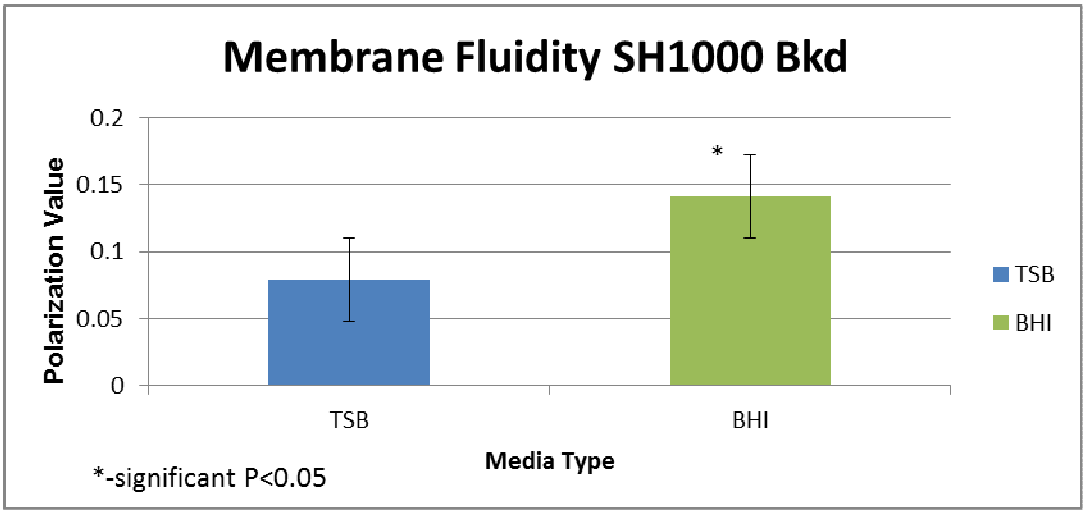


Figure 12. Membrane Fluidity of SH1000 BKD Cells Grown in Different Media



CHAPTER IV

DISCUSSION

The data from each portion of our study paints a confusing picture of the membrane dynamics of *S. aureus* cells grown in different media. The fatty acid analysis data from each portion of our study would indicate that *S. aureus* cells grown in MHB should have the most fluid membranes due to the high composition percentage of BCFAs. However, the fluidity data from each phase of the study indicates otherwise with MHB-grown *S. aureus* cells having some of the most rigid membranes of any media type. The picture becomes somewhat more clear when the carotenoid content of the cells are taken into account. Carotenoid pigments of *S. aureus* provide integrity to the cellular membrane and limit oxidative host defense mechanisms (Mishra et al., 2011a). Cells grown in MHB had the highest carotenoid levels of any media type for SH1000 and the Bkd mutant (Figures 2, 3). It is possible that the high carotenoid content of cells grown in MHB provides stability to the membrane counteracting the increased fluidity of the high BCFA content.

The data provides an interesting insight into the different effects that media composition can have on cellular membrane characteristics. A great deal of variation can be seen between media types in fatty acid composition, carotenoid content and membrane fluidity lending credence to our broader hypothesis that media composition affects these

characteristics of the cellular membrane. Certain trends can be identified in our data but without much statistically significant data to provide firm evidence. However, we were able to illustrate distinct differences in certain membrane characteristics for two of the most widely used media types in research and clinical laboratories TSB and MHB.

Of particular interest are the results of the SH1000 and BKD cells grown in bovine serum. These serum grown cells had fatty acid profiles quite unlike the cells grown in any other media with high content of odd numbered unsaturated fatty acids (Figures 8 and 9). These profiles raise the question of whether the compositions of nutrient media are altering the cellular membrane characteristics in significant ways that are not seen when the bacteria are growing in a host. The effect that different growth conditions might have on fatty acid biosynthesis was illustrated by the variation in the fatty acid profiles of the different growth media. The prevalence of certain media such as MHB to favor antieso fatty acid synthesis while media such as TSB favored a more even synthesis between anteiso and iso fatty acids cannot be discounted. That Serum grown cells would exhibit fatty acid profiles unlike either MHB (the commonly used clinical media) or TSB (a commonly used research media) is significant for its implications. More research is required to further investigate this question because the answers are of the most importance for the future of clinical lab testing of patient samples. This finding is important in consideration of *S. aureus* growing in vivo in an infection versus in vitro in a laboratory media.

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