WPI

Effects of Cranberries on Biofilms

A Major Qualifying Project Submitted to Faculty of Worcester

Polytechnic Institute in Partial Fulfillment of the Requirements for

The Degree of Bachelor of Science

April 19, 2013

Submitted by:

Cynthia Chelius

Catherine Duffty

Submitted to:

Professor Terri Camesano

Abstract

Biofilms are communities of bacteria that grow on living or nonliving surfaces and are typically encased in an extracellular matrix. This extracellular matrix is composed mainly of exopolysaccharides, proteins, and DNA. This matrix acts mainly to hold the biofilm together and can also provide the biofilm with stability and protection. The major problem associated with biofilms is their resistance to antibiotics and the matrix represents an initial barrier that can delay the penetration of antimicrobial agents. Previous research has suggested that cranberries have several health benefits, including prevention of recurrent urinary tract infections in women. Cranberry juice contains high concentrations of anthocyanidins, flavanol glycosides, phenolic acids and proanthocyanidins (PACs). PACs and anthocyanidins serve the purpose of anti-adhesion in the plant to protect it from potential infections. Previous studies have presented evidence that the A-linked PACs found in the American cranberry do have an impact on treating and preventing UTIs, however very few studies have evaluated the effects of only anthocyanidins on biofilm development. We have used a 96 well assay to compare the effects of PACs, anthocyanidins, and crude extracts on *S. aureus* and *E. coli*. Each of the bacteria were cultured 6 successive times in 120µg extract/mL broth. We found that PACs demonstrated an inhibiting effect on *S. aureus*, but there was not a significant trend in *E. coli*. The crude extract had no significant trend in *S. aureus*. Interestingly the crude and *E. coli* experiment resulted in significant data at all times, but the extract had more attachment to the wells than the only *E. coli* wells, which we believe to be in part due to the crude extract attaching to the walls as well. The anthocyanidins showed a significantly lower amount of attachment at the last time point however this is not enough to conclude any inhibition overall. Our results conclude that PACs has an effect on biofilm development, while the crude and anthocyanidins experiments remain inconclusive. The concentration of the extract could have an impact on biofilm inhibition and the next step in this research would be to increase the crude and anthocyanidin concentrations as well as to increase the number of replicates.

Acknowledgements

We would like to begin by thanking our advisor, Professor Terri Camesano, Department of Chemical Engineering at Worcester Polytechnic Institute, for giving us the opportunity to work on this project. She has supported our efforts during the duration of this MQP project and always kept a line of communication open to us.

We appreciate the guidance we received by Paola Pinzon in regards to the experimental procedure used for the 96 well assay. Her previous research and laboratory techniques were extremely helpful in guiding us towards our objectives and laboratory methodology.

The laboratory procedures concerning bacteria culture were an integral part of our MQP and our work would not have gone as smoothly without help from Mary Schwartz and Todd Alexander.

Finally we would like to thank Professor Neto of the University of Massachusetts Dartmouth Laboratory for kindly providing the isolated proanthocyanidins, anthocyanidins, and crude extracts necessary for this research.

Table of Contents

Table of Figures

Table of Tables

Chapter 1 – Introduction

Biofilms are communities of bacteria that grow on living or nonliving surfaces and often attach to tissues within the body or to medical implant devices and can cause infections. Typically medical devices that are infected with a biofilm must be completely removed from the body, as they have been shown to be antibiotic resistant. The surgery to replace medical devices takes a physical and economic toll on the patient. Biofilm development is being researched to find potential methods of eradication.

There are different types of bacteria, two of these are gram positive and gram negative. The main difference between these types is the presence or lack of a cell wall. This part of the cell may or may not affect how biofilms are formed and eradicated or prevented. Our study will focus on one representative of each type, *E. coli*, a gram negative, and *S. aureus*, a gram positive bacteria.

Bacteria can cause several health complications, especially in artificial organs or other body parts. One potential way to prevent these biofilms from forming in the body is by injecting certain chemical compounds which can prevent the biofilm from forming or adhering to surfaces. The American cranberry is a fruit produced mainly in New England and has many health benefits associated its consumption, including the prevention and potential treatment of urinary tract infections. There are several compounds found in cranberries which have health benefits associated with them. Anthocyanidins, proanthocyanidins, and flavonoids are three of the compounds with potential health benefits. Anthocyanidins and proanthocyanidins serve the purpose of protecting the plant from infections through anti-adhesion properties and there is research suggesting anthocyanidins also acts as an antioxidant and could have potential health benefits such as an anti-inflammatory agent and reducing risk of cardiovascular disease. Flavonoids have been used as folk remedies for hundred or years and have shown health benefits such as treating rheumatic pain. Other components in the cranberries are present but have less research associated with them, but it is likely other compounds can contribute health benefits when consumed.

Of the health benefits associated with consumption of cranberries, prevention of urinary tract infections is one of these. This benefit of cranberries is due to the proanthocyanidins present in the fruit. When studying the effect of cranberry juice and proanthocyanidins on biofilm formation, it was observed that cranberry juice cocktail was more effective then isolated proanthocyanidins at preventing biofilm formation. This suggests that other compounds may play a role in anti-biofilm properties.

The objective of our project was to develop a procedure to test different extracts received from the University of Massachusetts Dartmouth laboratory. This was accomplished through researching and replicating the experiments previously performed with light Ocean Spray cranberry juice cocktail and adjusting the procedure to implement the solid extract powders.

Chapter 2 - Literature Review

2.1 Biofilms

Biofilms have been classified as communities of microbes associated with a surface that are typically encased in an extracellular matrix (Ghannoum et al, 2004; Ghannoum et al, 2004) . Bacteria in biofilms have the ability to form on living as well as nonliving surfaces. Biofilms have been a problem in the medical field due to their capability of attaching to medical implants and their antibiotic resistance. Figure 2.1 shows a biofilm of *S. aureus* that has formed on an indwelling catheter.

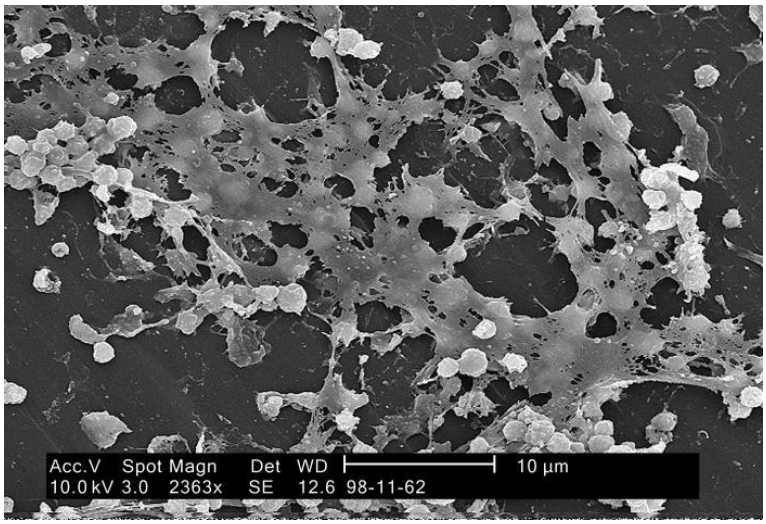


Figure 2.1. *S. aureus* bacteria formed into a biofilm with polysaccharides connecting the bacteria. (Carr, 2005)

2.2.1 Growth of Biofilms

Biofilms grow according to a biological cycle that includes initiation, maturation, maintenance, and dissolution. This cycle can be seen below in Figure 2.2. Individual planktonic cells can form cell-cell and cell-surface contacts, forming microcolonies (Costerton et al, 2004).

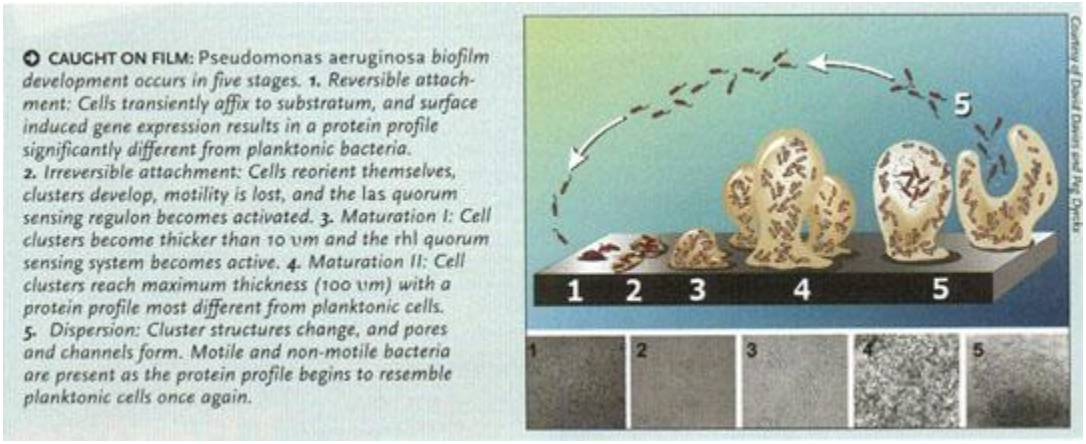


Figure 2.2: Stages of biofilm development as described by Johnston (Johnston, 2004)

2.1.1.1 Attachment

It has been thought that biofilm formation begins when bacteria sense environment conditions that trigger a transition to life on a surface. These environmental conditions include temperature, osmolarity, pH, iron, oxygen and the nutritional content of the medium (Costerton et al, 2004). The cell surface structure of a bacterium is important for attachment. The rate of bacterial attachment can be impacted by the presence of flagella, pili, fimbriae, or glycocalyx. Once a microbial cell is drawn to the surface, it must overcome the repulsive forces common to all surfaces and these appendages enable the cell to remain attached until more permanent attachment mechanisms are in place. Cell surface hydrophobicity has also been shown to be important for attachment (Donlan, 2001)).

For the gram-negative bacteria, *E.coli*, a variety of surface structures are involved in attachment to a surface, such as pili. Each surface structure may be specific to the properties of the attachment surface. The environment in which the bacterium is exposed to may change the expression for particular structures. *E.coli* has been observed to require flagellum-mediated motility in order to initiate attachment events. The gene responsible for the biogenesis of type-I pili is phase variable, meaning that only a fraction of the *E.coli* population will be primed for biofilm formation initiation which may be a selective advantage.

The gram-positive bacteria, *S.aureus*, initiates biofilm formation by cell-surface interactions which may be mediated by factors such as surface proteins, extracellular proteins, capsularpolysaccharide/adhesins (PS/A), and autolysins located on the cell surface. After subsequent cell-surface interactions the bacteria enter the "accumulation phase" of biofilm formation. Cell-cell interactions ensue and a formation of cell aggregates form on the attachment surface. At this point in development, a polysaccharide intercellular adhesion (ICA) has been identified as becoming implicated (Costerton et al, 2004).

2.1.1.2 Maturation

After initial attachment to a surface the biofilm matures and undergoes further adaption to life in a biofilm. Two events occur in both gram-positive and gram negative bacteria: increased synthesis of extracellular polysaccharides (EPS) and development of antibiotic resistance (Costerton et al, 2004). EPS provide the structure for the biofilm and are highly hydrated at 98% water, and are strongly bound to the underlying surface (Donlan, 2001).

In addition biofilms may develop properties such as increased resistance to UV light and increased rate of genetic exchange. Some species of *E.coli* have shown to develop characteristics such as microcolonies, water channels, heterogeneity in structure, and significant thickness (Costerton et al, 2004). Atomic force microscopy images revealed that the surface structure of a hydrated biofilm include 0.25µm pores and 0.50µm channels (Pavithra et al, 2008). The structure of the water channels allows transportation of essential nutrients and oxygen to all layers of a biofilm, maintaining the viability of the bacteria and their ability to proliferate (Costerton et al, 2004) . The major EPS of *E.coli*, colonic acid, is necessary in the development

of these structural characteristics, but is not involved in initial biofilm attachment. A genetic screening was performed on planktonic *E.coli* and *E.coli* in biofilms and it was observed that 40% of the genetic material of the bacteria had been altered (Costerton et al, 2004).

S.aureus also makes an EPS, but unlike *E.coli*, does so in a phase manner. The increased EPS corresponds to the ability to form a biofilm, however the signals regulating this process have not yet been identified. Forming these colonies involves integrating external and internal signals, determining the density and type of neighboring cells, and coordinating timed multicellular behaviors associated with morphological changes (Costerton et al, 2004).

2.1.1.3 Detachment

The release or detachment of bacterium from a biofilm is believed to be regulated by population density-dependent gene expression. The gene expression is controlled by the cell to cell signaling molecules which include acylaed homoserine lactones (AHLs) for gram-negative bacteria and specific peptides for gram-positive bacteria (Pavithra et al, 2008).

2.2.2 Surface Properties and Factors Influencing Bacterial Adhesion

There are three main factors that influence bacterial adhesion to a polymeric surface. These include the properties of the material, the type of microorganism and the nature of the environment. The environmental factors include temperature, time of exposure, fluid flow conditions, bacterial concentration, and the presence of antibiotics. Fluid flow near the surface of bacterial contact seems to be the dominant condition determining the number of attached bacteria as well as the structure and viability of the biofilm.

The second factor, organism properties, plays a role in attachment and the strength in which a bacterium does so. The surface charge of the bacterium, the presence of flagella of fimbriae, and the production of the EPS coat influence the attachment. Lastly the structure of the surface material impacts the attachment of biofilms. In comparing a rough surface to a smooth surface, smooth implants have been found to host less biofilm masses than rough surfaces. Rough surfaces contain irregularities and depressions that increase the surface area and provide a favorable site for adhesion. A diagram showing the factors influencing bacterial attachment can be seen below in **Error! Reference source not found.** (Pavithra et al, 2008).

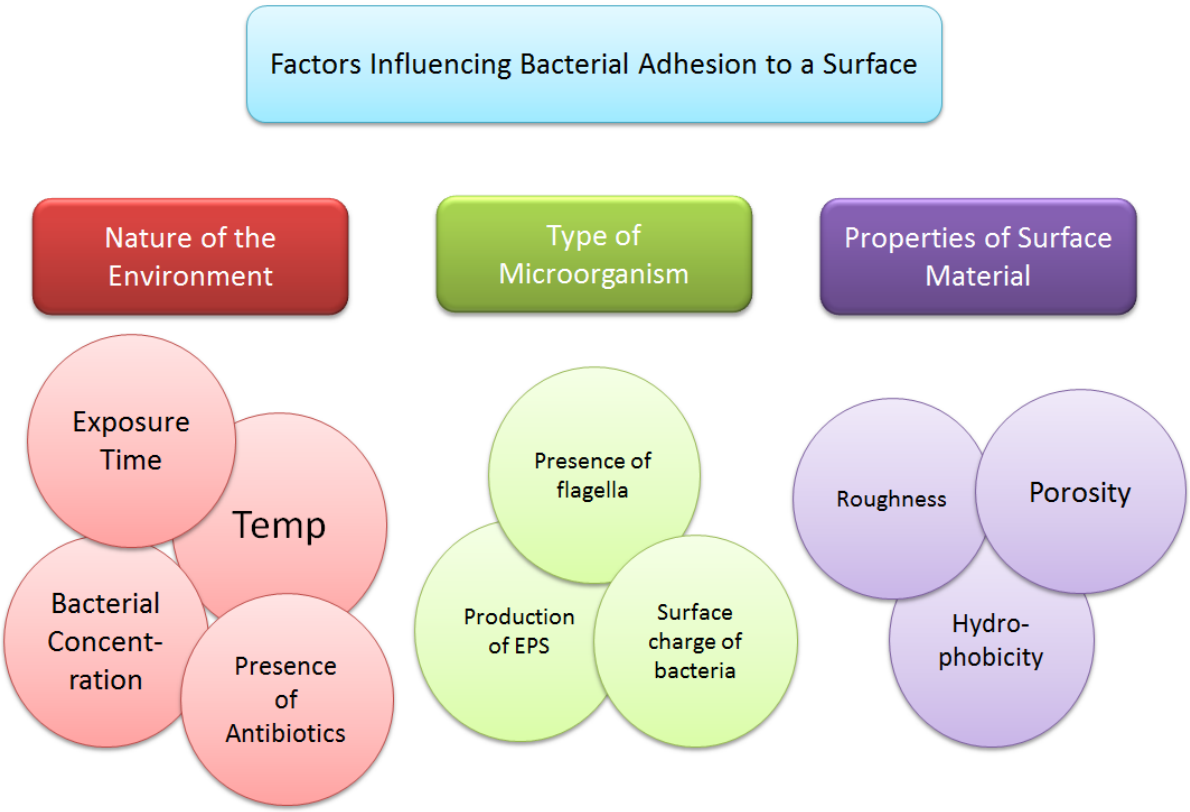


Figure 2.3: Factors Influencing Bacteria Attachment to Medical Devices (Based on Pavithra et al, 2008**)**

2.1.2 Problems Associated with Biofilms

Biofilms will grow on foreign body surfaces, such as prosthetic devices like catheters and stents. In addition to foreign bodies, biofilms can grow on natural surfaces such as teeth, heart valves, the lungs of cystic fibrosis patients, and in chronic wounds (Stewart et al, 2001). The major problem associated with biofilms is their resistance to antibiotics. Multiple factors contribute to their antibiotic resistance including their extracellular matrix, high frequency of mutation, high nosocomial infection rate, and growth properties such as thickness, rate of growth, and oxygen consumption.

There are several advantages to bacteria attaching to a surface to form a biofilm. One advantage is that surfaces absorb, meaning they concentrate the scarce nutrients in the surrounding liquid. Biofilms need nutrients to survive and surfaces contain a greater concentration. The surface also allows the biofilm, which usually contain heterologous species, an area to engage in metabolic interactions with other members of the biofilm (Ghannoum et al, 2004). Surfaces allow the bacteria to congregate to form biofilms, which are surrounded by an extracellular matrix that acts as the first line of defense against an antimicrobial agent.

The bacterial biofilm extracellular matrix consists of exopolysaccharides, proteins, and DNA. This matrix acts mainly to hold the biofilm together. Interestingly DNA makes up the majority of

the matrix, and an experiment was conducted in which deoxyribonuclease (DNAase) was added to a culture and biofilm growth was inhibited. This leads to the assumption that DNA plays a role in biofilm growth. The experiment was conducted on newly formed biofilms and mature biofilms. The mature biofilms were not dissolved by the DNAase, suggesting that mature biofilms are strengthened by other substances (Whitchurch et al, 2002). The extracellular matrix helps to provide the biofilm with stability and protection (Stewart et al, 2001). Although the matrix provides protection, it is not impenetrable. Antimicrobial agents are able to diffuse through the matrix, so the matrix represents an initial barrier that can delay the penetration of antimicrobial agents. Since the extracellular matrix does not completely protect the biofilm there must be other factors contributing to its high rate of survival (Mah et al, 2001).

Another factor contributing to the high rate of biofilm survival is the high frequency of bacterial mutation. Compared with planktonic (free-floating) bacteria, biofilms have a much higher rate of mutation. This can be attributed to the close living conditions of the bacteria and the resulting horizontal gene transmission that occurs (Stewart et al, 2001). The heterogeneous mixture of different bacterial species within a biofilm has been shown to be 10-1000 times more resistant to the effect of antimicrobial agents than planktonic cells (Mah et al, 2001). Also, oxygen consumption relates to biofilm survival. Oxygen concentration is high on the outside of the biofilm and very low in the middle, suggesting anaerobic conditions in the middle. Thus growth of the biofilm is stratified with high growth on the outside and very low activity on the inside. This oxygen limitation and metabolic rate are important factors relating to biofilm tolerance to antimicrobials (Stewart et al, 2001) because the slow growth is related to the fact that cell density increases during the late stage of exponential (Mah et al, 2001). The thickness of the biofilm also acts as a resistance factor. The thicker the biofilm the less the antimicrobial is able to penetrate. Thus the more mature the biofilm the harder it is to eradicate (Mah et al, 2001).

It is important to remove the biofilm from the body for multiple reasons. Biofilms are not always highly pathogenic, but they can trigger planktonic "showering". This is a condition in which the biofilm periodically releases bacterial cells into the host's bloodstream, which can cause sepsis (Ghannoum et al, 2004, 334). Sepsis is a condition in which the body overreacts to an infection that causes an overwhelming systemic inflammation leading to multiple organ failure and has a high mortality rate (Knapp, 2012, 1). The biofilm attached to the surface can also trigger a host inflammatory response which can produce more tissue damage than the biofilm itself (Ghannoum et al, 2004).

The bacteria associated with biofilms can either originate from the host's own microflora or from an outside source. It is very common for hospitalized patients to get infected. Bacteria have quite frequently been found to migrate along the exterior lumen of an intravenous catheter or are seeded internally from an outside source (Ghannoum et al, 2004). Once infected the biofilm has the ability to grow on many medical implants such as catheters, artificial hips, and contact lenses. Due to the high antimicrobial resistance the treatment usually requires the removal of the implant. This increases trauma to the patient and increases cost of treatment. 65% of nosocomial

infections are associated with biofilms. The treatment of these biofilm infections costs more than $1 billion annually (Mah et al, 2001). Biofilms not only have extreme physical consequences on a host, but also have economic consequences.

2.2 Classifying Bacteria Based on Structure

Most bacteria can be classified as Gram-positive or Gram–negative. In order to determine whether a bacterium is Gram-positive or Gram-negative a stain is applied. Gram positive bacteria turn a darker blue color whereas a negative become pink. The reason behind the difference in color is due to the makeup of the cell walls. Peptidoglycan absorbs the stain and in Gram-positive bacteria, the peptidoglycan makes up the outside layer of the cell wall. Gram-negative bacteria are surrounded by a phospholipid bilayer. *Staphylococcus aureus* and *Escherichia coli* are examples of the two different types of bacteria.

2.2.1 Gram-Negative Bacteria

The first type, Gram–negative bacteria, possesses a cell envelope with two phospholipid bilayers surrounding peptidoglycan layer, as seen in **Error! Reference source not found.**. The cytoplasmic membrane is symmetric, consisting mainly of phospholipids in both leaflets. The outer membrane on the other hand is asymmetric. The inner leaflet is mainly phospholipids, whereas the outer contains lipopolysaccharides (LPSs). Not shown in **Error! Reference source not found.** are capsular polysaccharides and the enterobacterial common antigen which are components of the outer leaflet of the gram-negative cell envelope (Moran et al,).

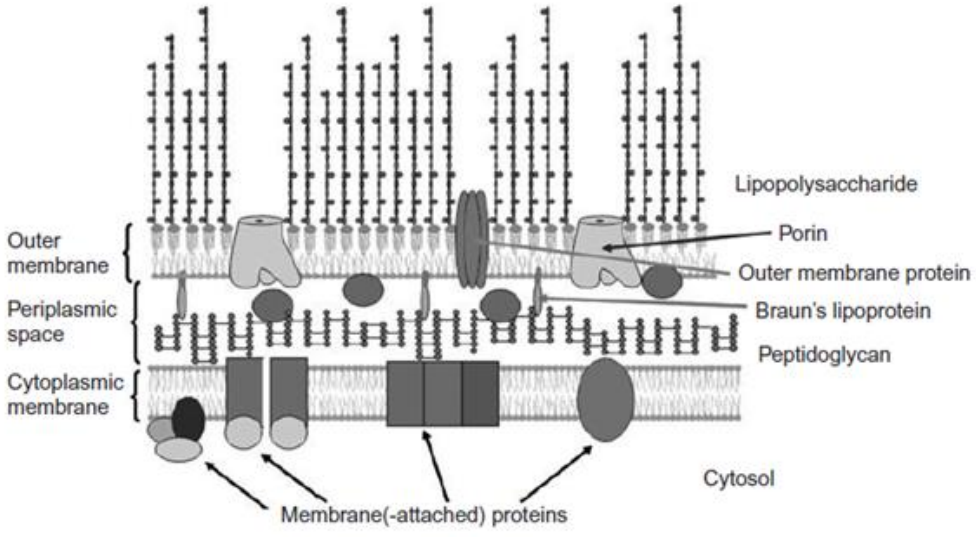


Figure 2.4: Gram-Negative Bacteria Cell Wall (Moran et al,)

LPS is a molecule of high importance for Gram-negative bacteria and for infected hosts. These molecules form a protective barrier for the bacterium, shielding them from their environment,

such as bile salts in the gut or antibiotics. The LPSs can also act as receptors for bacteriophages resulting in their death. For pathogenic bacteria LPSs are important virulence factors and their toxicity is based on their structure (Moran et al,). Lipopolysaccharides are amphiphillic and constitute 75% of the outer membrane leaflet. They are mainly involved in interactions with the external environment, in particular recognition, adhesion, and colonization. There are three regions that make up a LPS: a polysaccharide (may be the O-specific polysaccharide), a core oligosaccharide, and lipid A (Moran et al,). Other polysaccharide structures belong to the exopolysaccharides. These molecules are often found surrounding the bacteria and are important in biofilm formation and host interactions (Moran et al,).

One of the most common gram-negative bacteria that have been studied is *Escherichia coli*. *E. coli* is a non-sporing rod found as a normal commensal flora in the gastrointestinal tract and seen in **Error! Reference source not found.**. It is a facultative anaerobic bacterium with a metabolism of both fermentative and respiratory. E.coli can be motile or non-motile by use of peritrichous flagella. Most strains are not considered pathogens, but they can be opportunistic. As a pathogen, E.coli is most frequently the cause of bacterial infections such as, urinary tract infections, diarrheal disease and clinical infections such as neonatal meningitis, pneumonia, and bacteremia (Torres,1).

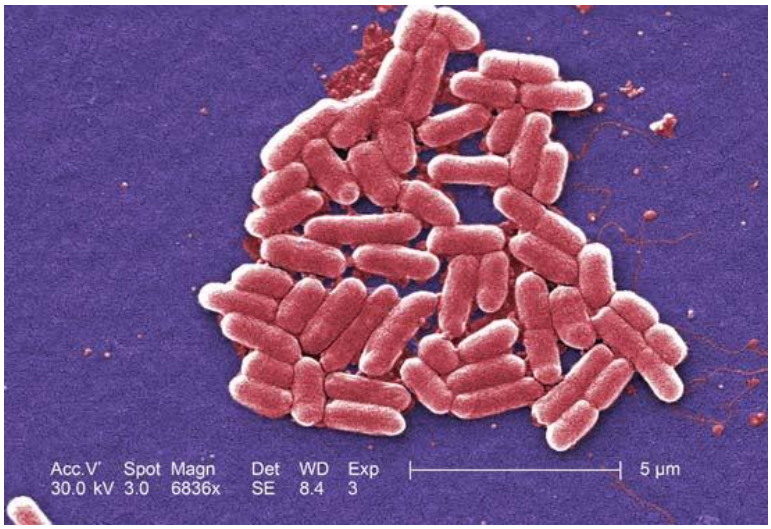


Figure 2.5: Scanning Electron Micrograph of Gram-negative *Escherichia Coli* bacteria (magnification 6,836×) (Carr, 2006)

2.2.2 Gram-Positive Bacteria

Gram-Positive bacteria are characterized by their cell walls that consist of a single phospholipid bilayer with a peptidoglycan layer surrounding it. **Error! Reference source not found.** is a model of the Gram-positive cell envelope. The envelope consists of a cytoplasmic membrane and a thick peptidoglycan layer. There are a number of proteins embedded within the membrane such as transport proteins and lipoproteins. In certain species, capsular polysaccharides and S-layer glycoproteins may be present. Various carbohydrate-based structures are attached to the cytoplasmic membrane such as teichoic and lipotechoic acids. The peptidoglycan layer of gram-

positive bacteria cell walls is generally 20-80 nm thick whereas the peptidoglycan layer is only about 7nm in Gram-negative (Moran et al,).

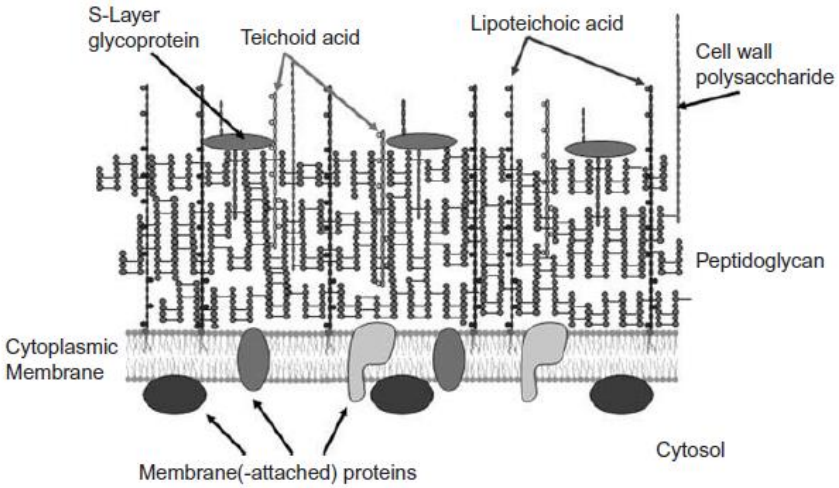


Figure 2.6: Gram-Positive Bacteria Cell Wall (Moran et al,)

An example of Gram-positive bacteria is *Staphylococcus aureus*. *Staphylococcus aureus* is a facultative anaerobic, non-motile bacterium that divides in more than one plane to form irregular clumps, as seen in **Error! Reference source not found.** (Blackburn et al,). Colonies usually have a golden yellow pigmentation and are mostly associated with community –acquired and nosocomial infections. Most staphylococci are responsible for skin infections such as boil, carbuncle, and furuncle (Bhunia, 2008).

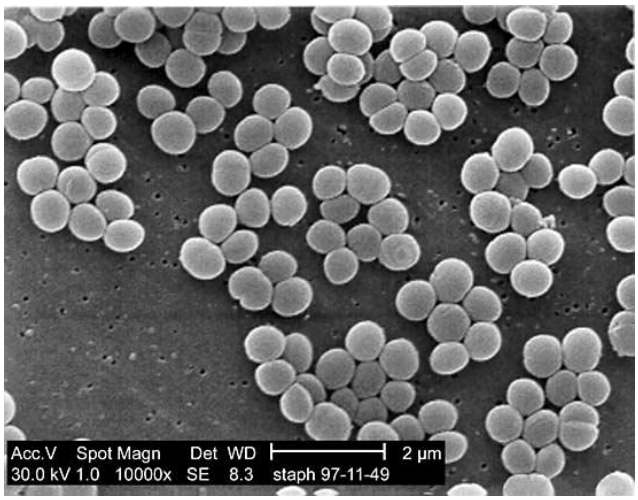


Figure 2.7: Microscopic image of *Staphylococcus aureus* (magnified 10,000x) (Carr, 2001)

The cell wall of *S. aureus* contain three main components: peptidoglycan comprising repeating units of N-acetyl glucosamine β-1,4 linked to N-acetyl muramic acid, a ribitol teichoic acid

bound via N-acetyl mannosaminyl- β-1,4-N-acetyl glucosamine to a muramyl-6-phosphate, and Protein A. Protein A is characterized by its ability to attach to immunoglobulin in plasma causing autoagglutination (Bhunia, 2008).

S. aureus also produces virulence factors such as adhesion proteins, enterotoxins, superantigens, and proteases. One such adhesion protein, Bap, is a biofilm-associated protein that is responsible for biofilm formation and colonization. The C-terminus of Bap contains a cell wall anchoring domain (Bhunia, 2008). Other binding proteins of *S. aureus* are ClfA, which activates platelet aggregation and plays a role in arthritis, Pls, a protein that binds to ganglioside GM3 of cells and promotes adhesion to nasal cells, and Can, which binds to collagenous tissues like cartilage (Bhunia, 2008).

2.3 Cranberries

Cranberries have been used for hundreds of years as a food source, and have been recognized for the health properties they possess. Native Americans used them to flavor and preserve meat and also as a medicine (Foo et al, 2000; Guay, 2009). Over 90% of the global cranberry production is done within the United States, with Wisconsin, Massachusetts and New Jersey growing much of it (Guay, 2009). The American Cranberry (Vaccinium macrocarpon) is grown primarily in these areas and is harvested in September and October.

For the past century cranberries have been recognized for several health benefits, including prevention of recurrent urinary tract infections in women (Avorn et al, 1994). One reason for this prevention is that cranberry juice cocktail has been demonstrated to prevent adherence of some species of bacteria to surfaces. Guay cited two studies which have evaluated the effect of cranberry on the adhesion of *Escherichia coli*, *Staphylococcus aureus*, and other bacteria species to glass coverslips (believed to have similar qualities to foreign surfaces in the urinary tract) and it was found that though white cranberry juice had no effect, red cranberry juice did reduce the adhesion of the *E. coli* and *S. aureus* bacteria to the coverslip (Guay, 2009).

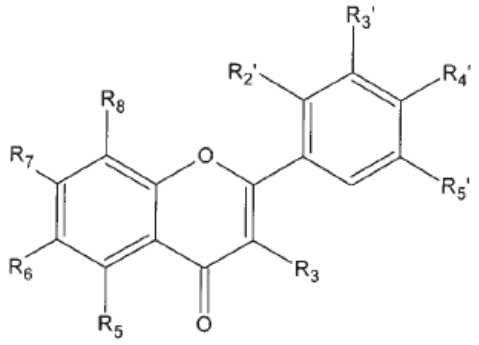
2.3.1 Components of Cranberries

Cranberries are composed of 88% water and a mixture of organic acids, vitamin C, flavonoids, anthocyanidins, proanthocyanidins (PACs), catechins, and triterpinoids (Guay, 2009). These components all serve different roles in the plant, leading to different potential health benefits from their consumption. PACs have recently been heavily studied due to health benefits that have been associated with them. Other components also have different potential health benefits, but varying amounts of research associated with them. Cranberry juice contains high concentrations of anthocyanidins, flavanol glycosides, phenolic acids and PACs.

Anthocyanidins and PACs are in a group of compounds called tannins, and found in vaccinium berries such as cranberries and blueberries. Both of these compounds serve the purpose of anti-adhesion in the plant to protect it from potential infections (Guay, 2009). As Thomasset et al. noted anthocyanidins are prone to quick chemical decomposition, which means that their

potential health benefits may not be fully utilized in pharmaceuticals which could potentially offer the benefits offered from consuming the compound (Thomasset et al, 2009). It has also been shown that anthocyanidins should be a compound that, when consumed, may aide in preventing cancer (Shih et al., 2005).

Flavonoids are a group made up of several thousand compounds which occur naturally in foods from plants. They have been in the plant kingdom most likely for over a billion years. As they are found in nearly all consumed fruits and vegetables and are consumed in large quantities. Plants and herbal remedies which contain flavonoids have been used in folk medicine around the world and are used for several different benefits such as preventative agents for peptic ulcers or gastric cancer in certain individuals, and treating rheumatic pains (Ren et al, 2003). There are four main groups of flavonoids, the type present in cranberries are called flavones which have the structure shown in Figure 2.7. Flavonoids have several potential clinical effects, including antiatherosclerotic, anti-inflammatory, antitumor, antithrombogenic, antiosteoporotic, and antiviral. Some of these effects, such as antitumor, are still up for discussion and the role of flavonoids in different effects is not fully known.



Flavone

Figure 2.8. Structure of flavones, which are found in cranberries. (Ren et al, 2003)

2.3.2 Proanthocyanidins

Proanthocyanidins (PACs) are one of many plant phenols, which are aromatic secondary metabolites found in the plant kingdom. PACs are also known as olgoflavanoids, and consist of monomer flavan-3-ol units. When linked through either C4 to C8 or C4 to C6 bonds, the linkages are called B-linked. When the linkages were through a C2 and C7 compound, they are called A-type (Hümmer, 2008). While B-linked PACs can be found in different fruit products including apple juice, purple grape juice, green tea, and dark chocolate, A-linked PACs are found in cranberries and it is a linkage with unique anti-adhesion properties associated with them (Howell et al., 2005). Several different chemical compounds make up the PAC compound as a whole,

including flav-3-ol, catechin and epicatechin. Figure 2.8 shows the makeup of the A-linked PACs compound found in cranberries (Dixon et al, 2005).

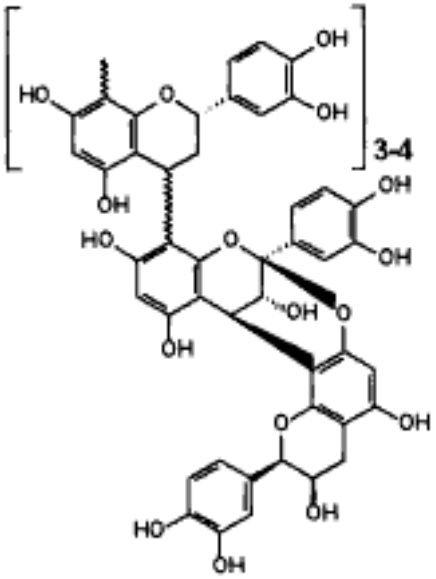


Figure 2.9. Cranberry A-type proanthocyanidin chemical makeup. (Dixon et al, 2005)

PAC compounds can be found in several parts of the plant in addition to the fruit, such as the bark, leaves, and seeds. The main function within the plant is to protect against microbial pathogens and pests (Dixon et al, 2005).] Literature throughout several years has described the many health benefits to crude and pure PAC compounds from parts of plants including the fruit, leaves, and bark. Most of these studies have focused on positive health effects on green tea, grape products including wine and juice, and cranberries (Dixon et al, 2005; Foo et al, 2000).

Studies have been done which have presented evidence that the A-linked PACs found in the American cranberry do have an impact on treating and preventing UTIs and suggest that the A-linked PACs do have bacterial anti-adhesion properties. In a study done by Cote, a daily dose of 500 mg of PAC-standardized whole cranberry powder was given to women who have had a past of UTIs. The daily dose of PAC standardized supplement caused a recurrence rate of UTIs 15% lower than those in the placebo group (Côté,). PACs from grapes, apple juice, green tea and dark chocolate were tested against A-linked PACs of cranberry and antiadhesion activity was observed after cranberry juice consumption but not in the other food (which contain PACs) (Howell et al., 2005). The anti-adhesion properties gained by consuming cranberry powder were dose dependent and were prolonged with higher amounts of PAC equivalents (Howell et al., 2010).

2.4 Gaps in Previous Research

Cranberry juice cocktail has been recognized for health benefits of maintaining urinary tract health. Preliminary clinical studies began in early 1920's. In 1984 Sotoba et al. found that preincubation of *E. coli* and uroepithelial cells in cranberry juice decreased bacterial adhesion. In

1994 Avorn et al. successfully demonstrated consumption of cranberry juice reduces the frequency of recurrent UTIs on elderly women. It has been shown that consumption may offer protection against both sensitive and resistant strains of *E. coli* (Howell et al., 2002). Liu et al showed that cranberry juice cocktail significantly decreases the adhesion forces on a nano-scale between P-fimbriated *E. coli* and uroepithelial cells.

PACs have been shown to be a compound in cranberry which has anti-adhesion effects. Three PAC trimers were isolated from cranberries and prevented adherence to P-fimbriated *E. coli* to urinary tract cell surfaces with sequences similar to uroepithelial cells (Foo et al, 2000). PAC standardized cranberry extracts inhibited the growth of *s. aureus* but not *E. coli*, and it did inhibit the biofilm production but did not eradicate the established biofilms. Cranberry may have beneficial effects against growth and biofilm producing capability of gram positive bacteria pathogens (LaPlante et al., 2011).

Pinzon-Arango demonstrated that bacteria which are grown in light cranberry juice cocktail (l-CJC) or PACs both resulted in a decrease of adhesion forces with an increasing number of cultures. The effect was reversible and once it was regrown in cranberry free media regained their attachment ability to uroepithelial cells. As the exposure to L-CJC increased, there was a decrease of bacterial attachment. The concentration of the cranberry products and the number of cultures the bacteria was exposed to determined how much the adhesion forces and attachment are altered (Pinzon-Arango et al, 2009; Pinzon-Arango et al, 2009). Pinzon-Arango et al. demonstrated although bacteria grown in PACs have anti-adhesion properties, these inhibitory effects were reversible, while grown in L-CJC were not. This implies that L-CJC is more effective than PACs at preventing biofilm formation, suggesting that other compounds in cranberry play a role in anti-biofilm activity (Pinzón-Arango, 2011).

Chapter 3 –Methodology

3.1 Preparing Broth

The appropriate mass of Luria-Bertani (LB) and Tryptic Soy broth was added to 400 mL of water and mixed using a magnetic stirrer. The mixture was stirred until all of the powder was completely dissolved and then the magnetic stirrer was removed. The broth was autoclaved in order to sterilize it and then was cooled to room temperature and then placed in the fridge for use.

3.2 Plating Bacteria

Two types of agar plates were made, LB and Tryptic Soy. Both *E. coli* and *S. aureus* grow in both media types. The appropriate mass of agar powder was added to ultra-pure water, mixed, then sterilized in the autoclave. The broth was left to cool until it was safe to hold and then poured into plates, coating the bottom and assuring that there were no bubbles in the media. The plates were then placed in the -20^{o}C freezer to be stored.

The bacteria strains (*E. coli* HB101 pDC1 and *S. aureus* ATCC 43866) were retrieved from the 80^{o}C freezer and defrosted until they were liquified. The hood was wiped down with ethanol and all the supplies were placed in the hood. Once the bacteria were defrosted, an inoculation loop was taken out of the container, being sure that no other loops touched. The loop end is placed in the bacteria and swiped onto an agar plate, beginning at one end, going about halfway and then switching directions, swiping across the plate and continuing the pattern, as shown in Figure 3.1.

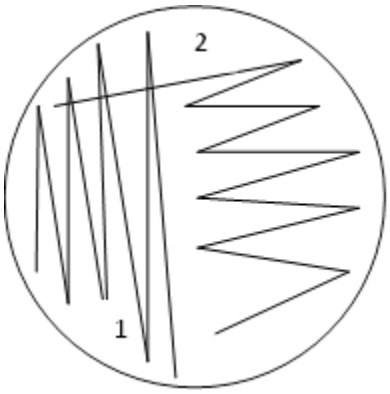


Figure 3.1: How to Plate Bacteria

Once plated, the petri dish is wrapped with parafilm and incubated at 37^{o}C for about 24 hours, then removed, and placed in the refrigerator.

3.3 Plotting a Growth Curve

A growth curve was made to determine how long it would take for the two types of bacteria to reach their late log phase. The hood was wiped down with ethanol and the culture was prepared in the hood. The appropriate broth (LB or TSB) was taken from the fridge, and 50 mL was poured into a clean flask. The agar plate with the appropriate bacteria on the same media as the broth is also taken from the fridge. With the prepared flask the parafilm was removed from the agar plate and a single inoculation loop was taken out, being sure not to touch any other loops. The loop was used to remove a single colony from the plate and then swirled in the broth for a few seconds. The flask was then put in the shaker for about 14 hours. The spectrophotometer is zeroed with fresh broth at 600nm and the absorbency of the culture is taken. The absorbency should be approximately 1, and if it is the growth curve can begin.

1 mL of the bacteria culture is added to 50 mL of fresh broth, and the absorbency is taken at 30 minute intervals until the bacteria growth had leveled off (about 8 hours). For the experiments the bacteria should be transferred in to new cultures in the late log phase, which is at the end of the exponential growth period.

3.4 LCJC Experiment

The experiment to test the effect of light cranberry juice cocktail (CJC) involved growing the bacteria in a broth and cranberry juice mixture. The *E. coli* HB101pDC1 were grown in 10wt% CJC for 11 consecutive cultures. The first step was to adjust the concentration of the cranberry

juice to a pH of 7.0 by adding NaOH. The bacteria were cultured in CJC at 37°C in 25g/L of LB broth supplemented with 10wt% CJC. The bacteria were then harvested in the late exponential growth phase which is at an absorbance of about 0.9 at 600nm. Once the bacteria reached this phase, 1 mL of the solution was transferred to a new flask and fresh media was added. Eleven transfers of bacteria in the broth/CJC solution were made successively.

After the eleven transfers (twelve cultures) a 96 well assay was completed. The bacteria of the eleventh transfer were diluted with a fresh LB broth and cranberry media in a 1:1 ratio. 200 µL of this new solution was added to the wells of the assay and were incubated at 37°C without agitation for 0, 3, 6, 24, 30, and 48 hours. At each of these time points the wells were stained using 20 µL of crystal violet and 180 µL of water. The wells were left to sit for 1 minute. After staining, the wells were washed three times with ultrapure water to remove planktonic bacteria and residual dye.

To determine the amount of biofilm formed, absorbency readings were then taken. To accomplish this, 200 µL of 20% ethanol/80% acetone was added to the previously stained wells. Ethanol/acetone is used to detach bacteria from the well walls. The ethanol/acetone was left to sit for 3-5 minutes and then placed in a cuvette. 800 µL of water was then added to the cuvette and an absorbency reading was taken at 600nm.

3.6 Extract Solution Preparation

To test the extracts instead of the LCJC the extracts had to be prepared. To prepare the extracts 2 mL of ultrapure water was added to a small flask. This was then zeroed and then the contents of the micro centrifuge tubes with the extract was carefully poured in, being sure to get as much as possible out of the tube and into the flask. Once all was poured the mass was recorded and the flask was taken out and swirled until the extract was dissolved. Then another mL was added to further dissolve the extract. After swirling to mix this water into the solution, the content of the flask was micropipetted into a larger centrifuge tube (14 mL tube). Another 2 mL of water was used to rinse out the flask and be sure all the extract was in the centrifuge tube. This was then wrapped in aluminum foil and stored in the fridge until use.

3.7 Cranberry Extract Experiment

The solid cranberry extracts were used after running experiments with L-CJC to determine if 6 cultures at 10 wt% were adequate to observe biofilm are inhibition. To each culture, the concentration of the extract as shown in Table 3.1 was added to broth. The concentration of PACs was equal to the amount of PACs that would be found in 10 wt% cranberry juice cocktail (Pinzon, 2011). The concentration of crude used to grow *S. aureus* was the same as the concentration for PACs while E. Coli used the rest of our supply which amounted to 147.1 mg/L. For anthocyanidins we used all of our supply in *S. aureus* which amounted to 0.383 mg/L. It is important to note however that even though this concentration is significantly lower the concentration of anthocyanidins in the cranberry is lower so it still may have the effect. This was

done 6 successive times and a 96 well plate assay was used following the procedure described above (3.4 LCJC Experiment).

Table 3.1. The concentration of extract used in each experiment.

Extract	Bacteria	Concentration
PACs	*E. coli*	120 mg/L
	S. aureus	
Crude	*E. coli*	147.1 mg/L
	S. aureus	120 mg/L
Anthocyanidins	*S. aureus*	0.383 mg/L

Table 3.2: 96 Well Plate Set-Up

Time	0		3		6		24		36		48	
Label	1	2	3	4	5	6	7	8	9	10	11	12
A	Control		Control		Control		Control		Control		Control	
B												
C	Extract 1		Extract 1									
D												
E	Extract 2		Extract 2									
F												
G	Extract 3		Extract 3									
H												

Chapter 4 – Results & Discussion

4.1 Growth Curves

4.1.1 Growth of Bacteria was measured every half-hour in LB Broth

The growth curve was performed in order to determine the proper time to grow bacteria in well plates or reseed in fresh bacteria when successive cultures are made. As shown in **Error! Reference source not found.**, it was determined that the late log phase is around 4-6 hours with absorbencies of about 1.2 to *S. aureus* and 0.8 for *E. coli*.

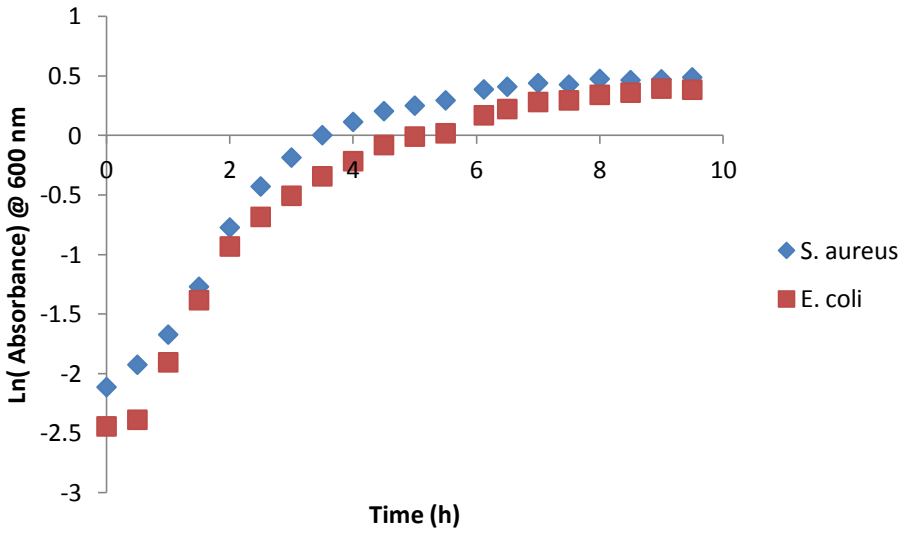


Figure 4.1: Growth Curve for *E. coli* and *S. aureus* in LB Broth

4.1.2 Growth Curve in LB Broth and 10%wt Cranberry Juice Cocktail

To prepare for the experiment outlined in 3.4 LCJC Experiment, we made a growth curve for *E. coli* to determine if the growth time changed when the bacteria was grown in cranberry juice. The media that was used was LB Broth and 10%wt CJC. We saw that the growth rate in cranberry juice was similar to bacteria grown only in broth (Figure 4.1). For all of the experiments, four hours was be used as the point in which to check the absorbency to determine whether the late log growth phase was reached.

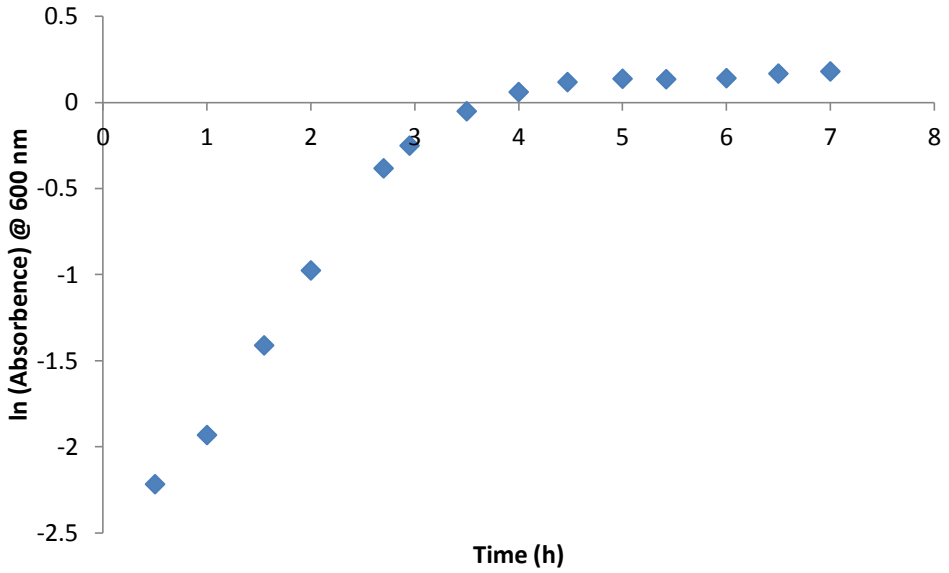


Figure 4.2: *E. coli* growth curve in 10wt% CJC

4.3 Bacteria Grown in 10 wt% LCJC with 11 Cultures

The cranberry juice cocktail experiment involved growing the bacteria in tryptic soy broth and 10 wt % cranberry juice. *E. coli* was cultured in the cranberry broth mixture 11 successive times. *S. aureus* was cultured 9 successive times. A 96 well assay was used to perform the biofilm analysis. Below, Figure 4.3, shows the absorbency measurements taken at time points 0, 3, 4, 24, 36, and 48 hours. For the first 3 time points the measurements were very low indicating that the bacteria did not attach to the well walls. For both experiments the data was statistically significant with a $P<0.004$ at time points 24, 36, and 48. The *E.coli* control rose significantly at the 24 hour time point and the *E.coli* that was grown in cranberry juice rose as well, but to a much lesser degree. This data indicates that the cranberry juice impacted biofilm formation of *E.coli*. *S. aureus* followed these same trends. The control group jumped in absorbency at 24 hours. The absorbance of the cranberry juice grown *S. aureus* stayed very low during the duration of the experiment. It appears that bacteria grown in cranberry juice did not attach to the walls of the wells as effectively as the control bacteria had. Attachment to the walls suggests biofilm formation.

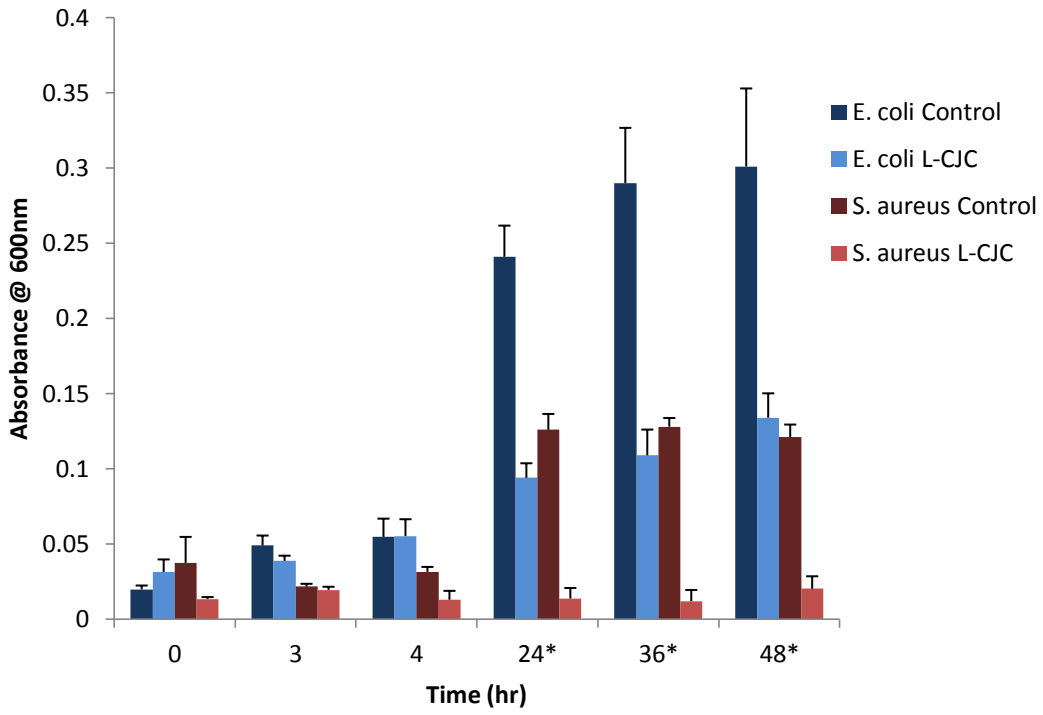


Figure 4.3: Experimental Results for 6 replicates of 12 10wt% Cranberry Juice Cultures (The error bars represent the standard error with P<0.997, P*<0.004 for the difference between the control and juice grown bacteria).

As expected due to results from experiments conducted by Pinzon et al. LCJC did appear to inhibit growth of biofilms in *E. coli* and *S. aureus*. Beginning at the 24 hour time point biofilms did form in the well plates, as shown from the dramatic increase in absorbency and the visual evidence present in the well plate. This shows that at bacteria grown in 11 cultures for *E. coli* and 9 for *S. aureus* with broth and 10 wt% LCJC grown. Although LCJC has been proven to inhibit biofilms, Johnson-White et al. has demonstrated that it is not due to the sugars such as fructose, glucose and D-mannose or the high acidity that is present in the juice (Johnson-White et al.).

4.4 Bacteria Grown in 5 wt% LCJC with 6 Cultures

Due to limited powder extract resources, we wanted to validate that biofilms will be inhibited by bacteria grown in 5 wt% LCJC in broth and 6 successive cultures, compared to the 10 wt% LCJC and 12 cultures previously tested. *S. aureus* displayed inhibited biofilm growth after 6 cultures (Figure 4.4).

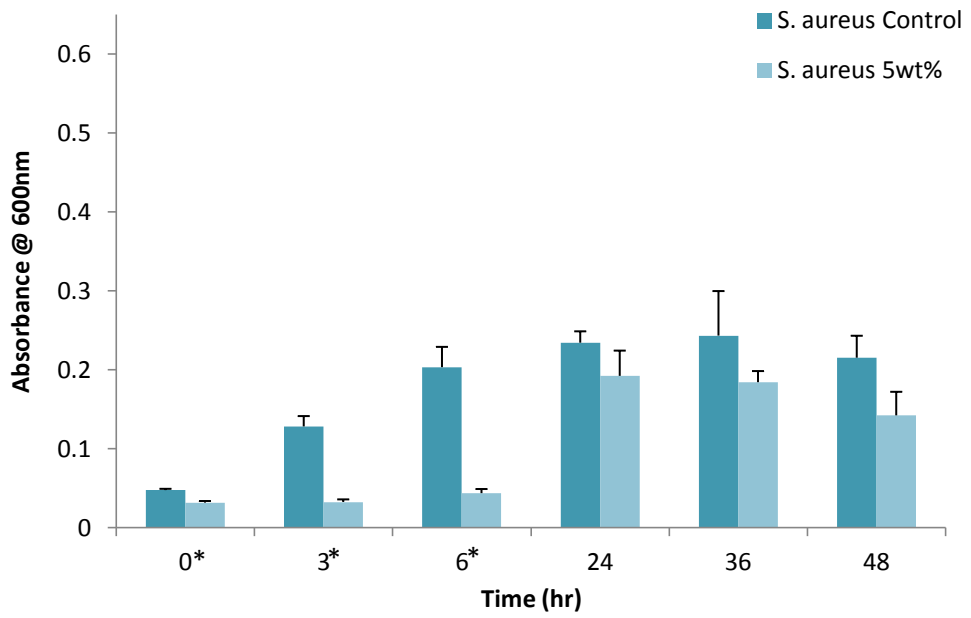


Figure 4.4: *S. aureus* grown in 5wt% LCJC for 6 cultures compared to a control with 4 replicates for each group. *S. aureus's* biolfilms were affected by the LCJC with error bars representing standard error between replicates. Significance used at the following P values (P<0.35, P*<.001).

This experiment confirmed that there was a lower absorbency when the bacteria were grown in the L-CJC instead of just in broth. A t-test was conducted and we found that the data was statistically significant at the 0, 3, and 6 hour time points. It is important to note that biofilms generally are not seen until the 24 hour time point (which is apparent in Figure 4.3). As seen in Figure 4.4, during the duration of the experiment, the control had higher absorbencies. The data is not statistically significant at the time points of biofilm development so, for further experiments with the extract 10 wt% or the maximum amount possible will be used.

The same experiment was conducted on *E. coli* (Figure 4.5).

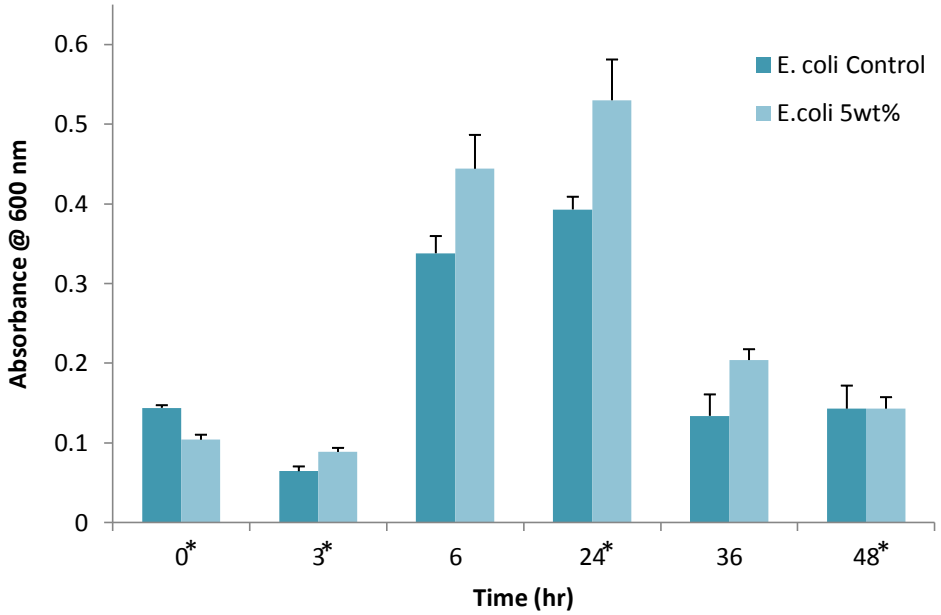


Figure 4.5: *E.coli* grown in 5wt% LCJC for 6 cultures with 4 replicates. Error bars are the standard error between replicates and the P values areP<.07 and P*<.045.

The biofilm assay to test bacteria grown in 5 wt% LCJC for 6 cultures resulted in the control being lower than the LCJC. Similar to the experiment above with *S. aureus*, the amount of cranberry juice in each culture was too low or the number of cultures was not enough to impact biofilm growth. This confirmed the need to increase cultures or concentration.

4.5 Bacteria Grown in PACs for 6 Cultures

In this experiment an extract solution of PACs was added to 6 successive cultures (Figure 4.6). When *E. coli* was grown in proanthocyanidins the absorbance measurements for the control and PACs grown bacteria were very similar from 0 to 6 hours. At the 24 hour time point, the absorbance of the bacteria grown in PACs was higher than the control. There were traces of crystal violet that had clumped together in these wells and we believe that made the absorbency higher. After 24 hours, the absorbance of the control bacteria was higher than the PACs. Although, this experiment is inconclusive because these time points were not statistically significant based on a rank sum test performed by Sigma Plot 12.3.

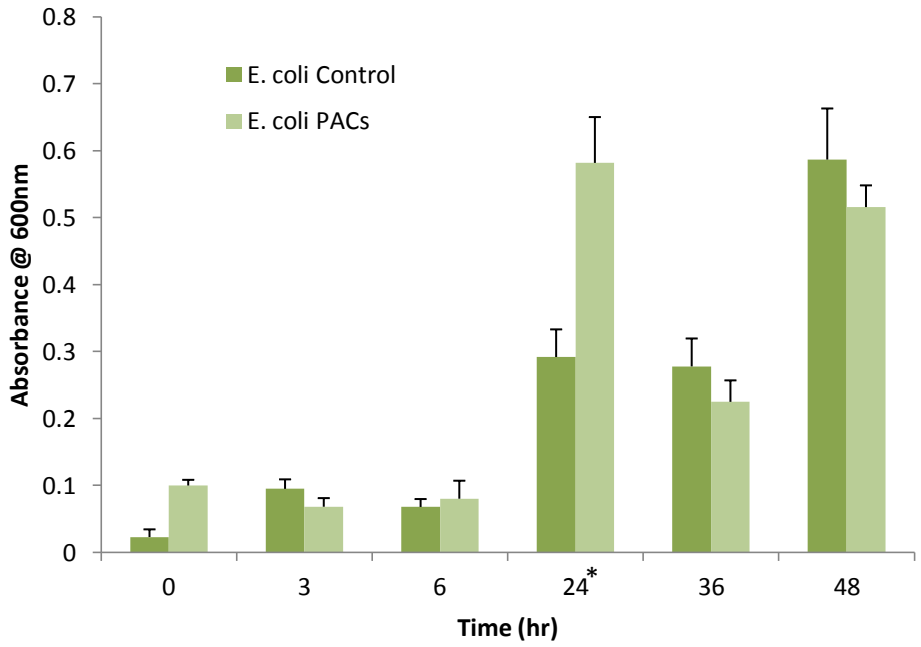


Figure 4.6: *E.coli* grown in 120 mg/L PACs for 6 cultures with 4 replicates compared to bacteria grown in only Tryptic Soy Broth. The error bars are the standard error between replicates and the significance was determined with the P values of P<0.7 and P*<0.011.

The same experimental procedure to test *E. coli* was then used to test *S. aureus* and the control had higher absorbencies except at 36 hours (as seen below in Figure 4.7). The absorbance of the 48 hour time point was low, suggesting limited biofilm growth near the end of the experiment. A possible reason for this could be that the bacteria were beginning to die. What is important is that the control absorbance was high midway through the experiment indicating the bacteria were still alive, allowing wall attachment. The rank sum test confirms this, because the 6 and 24 hour time points were both statistically significant when comparing the bacteria grown in only broth (the control) to the bacteria grown in PACs for 6 cultures ($P<0.033$ and $P<0.006$ respectively). PACs at 10 wt% and 6 cultures shows evidence of biofilm inhibition. This confirms previous experiments conducted by Pinzon et al. and is consistent with what was expected. PACs has been studied previously because of their anti-adhesion properties and it was known that PACs should

inhibit the biofilm formation of the bacteria tested (Pinzon et al., 2011). As reported by Eydelnant et al. antibacterial properties have been reported when PACs are present in a concentration greater than 200 mg/L, while antiadhesive effects have been observed at concentrations as low as 5 mg/L (Eydelnant et al., 2008). The distinction between antibacterial and antiadhesive is that antibacterial kills the bacteria, while antiadhesive (our focus) would prevent bacteria from attaching to surfaces. Our bacteria was grown in a concentration of 120 mg/L, which according to this study should be adequate to prevent bacterial adhesion. LaPlante et al. however has demonstrated that while gram positive biofilm formation was inhibited, gram negative biofilms were not. This is another reason to why the gram negative *E. coli* may not have been effected by the PACs (LaPlante et al, 2012).

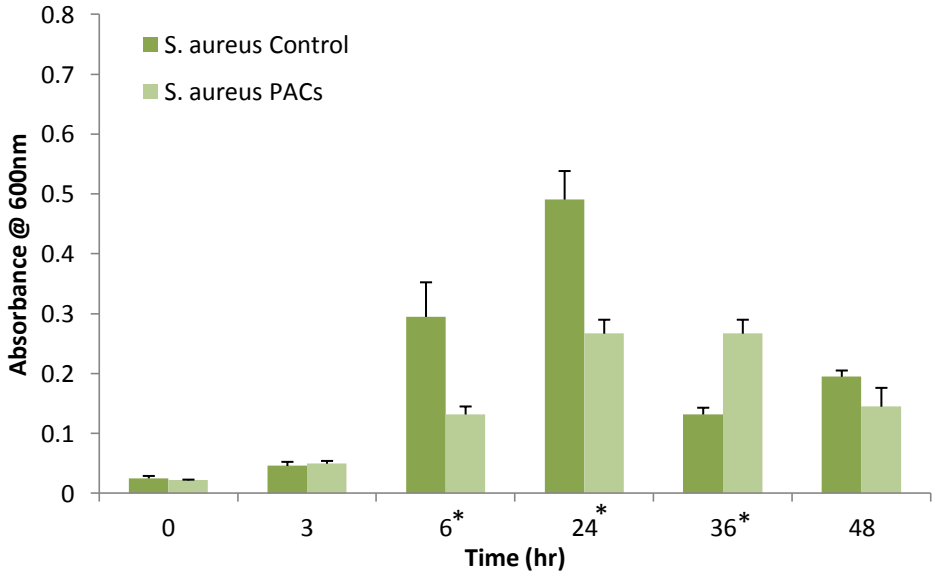


Figure 4.7. *S. aureus* grown for 6 cultures in PACs with 4 replicates compared to a control bacteria. The bacteria grown in PACs seem to have been inhibited in their biofilm growth. The error bars represent the standard error and the significance was based on P values of P <0.68 and P*<.035.

4.6 Bacteria Grown in Crude for 6 Cultures

The same experimental procedure was then conducted using *S. aureus* and crude extract. This crude extract experiment shows a similar trend to that of *S. aureus* and PACs. The 24 hour time point was higher for the control, which shows promise concerning its inhibitory effect, and approximately the same for the following time points. This test did not provide points that were considered statistically significant. The results we obtained for this are not conclusive and further testing would be necessary to determine if the crude extract at this concentration is high enough to inhibit biofilm formation.

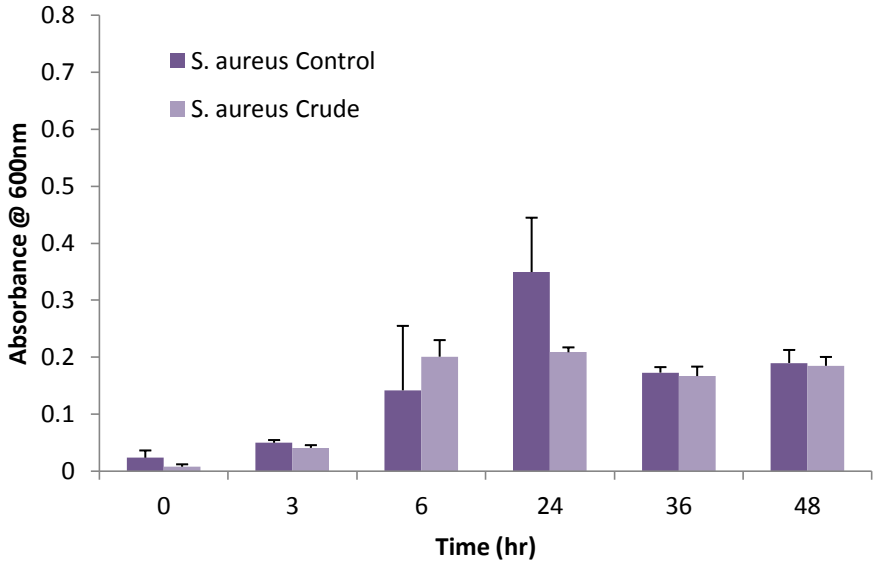


Figure 4.8: *S. aureus* grown for 6 cultures in Crude extract with 4 replicates compared to a Control. The error bars represent the standard error between replicated and the significance was calculated between the control and *S.aureus* grown in Crude extract with a P value of P<0.87.

E.coli grown in crude cranberry extract (Figure 4.9) did not exhibit strong indications of biofilm inhibition. One potential cause of this result is that crude was not dissolved fully in water or broth, causing the concentration to be decreased when it fell out of suspension in the solution. Another factor is that the concentration of crude used was the same as that of PACs extract. Crude contains PACs and other compounds, so this results in an overall lower PACs concentration. The crude extract was hypothesized to exhibit similar trends as the LCJC since the composition of the two should be similar.

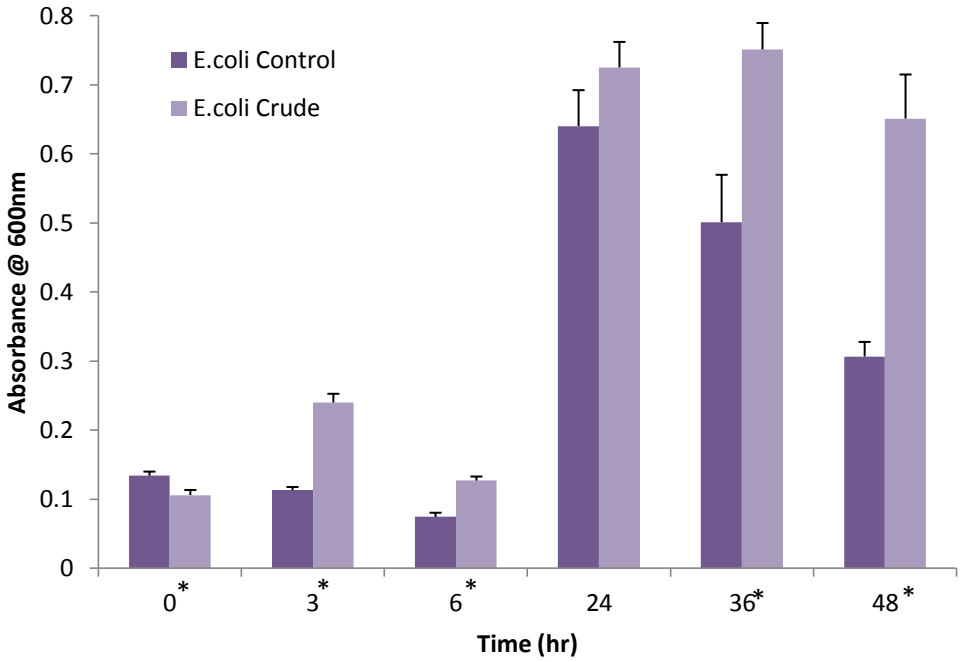


Figure 4.9: *E. coli* grown in crude extract and compared to a control (P<0.210, P*<0.01)

In a study completed by LaPlante et al., three different crude extracts provided by Ocean Spray were tested to see if they would inhibit *S. aureus* and all three inhibited growth. The third of these, Cran C, was the most active although it did not contain the highest wt% of PACs. This implies, as supported by other studies done by Pinzon et al. that other compounds are playing an active role in inhibiting biofilms formation (LaPlante et al., 2012).

4.7 Bacteria Grown in Anthocyanidins

S. aureus was then grown in anthocyanidins for 6 cultures. All of the available extract was used, amounting to 0.383 mg of anthocyanidin extract per culture. The data (Figure 4.10) is statistically significant at the 0, 3, and 48 hour time points. Unfortunately the biofilm should be prevalent during the 24, 36, and 48 hour time points. One explanation for this is that the concentration was just too low for it to have an impact on the bacteria. Anthocyanidins have not been isolated or tested on biofilms, so it may be that anthocyanidins do not impact biofilms. This experiment was inconclusive. The 48 hour time point does however give us promise that anthocyanidins may have an effect, but more testing is necessary to draw meaningful conclusions. Another important factor about anthocyanidins to note is that they are prone to quick chemical decomposition, and are sensitive to light, which means that their potential benefits may be difficult to utilize if they do inhibit biofilms (Thomasset et al, 2009).

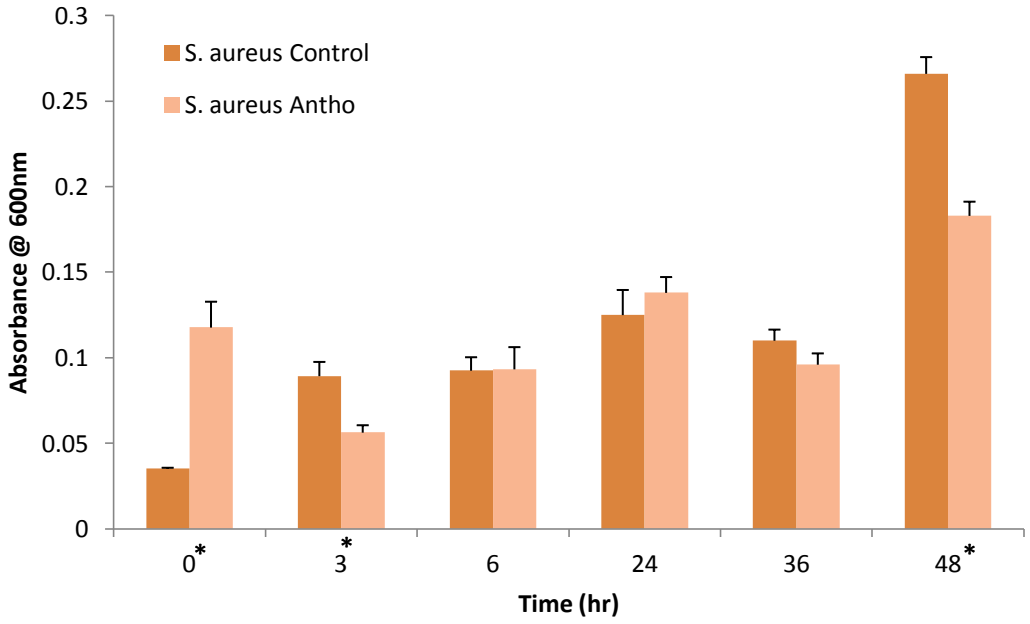


Figure 4.10: *S. aureus* grown in .383 mg of anthocyanidin per culture. (P<0.96, P*<.006)

Chapter 5 - Conclusions and Recommendations

Compounds that may have properties which inhibit biofilm development are important to study because they are potential natural remedy. Cranberries have been proven effective in reducing the occurrence of urinary tract infections, but the exact reason as to why is still relatively unknown. Although the proanthocyanidin compound has been shown to successfully inhibit biofilms, we are interested if other compounds also are likely to play a role. We performed experiments that looked at proanthocyanidins, anthocyanidins, and a crude extract.

In our experiments, it was demonstrated that PACs had an inhibiting effect on S. *aureus* biofilms, but the effect was seen less after 36 hours than it was 24 hours or before. In *E. Coli* the only statistically significant point was at the 24 hour mark and had the opposite effect, though this could have been due to the PACs settling and not being rinsed away, therefore causing staining with the crystal violet.

Crude extract was also tested in both *S. aureus* and *E. coli* and similar effects were seen. Although in *S. aureus* the data points generated no significant results, so it is not able to be determined the extent to which the crude had an effect. *E. coli* grown in crude extract generated significant points at the 0, 3, 6, 36, and 48 hour time points, but the effect appears to be the crude cause more biofilm formation. Part of this is due to the crude settling. Out of the extracts that were worked with (PACs, crude, and anthocyanidins), crude dissolved the least. It is likely that the incomplete dissolution affected the results and made it appear as though biofilms were formed even if they were not.

S. aureus was the only bacteria tested in anthocyanidins because there was only a small amount of anthocyanidin extract available. This was decided so the extract concentration could be high enough to potentially have an effect. In the experiment there were three significant points at times 0, 3, and 48 hours. Even though the 48 hour time point showed that the anthocyanidin treated bacteria had a significantly lower absorbance, overall we cannot conclude that anthocyanidins had an impact on biofilms since the other time points (24 and 36) were not significant.

Based on our results, we believe more replicates would be beneficial for generating a more complete data set. For some of the experiments we had only four wells for each time, meaning if any were thrown out there would be only three, which may not present accurate data that demonstrates the trends that occur. Another recommendation is to have a control monitoring the effect of the component settling in the well. It was difficult to know how much of the apparent biofilm growth in the extract treated bacteria was due to extract settling and sticking to the bottom of the well. Monitoring this by having wells at each time point with only broth and only extract in broth which can be used to zero the spectrophotometer at that time point, which may allow more accurate extract data.

It would be beneficial to test varying concentrations of each component. Due to a fixed supply of extract we were unable to retest the extract by varying the component concentration. It would be useful to know the concentrations of these different components in cranberry juice cocktail. This would enable one to directly compare the extract results to a known biofilm inhibitor (cranberry juice cocktail).

Continuing research on the effects of different compounds on biofilm inhibition would be beneficial. Biofilms are responsible for 65% of nosocomial infections. This widespread problem is one that has the potential to be preventable with continued research efforts.

Chapter 6 - References

Avorn et al. (1994). Reduction of bacteriuria and pyuria after ingestion of cranberry juice. *JAMA : The Journal of the American Medical Association, ,* 751.

Bhunia. (2008). Stapholococcus aureus. *Foodborne microbial pathogens* (pp. 125)

Blackburn et al.*Foodborne pathogens - hazards, risk analysis and control (2nd edition)* Woodhead Publishing. Retrieved from http://www.knovel.com/web/portal/browse/display?_EXT_KNOVEL_DISPLAY_bookid=2596

Carr, Janice. 2001. *11156: Under a very high magnification of 20,000x, this scanning electron micrograph (SEM) shows a strain of Staphylococcus aureus bacteria taken from a vancomycin intermediate resistant culture (VISA).* **Retrieved** December, 12, 2012, **from:** http://phil.cdc.gov/phil/imageidsearch.asp

Carr, Janice. 2006. *10086: Under a magnification of 6836x, this colorized scanning electron micrograph (SEM) depicted a number of Gram-negative Escherichia coli bacteria of the strain O157:H7, which is one of hundreds of strains of this bacterium. Although most strains are harmless, and live in the intestines of healthy humans and animals, this strain produces a powerful toxin, which can cause severe illness.* **Retrieved** December, 12, 2012, **from:** http://phil.cdc.gov/phil/imageidsearch.asp

Carr, Janice. 2005. *7486:This electron micrograph depicted large numbers of Staphylococcus aureus bacteria, which were fond on the luminal surface of an indwelling catheter. Of*

importance is the sticky-looking substance woven between the round cocci bacteria, which was composed of polysaccharides, and is known as "biofilm". This biofilm has been found to protect the bacteria that secrete the substance from attacks by antimicrobial agents such as antibiotics; Magnified 2363x. **Retrieved** December, 12, 2012, **from:** http://phil.cdc.gov/phil/imageidsearch.asp

Costerton et al. (2004). Bacterial biofilms: From the natural environment to infectious diseases. Nature Reviews Microbiology, 2, 95. Retrieved from http://go.galegroup.com.ezproxy.wpi.edu/ps/i.do?id=GALE%7CA188966026&v=2.1&u=mlin_c_worpoly&it=r&p=AONE&sw=w

Côté, J.In Caillet S., Doyon G., Sylvain J. and Lacroix M.(Eds.), Analyzing cranberry bioactive compounds Taylor & Francis. doi: - 10.1080/10408390903042069

Dixon et al. (2005). Proanthocyanidins: A final frontier in flavonoid research? New Phytologist, 165(1), 9-28. Retrieved from http://www.jstor.org/stable/1514582

Donlan, R. M. (2001). Biofilm Formation: A clinically relevent microbiological process. Clinical Infectious Diseases, 33(8), 1387-1392.

Eydelnant, I.A., Tufenkji, N (2008),
Cranberry Derived Proanthocyanidins Reduce Bacterial Adhesion to Selected Biomaterials
Langmuir200824 (18), 10273-10281

Foo et al. (2000). A-type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic P-fimbriated escherichia coli. American Chemical Society,

Ghannoum et al. (2004). Microbial biofilms (xiii ed.). Washington, DC: ASM Press.

Guay, D. R. P. (2009). Cranberry and urinary tract infections. Drugs, 69, 775. Retrieved from http://go.galegroup.com.ezproxy.wpi.edu/ps/i.do?id=GALE%7CA228249838&v=2.1&u=mlin_c_worpoly&it=r&p=AONE&sw=w

Howell et al. (2002). Cranberry juice and adhesion of antibiotic-resistant uropathogens. JAMA,

Howell et al. (2005). A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity. Phytochemistry, 66(18), 2281-2291. doi: 10.1016/j.phytochem.2005.05.022

Howell et al. (2010). Dosage effect on uropathogenic escherichia coli anti-adhesion activity in urine following consumption of cranebrry powder standardized for proanthocyanidin content: A multicentric randomized double blind stufy. BMC Infectious Diseases,

Hümmer, W. e. a. (2008). Analysis of proanthocyanidins. Molecular Nutrition & Food Research, 52(12), 1381-1398.

Johnson-White, B., Buquo L., Zeinali, M., Ligler, F.S. (2006). Prevention of Nonspecific Bacterial Cell Adhesion in Immunoassays by Use of Cranberry Juice *Analytical Chemistry200678 (3), 853-857*

Johnston, N. (2004, 08/02; 2012/9). Debaffling biofilms: Studies follow transformations and detail a major signal.18, 34. Retrieved from http://go.galegroup.com.ezproxy.wpi.edu/ps/i.do?id=GALE%7CA120845910&v=2.1&u=mlin_c_worpoly&it=r&p=AONE&sw=w

LaPlante et al. (2011). Effects of cranberry extracts on growth and biofilm production of escherichia coli and staphylococcus species. Phytotherapy Research,

LaPlante, K. L., Sarkisian, S. A., Woodmansee, S., Rowley, D. C. and Seeram, N. P. (2012), Effects of Cranberry Extracts on Growth and Biofilm Production of Escherichia coli and Staphylococcus species. Phytother. Res., 26: 1371–1374. doi: 10.1002/ptr.4592

Mah et al. (2001). Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology, 9(1), 34-39. doi: 10.1016/S0966-842X(00)01913-2

Moran et al.Microbial glycobiology - structures, relevance and applications Elsevier. Retrieved from http://www.knovel.com/web/portal/browse/display?_EXT_KNOVEL_DISPLAY_bookid=2771

Pavithra et al. (2008). Biofilms formation, bacterial adhesion and host response on polymeric implants - issues and prevention. IOP Science,

Pinzon-Arango et al. (2009). Role of cranberry on bacterial adhesion forces and implications for escherichia coli - uroepithelial cell attachment. Journal of Medicinal FOod,

Pinzón-Arango, P. A. (2011). In Holguin K., Camesano T. A.(Eds.), Impact of cranberry juice and proanthocyanidins on the ability ofescherichia coli to form biofilms The Korean Society of Food Science and Technology. doi: - 10.1007/s10068-011-0181-8

Ren et al. (2003). Flavonoids: Promising anticancer agents. Medicinal Research Reviews, 23(4), 519-534. doi: 10.1002/med.10033

Shih et al. (2005). Effects of anthocyanidin on the inhibition of proliferation and induction of apoptosis in human gastric adenocarcinoma cells. Food and Chemical Toxicology, 43(10), 1557-1566. doi: 10.1016/j.fct.2005.05.001

Stewart et al. (2001). Antibiotic resistance of bacteria in biofilms. The Lancet, 358(9276), 135-138. doi: 10.1016/S0140-6736(01)05321-1

Thomasset et al. (2009). Do anthocyanins and anthocyanidins, cancer chemopreventive pigments in the diet, merit development as potential drugs? Cancer Chemotherapy and Pharmacology, 64(1), 201. doi: 10.1007/s00280-009-0976-y

Whitchurch et al. (2002). Extracellular DNA required for bacterial biofilm formation. Science, 295(5559), p. 1487. Retrieved from http://www.jstor.org/stable/3076028

Chapter 7 - Appendices

6.1 LCJC Experiment

6.1.1 Raw Data

	0 1:45 PM		3 5:00 PM		4* 6:00 PM		24 2:00 PM		36 2:00 AM		48 2:00 PM	
A	0.02	0.027	0.045	0.04	0.089	0.049	0.208	0.205	0.317	0.185	0.435	0.289
B	0.015	0.017	0.095**	0.062	0.032	0.049	0.289	0.262	0.303	0.355	0.182	0.297
C	0.024	0.02	0.049	0.034	0.067	0.022	0.085	0.078	0.073	0.11	0.179	0.109
D	0.026	0.056	0.037	0.035	0.069	0.063	0.122	0.091	0.155	0.099	0.112	0.134
E	0.097	0.013	0.048**	0.023	0.039	0.032	0.156	0.122	0.113	0.135	0.102	0.143
F	0.022	0.018	0.024	0.018	0.023	0.032	0.11	0.114	0.139	0.125	0.122	0.118
G	0.013	0.014	0.018	0.025	0.001	0.029	0.034	0.002	0.034	0.005	0.033	0.036
H	0.01	0.017	0.02	0.015	0.009	0.013	0.008	0.011	0.008	0.001	0.007	0.005

** values have been disregarded in average calculation, wells were not as well washed

6.1.2 Average Absorbency Measurements

Condition		0	3	4	24	36	48
E.coli	Control	0.01975	0.049	0.043333	0.241	0.325	0.256
	CJC	0.0315	0.03875	0.066333	0.094	0.10925	0.1335
S.aureus	Control	0.0375	0.021667	0.0315	0.1255	0.128	0.12125
	CJC	0.0135	0.0195	0.007667	0.01375	0.012	0.02025

6.2 Data from 5wt%, PACs, Crude, and Anthocyanidin experiments

6.2.1 Grown in 5 wt% LCJC

Culture	Date/Time	Absorbency (*E. coli*)	Absorbency (S. Aureus)
1	2/3 10 am	1.386	1.497
2	2/3 2 pm	0.977	1.106
3	2/3 6 pm	0.774	1.181
4	2/3 10 pm	0.657	1.216
5	2/4 2 am	0.550	1.140
6	2/4 6 am	0.553	1.260
Control	2/4 6 am	0.662	1.347

E. coli	Time 0		Time 3		Time 6		Time 24		Time 36		Time 48	
Control	1	2	3	4	5	6	7	8	9	10	11	12
A	0.137	0.152	0.057	0.052	0.389	0.288	0.358	0.392	0.087	0.091	0.110	0.177
B	0.141	0.148	0.078	0.071	0.322	0.354	0.435	0.387	0.195	0.164	0.119	0.166
CJC												
E	0.121	0.095	0.074	0.090	0.436	0.361	0.645	0.555	0.242	0.182	0.245	0.191
F	0.107	0.094	0.095	0.096	0.562	0.415	0.398	0.524	0.187	0.204	0.243	0.190

S. Aureus	Time 0		Time 3		Time 6		Time 24		Time 36		Time 48	
Control	1	2	3	4	5	6	7	8	9	10	11	12
A	0.051	0.045	0.160	0.136	0.271	0.147	0.239	0.201	0.145	0.248	0.186	0.259
B	0.049	0.044	0.099	0.117	0.191	0.202	0.225	0.270	0.181	0.400	0.150	0.263
CJC												
E	0.031	0.026	0.032	0.024	0.057	0.047	0.188	0.158	0.186	0.214	0.099	0.161
F	0.034	0.035	0.040	0.033	0.033	0.037	0.283	0.139	0.146	0.189	0.089	0.217

6.2.2 Grown in PACs (*S. aureus*)

Culture	Date/Time	Absorbency (S.Aureus)
1	1:45 am	1.140
2	6am	1.238
3	10am	1.147
4	2pm	0.951
5	6pm	0.943
6	10pm	0.912
Control	10pm	1.723

Control	Time 0		Time 3		Time 6		Time 24		Time 36		Time 48	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.029	0.015	0.049	0.033	0.200	0.208	0.466	0.458	0.121	0.159	0.204	0.209
B	0.034	0.023	0.038	0.064	0.326	0.445	0.629	0.410	0.139	0.107	0.204	0.164

PACs	Time 0		Time 3		Time 6		Time 24		Time 36		Time 48	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.020	0.025	0.039	0.058	0.139	0.166	0.326	0.248	0.146	0.111	0.103	0.141
B	0.022	0.022	0.057	0.045	0.115	0.106	0.216	0.276	0091	0.084	0.100	0.235

6.2.3 Grown in PACs (*E. coli*)

Culture	Date/Time	Absorbency (*E. coli*)	*Notes*
1	2/19 2 PM	1.034	
2	2/19 6 PM	0.751	
3	2/20 2 AM	1.181	Left for extra time as after 4 hours the absorbency was only 0.676
4	2/20 8 AM	1.002	
5	2/20 2 PM	1.070	
6	2/20 8 PM	1.135	
Control	2/20 8 PM	0.692	

Control	**Time 0**		**Time 3**		**Time 6**		**Time 24**		**Time 36**		**Time 48**	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.048	0.001	0.087	0.101	0.101	0.050	0.264	0.409	0.153	0.319	0.373	0.728
B	0.006	0.037	0.062	0.130	0.069	0.052	0.216	0.278	0.318	0.322	0.651	0.596

PACs	**Time 0**		**Time 3**		**Time 6**		**Time 24**		**Time 36**		**Time 48**	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.027	0.002	0.044	0.093	0.068	0.034	0.749	0.559	0.308	0.187	0.480	0.605
B	0.067*	0.001	0.088	0.048	0.158	0.061	0.602**	0.416	0.163	0.240	0.522	0.458
Notes	Cuvette very cloudy, result may be off						** had crystal violet in it previous to testing					

6.2.3 Grown in Crude (*S. aureus*)

Culture	Date/Time	Absorbency (*E. coli*)
1	2/23 10 AM	1.397
2	2/23 2 PM	1.184
3	2/23 6 PM	1.215
4	2/23 10 PM	1.134
5	2/24 2 AM	1.140
6	2/24 6 AM	1.254
Control	2/24 6 AM	1.190

Control	Time 0		Time 3		Time 6		Time 24		Time 36		Time 48	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.011	0.012	0.062	0.051	0.17	0.118	0.187	0.408	0.187	0.144	0.128	0.234
B	0.031	0.041	0.048	0.039	0.131	0.15	0.378	0.425	0.18	0.18	0.211	0.187

Crude	Time 0		Time 3		Time 6		Time 24		Time 36		Time 48	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.011	0.032	0.029	0.046	0.272	0.182	0.212	0.215	0.206	0.154	0.155	0.163
B	0.028	0.019	0.051	0.038	0.134	0.217	0.194	0.214	0.129	0.18	0.2	0.202

6.2.4 Grown in Crude Extract (*E. coli*)

Culture	Date/Time	Absorbency (E. coli)
1	3/14 2 pm	1.234
2	3/14 8 pm	0.874
3	3/15 2 am	0.796
4	3/15 8 am	0.717
5	3/15 2 pm	1.112
6	3/15 8 pm	1.221
Control	3/15 8 pm	1.079

***E. coli* control**

	Time 0		Time 3		Time 6		Time 24		Time 36		Time 48	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.111	0.141	0.115	0.097	0.085	0.085	0.511	0.727	0.290	0.305	0.368	0.304
B	0.136	0.160	0.089	0.120	0.096	0.078	0.607	0.906	0.671	0.848	0.291	0.226
C	0.121	0.118	0.111	0.124	0.056	0.073	0.577	0.752	0.552	0.483	0.374	0.277
D	0.135	0.153	0.120	0.127	0.043	0.079	0.598	0.446	0.441	0.468	0.150	0.121

***E. coli* crude**

	Time 0		Time 3 *Crude particles settled a lot		Time 6		Time 24		Time 36		Time 48	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.149	0.121	0.205	0.248	0.132	0.096	0.753	0.766	0.714	0.709	0.797	0.626
B	0.100	0.102	0.253	0.256	0.138	0.143	0.574	0.772	0.814	0.913	0.530	0.268
C	0.107	0.087	0.194	0.225	0.139	0.134	0.612	0.805	0.770	0.810	0.211	0.313
D	0.095	0.085	0.309	0.233	0.122	0.108	0.643	0.876	0.540	0.735	0.337	0.333

6.2.4 Grown in Anthocyanidin Extract (*S. aureus*)

Notes

- 1 ml anthocyanidin (0.383 mg per culture)

Culture	Date/Time	Absorbency (*S. aureus*)
1	3/16 10 am	1.147
2a	3/16 2 pm	0.509 * too low, left longer
2b	3/16 6 pm	1.297
3	3/17 10 pm	1.105
4	3/17 2 am	0.821
5	3/18 6 am	0.913
Control	3 18 6 am	1.336

***S. aureus* Control**

	Time 0		Time 3		Time 6		Time 24		Time 36		Time 48	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.036	0.0.36	0.069	0.071	0.092	0.095	0.174	0.117	0.101	0.104	0.303	0.272
B	0.033	0.036	0.077	0.116	0.053	0.080	0.136	0.121	0.089	0.134	0.237	0.277
C	0.036	0.035	0.092	0.111	0.090	0.150	0.136	0.064	0.122	0.109	0.244	0.260
Broth only (no bacteria)		0.034		0.034		0.067		0.044		0.091		

***S. aureus* anthocyanidin**

	Time 0		Time 3		Time 6		Time 24		Time 36		Time 48	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.097	0.069	0.069	0.048	0.127	0.089	0.171	0.108	0.109	0.097	0.179	0.186
B	0.171	0.135	0.059	0.050	0.078	0.086	0.134	0.127	0.093	0.095	0.193	0.181
C	0.151	0.083	0.068	0.044	0.101	0.074	0.158	0.131	0.087	0.119	0.149	0.211
Broth and a. (no bacteria)		0.025		0.056		0.125		0.069		0.086		0.178

6.4 Statistical Calculations for Rank Sum Test

6.4.1 5 wt% LCJC

S. aureus	**5wt%**				
Time	Control	SEM	5wt%	SEM	P value
0*	0.0473	0.00165	0.0315	0.00202	0.001
3*	0.128	0.0131	0.0323	0.00328	0.001
6*	0.203	0.0257	0.0435	0.005238	0.001
24	0.234	0.0144	0.192	0.032	0.279
36	0.243	0.0564	0.184	0.0141	0.343
48	0.215	0.0278	0.142	0.0298	0.124

E. coli			**5wt%**		
Time	Control	SEM	5wt%	SEM	P value
0*	0.144	0.00338	0.104	0.00632	0.001
3*	0.0645	0.00603	0.0887	0.00509	0.022
6	0.338	0.0216	0.444	0.0425	0.07
24*	0.393	0.0159	0.53	0.0511	0.042
36	0.134	0.0269	0.204	0.0136	0.061
48*	0.143	0.02894	0.143	0.01447	0.029

6.4.2 PACs

S. aureus	**PACs**				
Time	Control	SEM	PACs	SEM	P value
0	0.0253	0.00409	0.0222	0.00103	0.504
3	0.046	0.00687	0.0498	0.00464	0.667
6*	0.295	0.0578	0.132	0.0134	0.033
24*	0.491	0.0477	0.267	0.0233	0.006
36*	0.132	0.0113	0.267	0.0233	0.002
48	0.195	0.0105	0.145	0.0315	0.179

E. coli	**PACs**				
Time	Control	SEM	PACs	SEM	P value
0	0.023	0.0115	0.1	0.0085	0.437
3	0.095	0.0142	0.0683	0.0129	0.213
6	0.068	0.0118	0.0803	0.0269	0.691
24*	0.292	0.0413	0.582	0.0685	0.011
36	0.278	0.0417	0.225	0.0321	0.349
48	0.587	0.0763	0.516	0.0324	0.426

6.4.3 Crude

S. aureus	**Crude**				
Time	Control	SEM	Crude	SEM	P value
0	0.02375	0.012755	0.008	0.004183	0.695
3	0.05	0.00474	0.041	0.00481	0.231
6	0.142	0.113	0.201	0.0291	0.108
24	0.3495	0.095317	0.20875	0.008584	0.343
36	0.173	0.00972	0.167	0.0166	0.784
48	0.19	0.0228	0.185	0.0155	0.862

E. coli	**Crude**				
Time	Control	SEM	Crude	SEM	P value
0*	0.134	0.00604	0.106	0.00738	0.01
3*	0.113	0.00473	0.24	0.0126	0.001
6*	0.0744	0.00605	0.127	0.00589	0.001
24	0.64	0.0521	0.725	0.0369	0.206
36*	0.501	0.0686	0.751	0.0383	0.007
48*	0.306666667	0.021039	0.651	0.063755	0.024

6.4.4 Anthocyanidins

S. aureus	**Anthocyanidin**				
Time	Control	SEM	Antho	SEM	P value
0*	0.03533	0.000451	0.1177	0.015141	0.002
3*	0.0893	0.00835	0.0563	0.00434	0.006
6	0.0925	0.00789	0.0933	0.013	0.957
24	0.125	0.0147	0.138	0.00926	0.454
36	0.11	0.00653	0.096	0.00663	0.168
48*	0.266	0.00981	0.183	0.0083	0.001