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Investigating the effects of cranberry juice on the physicochemical properties of Escherichia coli for the prevention of urinary tract infections

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INVESTIGATING THE EFFECTS OF CRANBERRY JUICE ON THE PHYSICOCHEMICAL PROPERTIES OF *ESCHERICHIA COLI* FOR THE PREVENTION OF URINARY TRACT INFECTIONS

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Abstract

The adhesion of bacteria to uroepithelial cells or urinary catheters is the first step in the development of biofilm formation and urinary tract infections (UTIs). Previous research has suggested that consumption of cranberry juice can prevent the recurrence of UTIs by decreasing bacterial adhesion since isolated compounds in cranberries, known as A-type proanthocyanidins (PACs), change the conformation of proteinaceous fimbriae that help attach bacteria to epithelial cell receptors. Most clinical and laboratory studies have shown the effects of cranberry juice cocktail (CJC) on large communities of bacteria; however, very few studies have evaluated how cranberry affects the adhesion forces of a single bacterium as well as effects on cellular composition and biofilm formation. We used atomic force microscopy (AFM) to investigate the effects of CJC and PACs on the adhesion forces between *E. coli* and a silicon nitride tip. Bacterial cultures were grown in tryptic soy broth (TSB), supplemented with 0 and 10 wt.% light cranberry juice cocktail (L-CJC) or 128 μg/mL PACs. *E. coli* bacteria were continuously cultured in the presence of cranberry products up to twelve times. Experiments were conducted at different scales to test bacterial attachment and adhesion forces. At the macroscale, bacteria were incubated with uroepithelial cells and the number of bacteria attached per uroepithelial cell was determined. In nanoscale experiments, the forces of adhesion between *E. coli* and a silicon nitride AFM tip were probed for bacteria grown in L-CJC or PACs for different numbers of culture times. Successive replacement of media and continued culture in L-CJC and PACs resulted in a significant decrease in adhesion forces for *E. coli* strains. Finally, during the continuous exposure of L-CJC to bacteria we examined the growth, morphology, and ability to form biofilms of *E. coli*. We found a decrease in growth rates related to changes in Gram staining with increasing number of cultures in L-CJC. Growth of bacteria in L-CJC or PACs

inhibited the development of biofilms on polyvinyl-chloride, which can model biofilm formation on urinary catheters. We also determined that growth of *E. coli* in L-CJC results in prevention of the expression of indole which can be linked to the inhibition of biofilm formation. Our results help support the molecular mechanisms for the role of cranberry in preventing the adhesion of *E. coli* to biotic and abiotic surfaces, thus helping to scientifically validate the use of cranberry juice as a prophylactic treatment for the prevention of UTIs.

Authorship

The contents of this thesis are a representation of the work done by the main author. Contributions to this project were made by Yatao Liu, a Ph.D. candidate in the department of chemical engineering at Worcester Polytechnic Institute. Yatao was of great assistance on the bacteria bonding to clean glass slides for the atomic force microscopy experiments. He also contributed to this project by growing the uroepithelial cells used for the attachment study.

Kerrie Holguin was an undergraduate student from the materials science and engineering department at Carnegie Mellon University, PA. Kerrie participated in the 2007 REU program at WPI. She assisted in the preliminary experiments on biofilm formation.

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Chapter 1: Overview

1.1 Research Motivation

Urinary tract infections (UTIs) are among the most common infections affecting humans today. The presence of bacteria in the urinary system results in serious infections that can cause kidney failure, and in some cases death[1-3]. The first step in infection is the adhesion of bacteria to uroepithelium where bacterial cells can multiply and colonize different organs of the urinary system[4]. Conventional methods to treat UTIs rely on the use of antibiotics. However, many bacteria can develop antibiotic resistance[5]. The use of antibiotics to treat infections can also become a major environmental problem since the human body does not process these substances completely, and therefore they persist in wastewater.

Other therapeutic methods are currently being investigated for the prevention and treatment of UTIs, where the use of natural products has shown signs of becoming a promising alternative. For example, cranberries have been used as a remedy to prevent and treat UTIs for many years. Limited clinical evidence has suggested that cranberries can be used as a prophylactic treatment to prevent recurrent UTIs, since they may prevent bacteria from adhering to uroepithelium[6,7]. Limited *in vitro* studies have been done to investigate the mechanisms responsible for this anti-adhesive activity[8,9].

Escherichia coli is the most common infecting agent in the urinary tract, targeting most frequently neonates, preschool girls, sexually active women and elderly women[1]. While UTIs can be caused by other bacterial strains, including *Pseudomonas aeruginosa, Klebsiella* spp., *Enterococcus* spp., and *Proteus mirabilis, Escherichia coli* is identified as the etiologic agent in at least 75% of women who present symptoms of cystitis. *E. coli* also accounts for 90 to 100% of bacterial infection in the kidneys or acute pyelonephritis[4,10].

The first step in the development of a UTI is the adhesion of bacteria to uroepithelium[4]. In the case of *E. coli*, these bacteria have extracellular compounds that interact with the surface of uroepithelial cells[1]. These compounds, known as fimbriae, are protein structures that act as adhesins that bind to receptors of different cells. *E. coli* can express several types of fimbriae that bind to specific receptors. Acute pyelonephritis has been found to be caused by *E. coli* that have fimbriae of type P[11,12]. This type of fimbriae binds to receptors in P blood antigens that are found on the surface of uroepithelial cells. The P blood antigen is present in over 99.9% of the world's population[1].

For hundreds of years cranberries have been used for medical purposes. Native Americans used this plant to treat several illnesses such as diarrhea, diabetes, urinary disorders, blood poisoning and wounds[13]. The role of cranberry juice to prevent and treat UTIs is now being studied[13-16]. Several clinical studies have taken place where patients with pyuria and bacteriuria have consumed cranberry juice and the effects of this juice on urinary infections have been investigated[6,13]. *In vitro* studies have taken place to isolate the compounds that cause an anti-adhesive activity, and that inhibits the adhesion of *E. coli* to the receptors found on uroepithelial cells[9,16-18]. Proanthocyanidins are examples of these compounds, since they differ from other fruits in their chemical structure[19].

The research described in this document is based on the principle that the development of UTIs cannot occur unless there is attachment of *E. coli* bacteria to uroepithelial cells or biomaterials such as urethral and ureteral catheters. The following sections of this document will focus primarily on a basic understanding of the mechanism of action of cranberry juice and proanthocyanidins against bacterial attachment to biotic and abiotic surfaces. Furthermore, a basic understanding on the effects that this fruit imparts on the morphology of *E. coli* and excretion of extracellular signals is presented.

1.2 Research Summary

Chapter 2 comprises of an extended literature review on the development of urinary tract infections (UTIs) and formation of biofilms on urinary catheters. Basic understanding on the process of

bacterial adhesion is summarized and current mathematical models that have been used by researchers to explain this phenomenon are presented. An extended background on the uropathogen *E. coli* is presented, as is the role of surface polymers on bacterial adhesion. The use of cranberries as a prophylactic treatment against UTIs is reviewed and active compounds found in cranberries are presented. A detailed compilation of *in vitro* and clinical studies of cranberries and UTIs is also reviewed. Finally, the various microscopy techniques used in this study are explained.

Chapter 3 is based upon a research project performed in collaboration with Yatao Liu, a Ph.D. candidate of the Department of Chemical Engineering at WPI and Kerrie Holguin who was part of the 2007 Research Experience for Undergraduates (REU) program that was funded by the National Science Foundation. We specifically investigated the effects of increasing concentrations of cranberry juice and proanthocyanidins on bacterial attachment to uroepithelial cells. We also investigated the effects of continuous culture of *E. coli* bacteria in a cranberry-rich environment on bacterial attachment and formation of biofilms on a polyvinyl chloride surface. The benefits of long term exposure to cranberry products are explained.

Chapter 4 describes a project completed in collaboration with Yatao Liu from the Dept. of Chemical Engineering at WPI. The adhesion force interactions between *E. coli* and a silicon nitride probe were investigated, and we evaluated how cranberry products affect these forces. Atomic force microscopy (AFM) was the main tool used during this investigation where continuous culture of *E. coli* in cranberry juice and proanthocyanidins (PACs) was assessed. An investigation on the effects of cranberry constituents on changes of bacterial surface charge was also carried out. Initial results describing the differences between PACs and cranberry juice are reported. Significance of this research on the medical field is hypothesized.

Chapter 5 comprises an ongoing investigation on the effects of cranberry products on the morphology of *E. coli* and changes in the expression of extracellular signals. Variability in Gram staining

due to growth of bacteria in cranberry juice is described as well as changes in binary fission. Changes in Gram staining are correlated with changes in the production of extracellular signals that affect biofilm development. This type of study is the first in its field, since no other researcher has attempted to understand how cranberry products affect the morphology of bacteria and expression of essential cellular signals over such long time periods. This study was interpreted and combined with previous work performed in our laboratory in an attempt to understand the benefits of the consumption of cranberries.

Chapter 6 and **Chapter 7** describe the overall conclusions of this project and experiments that are currently under investigation. Recommendations are made in this section for future examination on the benefits of consumption of cranberry products for the prevention of UTIs.

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Chapter 2: Literature Review

The development of new therapeutic methods to control infections requires a comprehensive understanding of the mechanisms of bacterial adhesion to human epithelial cells and to medical biomaterials. Conventional treatments, such as the use of antibiotics and analgesics to eradicate urinary tract infections are reviewed, as well as the frequent development of antibiotic resistance in biofilm settings. In this chapter, an extensive background on the uropathogenic bacteria *Escherichia coli* is presented, as well as the role of surface molecules on the development of infections. The use of cranberries and its constituents, as a prophylactic treatment to prevent infections is presented. Various cranberry *in vitro* and clinical studies are also reviewed. Finally, the fundamental physics of atomic force microscopy, and how this technique can be used to understand biological settings including bacterial adhesion, are also presented.

2.1 Bacterial Adhesion

Bacterial adhesion to different surfaces is known to be influenced by physicochemical properties of polymers on the surface of a bacterium[1]. Adhesive properties of bacteria are due to extrapolymeric substances (EPS), such as lipopolysaccharides, capsule, hemolysins, flagella, and fimbriae[2-4]. The expression of these components allow bacteria to strongly attach to different surfaces, such as biomedical catheters and mammalian cells, which often results in multiplication and growth of bacteria species and development of biofilms[5]. Biofilms are usually seen associated with surfaces that provide a rich environment for the community of bacterial

cells[6]. Formation of biofilms is of particular interest in the industrial and medical field. For instance, fouling of ship hulls by microorganisms in marine environments is recognized as a serious problems in industry[7], as well as development of ocular infections due to biofilm formation on contact lenses[8], maturity of *Pseudomonas aeruginosa* biofilms on tissues of cystic fibrosis lungs[9], development of meningitis and septicemia due to formation of *Listeria monocytogenes* biofilms[10], and the damage of oral tissues caused by attachment of different *Porphyromonas* species to tooth surfaces[11].

The interaction of bacteria and a surface is governed by long range forces such as steric and electrostatic forces, and by short range forces that include hydrophobicity, free energy, surface charge and van der Waals interactions[4].

Many researchers have described bacterial adhesion in terms of surface free energy to predict if adhesion is favorable to a determined surface[12,13]. Thermodynamic models such as the van-Oss-Chaudhury-Good (VCG) approach, have been developed to account for the non-specific Lifshitz-van der Waals (LW) and acid/base (AB) interactions[14,15]. Gibbs free energy of adhesion can be calculated based on the contact angle of several probe liquids on a given surface or a layer of bacterial cells[14]. Contact angles are a function of three different interfacial free energies or surface tensions, including bacteria/substrate, bacteria/water, and substrate/water (Figure 2.1).

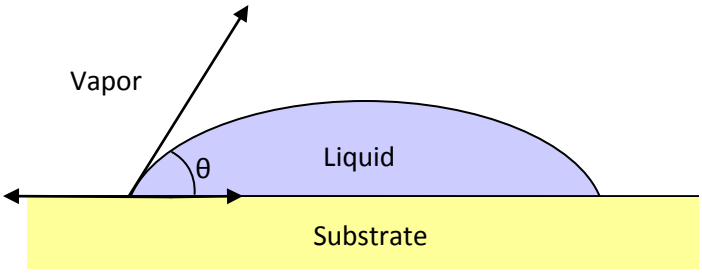

Figure 2.1: Contact angle and interfacial tensions

(Adapted from van Loosdrecht et al.[14])

Contact angles measure the hydrophobicity of the surface, which could be a biomaterial or a layer of bacteria. In most cases, hydrophobicity can be directly correlated with surface free energy (hydrophobicity decreases with increasing surface free energy)[14].

Electrostatic interactions are also of great importance to bacterial attachment. The surface of bacteria is surrounded by protein structures such as lipopolysacharides that contain carboxylate, phosphate, and amino groups[16]. These assembly of charged molecules ionize as a function of the pH of the surrounding environment, thus giving an electrostatic charge to the surface of the bacterium[17]. Ionic strength affects the probability of a bacterium to attach to surfaces[1], where low ionic strength solutions result in less attachment because of increased electrostatic repulsion. Electrostatic interactions can be measured through electrophoresis and zeta potential.

Zeta potential is the electrical potential of the interfacial regions between the surface of the bacteria and the surrounding aqueous environment[18]. At physiological pH, bacteria possess a negative electrostatic surface charge that forms an interfacial electrical double layer[17] (Figure 2.2).

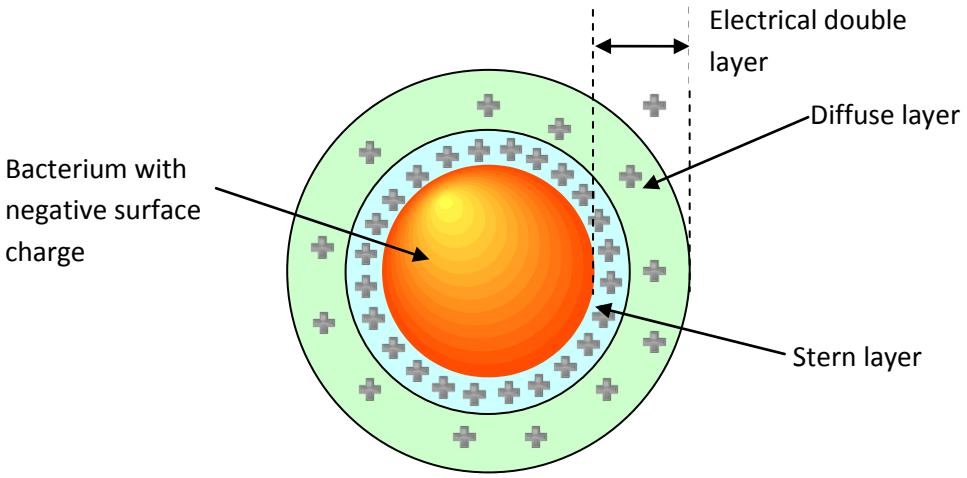

Figure 2.2: Representation of solvent layers surrounding bacterial cells

The liquid surrounding the bacterium exists in two parts: an inner region, also known as the Stern layer, where ions are strongly bound to the surface of the bacterial cell and an outer region, known as diffuse layer, where anions and cations are more diffuse and less firmly attached. Ions and particles form a stable entity or boundary within the diffuse layer. When bacterial cells move, ions within the boundary move with it, but any ions beyond this boundary do not travel with the cell. Zeta potential calculates the electrical potential that exists at this boundary[17]. Zeta potential is then calculated from the electrophoretic mobility, using a relationship such as (Eq. 1)

$$U_E = \frac{2\varepsilon z f(ka)}{3\eta} \qquad (1)$$

where U_E is the electrophoretic mobility, z is the zeta potential, ε is the dielectric constant of the aqueous media, η is the viscosity and *f(ka)* is Henry's function. Since bacterial cells are larger than 0.2 μm, then the Henry's function or *f(ka)* value is 1.5. This is referred to as the Smoluchowski approximation and it is used for samples in aqueous media[17].

The electrophoretic mobility is obtained by applying an electric field across an electrolyte (Figure 2.3).

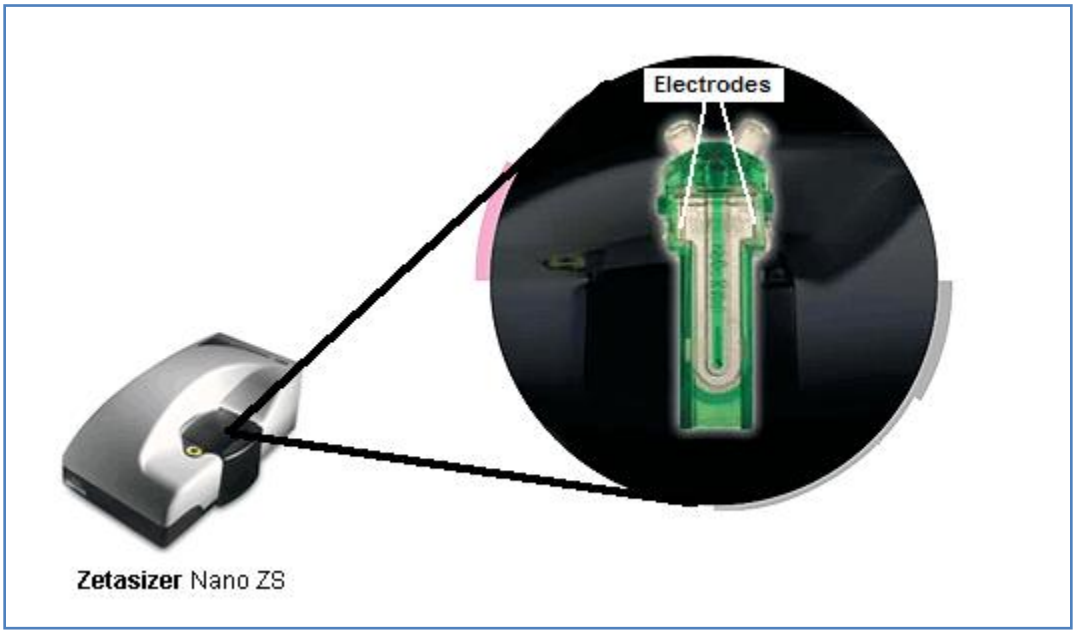

Figure 2.3: Zeta potential measurement device

Bacteria suspended in a cell are attracted towards the electrode of opposite charge because of their charged particles at the surface. The velocity at which these particles move over a given distance is then calculated and expressed in unit field strength as their mobility.

Bacteria and surface interactions can also be modeled as a function of separation distance. Classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloidal stability can be applied to determine the interaction energy between a bacterium and a surface of interest[1]. DLVO theory is the sum of van der Waals and electrostatic double layer interactions, which takes into account non-specific forces such as hydrogen bonding and acid/base interactions[13]. While these thermodynamic models help researchers better

understand the probability and mechanisms of bacterial adhesion, there are several factors that are not taken into account and that might be of great importance in understanding adhesion. For instance, flagellar rotation is not typically address by DLVO or VCG theories as well as ion penetrability[1]. Many researchers believe that motility due to flagellar rotation is a requirement in the first stages of biofilm formation since the expression of flagella increases the chances of bacterial attaching to a surface and develop a biofilm[19,20].

In other cases, bacterial adhesion is governed by the specific interaction of adhesins on the surface of bacteria and receptors on uroepithelial cells[21].

Bacterial adhesion is a complicated process that needs special attention in the environmental and medical fields. A combination of microscopy techniques, such as atomic force microscopy, scanning force microscopy, phase contrast microscopy and zeta potential measurements can help better understand the mechanisms of bacterial adhesion and how to prevent the attachment of bacterial cells to medical devices such as catheters or to mammalian cells. The understanding of this adhesion step is the main focus to finding prophylactic treatments to prevent the development of infections.

2.2 Urinary Tract Infections

One of the most common diseases encountered in the practice of medicine today is caused by the presence of bacteria in the urinary tract[21]. Urine is normally sterile with fewer than 10,000 colony forming units (CFU). When bacteria cling to uroepithelial cells they start multiplying and developing infections in the urinary tract. Contamination of urine is usually characterized by the presence of 10,000 to 100,000 CFUs where bacteria

do not multiply at high growth rates; the presence of 100,000 or more CFUs in urine is considered as true bacteriuria and infection[22]. Studies have suggested that 95% of all urinary tract infections (UTIs) develop through an ascending route of infections, caused mostly by Gram-negative bacteria, while the other 5% develops by a descending route (hematogenous infection). The latter is usually caused by Gram-positive organisms[23]. Ascending route UTIs begin with the colonization of bacteria in the periurethral area, followed by an upward progression of bacteria to infect the bladder (Figure 2.4). If conditions of infection persist, this progression of bacteria could continue to the ureters and finally to the kidneys[24].

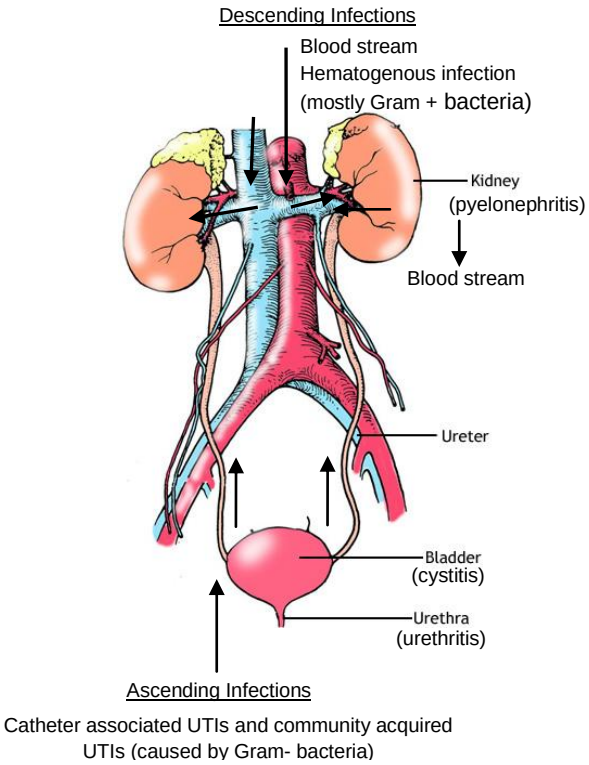

Figure 2.4: Structures and infections of the urinary tract[25]

Depending on where the infection is located in the urinary system, the disorder might have mild consequences. For instance, if the infection is in the bladder this disorder is known as *cystitis* while if it is in one or both kidneys, it is called *pyelonephritis*, which is a more serious disorder that if not treated properly could cause kidney failure[26]. If there is passage of bacilli with the urine, this condition is known as *bacilluria* and the presence of bacteria in the urine is known as *bacteriuria*[22].

UTIs are usually categorized as being uncomplicated or complicated infections[27]. Uncomplicated UTIs usually refers to development of cystitis and pyelonephritis that occur in young healthy women that are not pregnant and that do not have any anatomic abnormalities of the urinary tract, as well as episodes of cystitis in healthy young men[28]. Complicated UTIs are associated with elderly patients, development of infections due to instrumentation such as insertion of catheters, antimicrobial treatments, infections that are also associated with anatomic abnormalities of the genitourinary tract, infections in diabetic patients and urethritis due to inflammation of the prostate gland in men[29].

Escherichia coli predominate as the most common urinary pathogen accounting for at least 85% of community-acquired urinary tract infections[30]. These bacteria are part of the normal flora of the human intestinal tract where they are essential during digestion since they produce vitamin K from undigested material in the large intestine[31]. However, *E. coli* become a hazard when the bacteria cling to the urethra opening and start multiplying. Other bacteria species that cause UTIs include Gram-positive organisms such as *Staphylococcus saprophyticus* accounting for ~5-10% of uncomplicated cystitis, *Staphylococcus aureus*, and Gram-negative organisms such as *Proteus mirabilis*, *Klebsiella* sp, *Citrobacter* sp and *P. aeruginosa*[27].

The symptoms associated with UTIs have been characterized according to the site of infections. Patients who have been diagnosed with acute cystitis usually present symptoms of micturition (urge to urinate frequently), dysuria (difficulty or painful discharge of urine), discomfort or pressure in the lower abdomen, cramping of pelvic area and strong odor of urine accompanied by the presence of white blood cells (pyuria)[23,29,32]. Patients with acute pyelonephritis usually present the same symptoms of lower UTIs lasting more than one week. In addition to these symptoms, patients with pyelonephritis also develop nocturia (excessive urination at night), as well as chills, persistent fever, pain at the waist area, vomiting and nausea[28].

Infections in the urinary tract can also be asymptomatic[22,32]. Asymptomatic bacteriuria is often defined as significant isolation of bacteria from urine that is consistent with infection, but lacks any signs of illness or symptoms that are usually associated with UTIs[32]. Asymptomatic UTIs are often seen in diabetic females, patients with previous history of UTIs, women with cystoceles (hernias in urinary bladder), and in about 50% of geriatric women[22]. Asymptomatic UTIs have been of great interest since these 'silent' infections can result in sepsis or even death[32]. For instance, pyelonephritis has been found in 12 to 20% of autopsies where only one-fifth of the people were diagnosed with the UTI[22]. Special attention has been paid to pregnant women, since UTIs are the most common bacterial infection during pregnancy[33], and if untreated could lead to chronic pyelonephritis, premature delivery, low birth weight, anemia, preeclampsia and fetal mortality[32].

2.2.1 Statistics and Treatments

Due to high incidence of UTIs, the financial implications of the eradication of this infection are considerable. The total yearly cost of community-acquired UTIs plus nosocomial UTIs due to insertion of indwelling catheters is close to $2 billion[32]. This accounts for direct costs such as doctor visits, antimicrobial prescriptions, hospitalization expenses, travel and sick days. The Center for Disease Control and Prevention (CDC) estimates that approximately 7 million cases of acute cystitis and at least 250,000 cases of acute pyelonephritis occur in the United States annually[34]. In addition, approximately 8 million doctor visits and hospital admissions result in the prescription of antibiotics to eradicate UTIs, and 1.3 million procedures are ordered[35]. Women are more prone to UTIs due to the anatomy of their urinary system[28]. It has been estimated that ~50% of women will experience at least one UTI in their lifetime[32].

Most lower UTIs clear spontaneously without the need of any antibiotics; however, symptoms can persist for several months[27]. If treatment is needed it will depend on the patient's recurrence of having UTIs, as well as the location of the infecting bacteria. Commonly used antimicrobials and analgesics include, but are not limited to trimethoprim-sulfamethoxazole (TMP-SMX), trimethoprim, norfloxacin, ciprofloxacin, amoxicillin, gentamicin, nitrofurantion, phenalzopyridine and various generations of cephalosporins[28,36]. The prescription of any of these antibiotics depends on the severity of the UTI; however, common factors that should be considered when selecting these drugs include the prevalence of resistant uropathogens, the effect of the antibiotic on fecal and vaginal flora, potential side effects, and cost of treatment among others[36]. While the majority of treatments are effective, the development of antibiotic resistance is inevitable

for some cases[37]. For instance, as many as 25% to 70% of *E. coli* isolates have shown signs of resistance to sulfonamines and ampicillin, while resistance to nitrofurantion has only been reported in less than 5% of patients with uncomplicated UTIs[28]. The use of antibiotics to treat infections can also become a major environmental problem since the human body does not absorb these substances completely, where 25% to 75% of unaltered compounds leave the body via urine or feces; therefore persisting in wastewater[38,39].

2.3 Biofilms

In the past few decades, researchers have realized that bacteria are not usually found in a planktonic or free living state but in a more organized multi-cellular surface attached community, also known as biofilm[40]. These communities of bacterial cells can form on biotic and abiotic surfaces if the proper environmental conditions are present. Formation of biofilms in urethral and ureteral catheters is almost inevitable. More than 1 million cases of catheter associated UTIs are reported each year in the United States, which accounts for approximately $500 million in medical expenses[32]. The risk of UTIs due to insertion of urethral catheter increases with increasing duration of catheterization[41]. It has been estimated that once a catheter is in place, the incidence of bacteriuria increases by 3-10% and patients with catheters are 60 times more likely to develop bacteremia than patients without catheters[41]. As a preventative measure, catheterized patients are administered antibiotics; however, these compounds have a tendency to lose potency several days after the insertion of the catheter, since resistant organisms start developing, multiplying, and forming biofilms on the abiotic surface [22,41].

2.3.1 Development of Biofilms

Formation of biofilms is ruled by a stable biological cycle that includes initiation, maturation, maintenance, and dissolution of the biofilm (Figure 2.5)[42]. Biofilm development initializes with the attachment of planktonic bacterial cells onto a living (biotic) or abiotic surface[6]. This process is a response to different variables including environmental factors, bacterial species, surface composition and essential gene products[19,43].

Formation of biofilms in abiotic catheters usually occurs when the biomedical device is placed and the surface is modified by absorption of different proteins such as fibronectin, extracellular matrix molecules, albumin, lipids and absorption of water[44]. Bacteria can interact with some of these proteins and attach to the new conditioned surface. The approximation of bacterial cells to a surface is often a response of environmental conditions, especially in non-motile bacteria. For instance, fluid flow and Brownian motion are important factors that account for the attachment of non-motile bacteria to abiotic surfaces, while bacteria that express flagella have more probabilities of encountering a new surface to attach to[6].

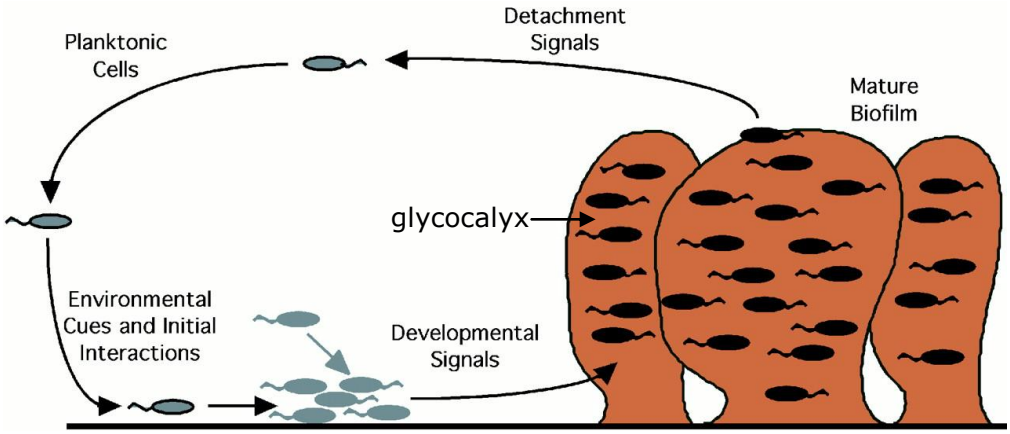

Figure 2.5: Biofilm development cycle. Adapted from O'Toole et al.[42]

Initial attachment to tissue and epithelial cells is also mediated by specific molecular docking systems, such as the assembly of adhesins located at the distal end of fimbriae surface proteins, and receptors often found on the surface of epithelial cells[23,43]. The expression of these adhesins on bacterial surfaces can overcome electrostatic repulsive forces that are often seen between bacteria and epithelial cells since both surfaces are negatively charged.

Once single organisms have attached to a surface, the second stage of the cycle begins, which is characterized by formation of microcolonies[45]. Microcolonies form as a result of the production of extracellular substances including exopolysaccharides and capsular polysaccharides. These substances strengthen the attachment of bacteria to a surface or to tissue, and this adhesion process becomes irreversible[43]. In this new environment, planktonic cells can also stick to each other, thus increasing the density of the biofilm.

Maturation of the biofilms starts after permanent attachment of bacteria to the surface. The continuing production of exopolysaccharides provides a stable architecture

of the biofilms that will protect the bacterial cells from environmental insults such as immune responses and antibiotics[5]. In this extracellular matrix, bacteria cells start replicating and generating other components that interact with inorganic and organic materials in the immediate environment. For instance, infected biomaterials will cause an inflammatory response from the immune system where proteins such as fibrinogen and fibronectin will be enclosed in the biofilm matrix[43]. Differences in the biofilm architecture arise from changes in the environmental conditions as well as differences among bacteria species[45]. Other polymers found in the matrix of biofilms include cellulose, which has been determined as a crucial component in the architecture of *E. coli* biofilms, as well as polysaccharide intercellular adhesins encoded by the *pga* gene that are similar in *Staphylococcus* species[43].

Further growth of biofilm and increases in bacterial density are a response to nutrients available around the biofilm. Other factors that play an important role in the maintenance of biofilms are the regulation and perfusion of nutrients within the matrix, as well as the removal of waste[43]. When the biofilm has reached its critical mass, the outermost layer of growth begins to rupture and planktonic cells are released from the matrix[42]. These cells can then move to uninfected surfaces and start the biofilm cycle again.

Recent research has suggested that there is a connection between population density and production of extracellular molecules[43,45]. *Quorum sensing* is the process by which bacteria communicate with each other through chemical signals[46]. Extracellular signals can activate different genes in bacteria that can alter biofilm development. For instance, Gram-negative bacteria are known to produce *N*-acyl derivatives of homoserine

lactone, cyclic dipeptides and quinolones[46]. These signals regulate biofilm maturation through genetic pathways and keep water channels open in the architecture of the biofilm so the matrix can develop, as for the formation of *Pseudomonas aeruginosa* biofilms[45].

2.3.2 Antibiotic Resistance in Biofilms

The complex architecture of biofilms provides the bacteria enclosed in this matrix a defense mechanism against antimicrobial agents. In urethral catheters the formation of biofilms is almost inevitable[47]. Insertion of a catheter might carry uropathogens into the bladder than can ascend into the ureterers and infect the kidneys. While planktonic bacteria can be eradicated with common antibiotics, sessile bacteria in biofilms are more resistant to these biocides[5]. Scientists have shown that biofilms can be hundreds or even a thousand times more resistant than bacteria grown in suspension[48]. This means that the minimum inhibitory concentration of antibiotics needed to eradicate a biofilm can be highly toxic if it was administered to a patient. Therefore, biofilms usually persist in the catheter until the device is surgically removed and replaced by a new one[5,41].

There are several theories that have tried to explain the mechanisms of antibiotic resistance in biofilms[5,41,49]. The polysaccharide-rich matrix or glycocalix enclosing bacteria can act as a molecular filter since it can prevent the perfusion of antimicrobials in the biofilm[43]. Researchers have also proposed that bacterial resistance in a biofilm can be explained by a delay reaction and penetration of the antimicrobial into the matrix of the biofilm[49]. If the antibiotic is deactivated in the biofilm, penetration can then be retarded. Dunne suggested that environmental factors within the biofilm can produce undesirable effects[43]. Factors such as pH, hydration level and divalent cation concentration can affect the activity of biocides since acidic and anaerobic environments

persist at the deepest layers of the biofilm. Other organisms might deactivate the antibiotic at the surface layers of the matrix[5].

Another hypothesis depends on bacteria growth rates within the biofilm[43]. Accumulation of waste within the glycocalyx can result in significantly slower bacterial growth rates, diminishing the uptake of antimicrobial molecules. Some bacterial cells can enter a non-growing state in which they are protected from killing[5,49]. For instance, penicillin antibiotics kill only growing bacteria, and are therefore ineffective for dormant bacteria[5]. Other theories depend on bacterial cells within the biofilm that could undergo phenotypic changes that will protect the cell against biocides; similar to the process of sporulation[5].

One promising strategy to prevent development of biofilms is to prevent the initial adhesion of bacteria onto catheters or mammalian cells. In lieu of increasing antibiotic usage and biofilm antibiotic resistance, natural products that are anti-septic and environmentally friendly are being sought. Development of UTIs due to the adhesion and development of *Escherichia coli* biofilms is a problem that is financially considerable and in need of prophylactic treatments. *E. coli,* being the most common urinary pathogen, needs to be better understood in order to study different solutions for the prevention of UTIs.

2.4 Escherichia coli

Escherichia coli is the most common infecting agent in the urinary tract, targeting most frequently neonates, preschool girls, sexually active women and elderly women[23]. While UTIs can be caused by other bacterial strains, including *Pseudomonas aeruginosa*,

Klebsiella spp., *Enterococcus* spp., and *Proteus mirabilis*, *E. coli* is identified as the etiologic agent in at least 75% of women who present symptoms of cystitis. *E. coli* also accounts for 90 to 100% of bacterial infection in the kidneys or acute pyelonephritis[21,50].

In vitro, the reproduction of *E. coli* is rather impressive. They can grow in the complete absence of oxygen or in air and under minimal media conditions, as they encode all the enzymes they need for amino acid and nucleotide biosynthesis[51]. In liquid media, *E. coli* grows to 10^9 cells/mL, while in agar-solidified medium a single cell can multiply to a visible colony with 10^7-10^8 cells in less than 24 hours. *E. coli* can grow in sterile urine to about 10^8 bacteria/mL even without the addition of glucose or any other carbon source[22].

Uropathogenic *E. coli* differs from commensal *E. coli* strains, like the ones found in the large intestine, in that the uropathogens have extra genetic material that encodes for the production of genes that contribute to pathogenesis[23]. The production of these genes causes the bacteria to express certain factors that play roles in the initiation of infections. These factors include membrane-bound and secreted proteins, lipopolysaccharides, capsule, hemolysins, and fimbriae. The cell membrane and wall of *E. coli* are comprised of an inner membrane, where phospholipid chains and proteins can be found, and an outer membrane that is composed of lipopolysaccharide (LPS), peptidoglycan, and periplasm (Figure 2.6)[3]. The LPS structure is responsible for the protection of the bacterial cell wall since it acts as a selective permeability barrier for *E. coli* and other Gram-negative bacteria [52]. The loss or damage of LPS molecules would result in an increase of the permeability of the cell wall.

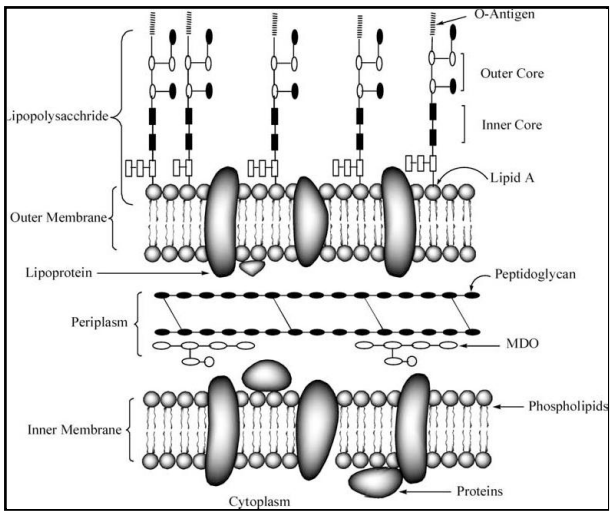

Figure 2.6: *E. coli* membrane a factors that can contribute to pathogenesis, adapted from Bahrani-Mouget et al.[23]

2.4.1 Role of Fimbriae on Urinary Tract Infections

Fimbriae or pili are protein structures that project beyond the bacterial surface. They are projected from and are distributed over the entire surface of the cell, where they act as adhesins that bind to receptors or targets on the host cell surface[23]. As the first step in infection, adherence of these structures to tissue is crucial. Fimbriae accelerates initial adhesion of bacteria to the surface[45].

E. coli can express several types of fimbriae, including P, F1C, S, M, Dr, and type 1[23,53]. Each type binds to different receptors and target different areas of the urinary tract, but they are mainly divided into two groups based on their ability to hemagglutinate erythrocytes in the presence of mannose: mannose-sensitive (MSHA) and mannose-resistant (MRHA)[23,53-60]. Type 1 fimbriae do not hemagglutinate erythrocytes in the presence of mannose and therefore are considered as mannose-sensitive, while the other types demonstrate mannose-resistance phenotypes. It is hypothesized that after type 1

fimbriae have attached to uroepithelium or bladder mucosa, bacteria induce apoptosis in uroepithelial cells and after invading deeper tissues recurrence of UTIs are inevitable[61].

P-fimbriae were the first uropathogenic virulence factors described in the context of UTIs[62]. After isolating *E. coli* from patients with pyelonephritis, scientists determined that these bacterial cells could agglutinate human type O erythrocytes in the presence of mannose. Later, the P blood antigen, which is present in over 99.9% of the world's population, was identified as the eukaryotic receptor for P-fimbriae[23]. Therefore, it is believed that the presence of these fimbriae accounts for 90-100% of *E. coli* strains that cause acute pyelonephritis[21].

The P blood antigen was found to be a glycophospholipid that consisted of a lipid section anchored in the cell membrane and a carbohydrate chain that is exposed on the surface of the erythrocytes[23]. The digalactoside αGal(1→4)βGal was identified as the oligosaccharide receptor in this antigen, which was capable of inhibiting P-fimbrial adherence[60,63-65]. This receptor, which is also found on uroepithelial cells, is recognized by most uropathogenic *E. coli*, and only the cells that contain this structure can be bound by P-fimbriated bacterial cells[66]. However, the addition of the receptor to cells that lack or synthesize small amount of globoseries of glycolipids results in the ability of bacteria to adhere to these cells.

Glycolipid binding is mediated by G adhesins located at the distal end of P fimbriae[21]. Three different types of these adhesins, which are encoded by *pap* or *prs* gene clusters, have been identified. All of them recognize glycolipids in the globoseries but differ in their ability to bind to different receptors of the αGal (1→4) βGal glycolipid

family. Human pyelonephritis caused by *E. coli* J96 is produced by P-fimbriae that bear Class I G adhesin (PapGJ96). The Class II G adhesin is linked by P-fimbriated *E. coli*, which is associated with acute pyelonephritis. The Class III G adhesins, also known as F adhesins, have been found in *E. coli* strains from patients with acute cystitis.

Other types of fimbriae (Dr, F1C and S) have been found to bind to bladder epithelium and type IV collagen. However, the role of these types of structures in the pathogenesis of UTIs is unclear. F1C and S fimbriae have been linked to extraintestinal infections, particularly neonatal meningitis[23].

The expression of fimbriae in *E. coli* cells has been studied during *in vitro* experiments and it has been determined that this expression varies with temperature, pH, and nutrition conditions[67]. The pathogenicity of these structures must be assessed to understand the mechanism of bacterial adhesion to uroepithelial cells. Addressing the adhesion forces between these cells and uroepithelial cells is an essential step towards the development and understanding of prospective treatments against this disorder.

2.5 Impact of Natural Products on Infections

With increasing problems of antibiotic resistance, the use of natural products as a prophylactic treatment provides researchers with alternative methods to use for the prevention and possibly treatment of infections. For hundreds of years natural products have been used to treat infections and for wound healing[68]. For instance, the use of honey in medicine has been used for centuries by Assyrians, Greeks, Egyptians and Chinese to heal wounds and cure diseases[69]. *In vitro* studies have shown that honey prevents the attachment of *Salmonella interitidis* to isolated intestinal epithelial cells. The use of

thyme as an antimicrobial agent has also been recognized since it can target several species of bacteria, viruses and fungi[70].

2.5.1 Cranberries and UTIs

It has been suggested that ingestion of *Vaccinium macrocarpon* or cranberries is beneficial for the prevention and treatment of urinary tract infections, especially for patients with recurrent UTIs. Compounds in cranberries interact with certain organelles on the bacterium that impede the adherence to uroepithelial cells[68]. In research conducted as early as 1923, scientists examined the effects of consumption of prunes and cranberries on the acidification of urine due to the synthesis and excretion of hippuric acid, which is formed in the body from benzoic acid and other substances found in several fruits[71]. They hypothesized that fruits like cranberries acidify the urine which could prevent bacteria from adhering to uroepithelium and thus prevent infections. However, this hypothesis was later dispelled by other researchers when clinical studies demonstrated that consumption of cranberry juice does not lower the acidity of urine. In fact, subjects who were consuming cranberry juice for a 6-month period presented a higher pH in their urine than subjects who were taking a placebo that contained no cranberry[72].

Even though the mechanism of action of how cranberries disrupt the adhesion process of bacteria to uroepithelial cells is unknown, several researchers have attempted to isolate the compounds in this fruit that possess this anti-adhesive activity[73-75]. Since there is variability within the proteins expressed on the surface of *E. coli*, different compounds in cranberry have been found to have this anti-adhesion activity. For instance, *E. coli* that express type 1 fimbriae are sensitive to fructose, which is present in many

fruit juices[76]; while the adhesion activity of P-fimbriated *E. coli* has been found to be disrupted by compounds that are specific to cranberries, such as proanthocyanidins[73,77].

Proanthocyanidins are high molecular weight polymers that are part of a group of polyphenolic compounds called flavonoids. These compounds can be found in different food products including apple juice, grape juice, tea, dark chocolate, avocados, apricot, bananas, pears, and cranberries, among others[78]. Members of the proanthocyanidin family are distinguished by slight changes in shape and attachments of their polyphenol rings. Through column chromatography, ethyl acetate extract from cranberries was fractioned. Several proanthocyanidin compounds were identified through electrospray ion mass spectroscopy[74,75]. Cranberry proanthocyanidins differ from those of other tannin-rich foods in their chemical structure since these compounds present unusual A-type linkages as opposed to the common B-type linkages that are found in other food products (Figure 2.7).

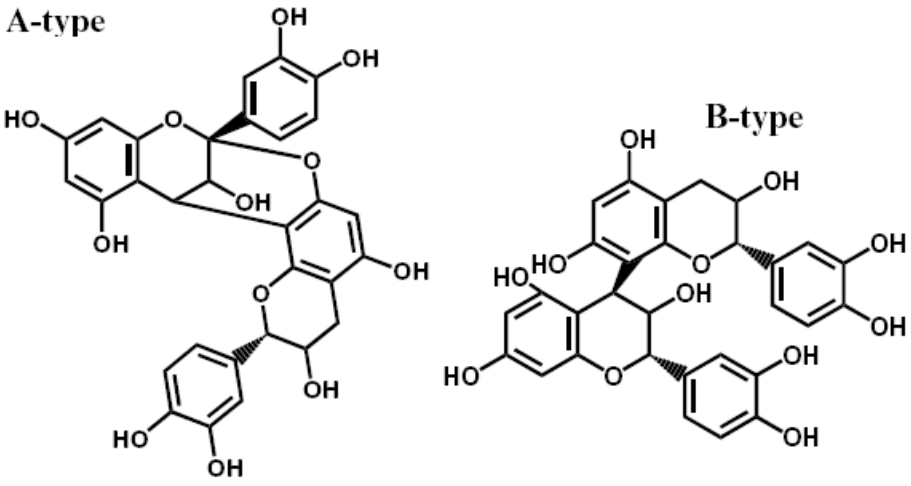

Figure 2.7: Common linkages found in proanthocyanidins extracted from foods. Adapted from NDL[78]

Most common proanthocyanidin linkages have C-C bonds (B-type, 4→6 or 4→8), but certain plants like cranberries present proanthocyanidins where double linkages occur (A-type, 4→8 and 2→7)[78].

Through the analysis of urine of volunteers who had consumed cranberry juice, researchers found that consumption of juice with A-type linkages had a bacterial anti-adhesion activity that was not present in tannin-rich foods with B-type linkages. Bacteria from urine of individuals who had consumed cranberry juice, apple juice, dark chocolate, tea or grape juice, were collected every two hours and tested for anti-adhesion activity. Bacteria present in the urine of the individuals who had consumed cranberry juice did not attach to mannose-resistant human red blood cells[73]. Further, this study showed that proanthocyanidins are not completely degraded by the digestive system since bacteria from urine were still unable to attach to red blood cells.

Another compound that has been isolated from cranberries is the high molecular weight, non-dialyzable material (NDM)[79]. This material exhibits tannin-like properties, is soluble in water, contains 56.6% carbon, 4.14% hydrogen, and it lacks proteins, carbohydrates and fatty acids[80]. Bodet et al. concluded that this NDM fraction contains 0.35% anthocyanidins and 65.1% proanthocyanidins[81]. This fraction isolated from cranberries prevents the adhesion of a number of bacteria species and inhibits the aggregation of oral bacteria on teeth[82]. However, no studies have addressed the effects of NDMs on UTIs.

2.5.2 *In vitro* Studies of Cranberries and UTIs

There are a limited number of *in vitro* studies that have investigated the effects of cranberries on the adhesion of *E. coli* to uroepithelial cells or erythrocytes. Erythrocytes

are often used as model cells in binding assays since they have cell surface carbohydrates that are recognized by *E. coli* and play a role in bacterial adherence[55].

One such study explored the ability of P-fimbriated *E. coli* to agglutinate erythrocytes after being exposed to cranberry juice[54]. Two isogenic strains of *E. coli*, one that expresses P-fimbriae and one that lacks these surface structures, were used. Both strains were cultured on tryptic soy agar (TSA) plates that were supplemented with different concentrations of cranberry juice (without additives). After both strains had grown for 24 to 48 hours they were tested for hemagglutination activity by exposing the bacterial cells to human erythrocytes in phosphate buffered saline (PBS) solution. A minimum concentration of 5% cranberry juice in the media was found to weaken the ability of P-fimbriated *E. coli* to adhere to red blood cells. When cranberry juice concentrations exceeded 20%, P-fimbriated bacteria were unable to elicit any detectable levels of hemagglutiation. The non-fimbriated *E. coli* did not hemagglutinate before or after being exposed to cranberry juice.

One of the first cranberry juice-bacteria studies was done by Ahuja et al. where *E. coli* was cultured on agar plates in the presence of 25% cranberry juice[83]. While the morphology of the colonies that grew in the cranberry juice-rich media were identical to the control experiments, the ability of bacteria cultured in this juice to agglutinate P-receptor specific latex beads was partially inhibited. Complete inhibition was observed after the third plating. However, this ability to agglutinate was regained after the bacterial cells were replated on agar media that did not contain any cranberry juice. Electron micrographs of *E. coli* showed that cells grown in cranberry juice underwent morphological changes. The results of this study suggested that there is a phenotypic

variation on bacteria where the cells become elongated. From the electron micrographs it was also observed that *E. coli* lose their fimbriae after culture in cranberry juice. These changes then lead to prevention of bacteria binding to uroepithelium.

Another study showed that cranberry juice inhibited the adhesion of more than 60% of *E. coli* clinical isolates obtained from patients with UTIs[84].

Recently in our laboratory, the effects of cranberry juice on *E. coli* were investigated at a molecular-scale level by the use of atomic force microscopy (AFM) [85]. *E. coli*-coated slides were immersed in ultrapure water or in solutions with different concentrations of cranberry juice cocktail. Bacterial cells were individually probed with a bare silicon nitride tip and the forces of adhesion between the bacteria and the tip were characterized. The adhesion forces decreased as the concentration of cranberry juice cocktail increased, and this decrease in adhesion was attributed to alteration of the conformation of P-fimbriae on *E. coli* HB101pDC1. Liu et al. also measured the adhesion forces between P-fimbriated *E. coli* and uroepithelial cells[86]. After exposure of the P-fimbriated *E. coli* HB101pDC1 and uroepithelial cells to different concentrations of cranberry juice cocktail, the adhesion forces decreased from 9.64 nN to 0.5 nN with increasing concentration of cranberry juice. After cranberry treatment, the length of the fimbriae of *E. coli* HB101pDC1 decreased from 148 nm to 48 nm[85,86].

Our laboratory also used a thermodynamic model to calculate the Gibbs free energy of adhesion (ΔG_{adh}) between *E. coli* HB101pDC1 and human uroepithelial cells, based on contact angle measurements[12]. Bacteria and uroepithelial cells were exposed to increasing concentrations of neutralized cranberry juice cocktail and interfacial tensions

were calculated. We found that with a 27 wt.% cranberry juice concentration, the Gibbs free energy of adhesion became positive; suggesting that bacterial adhesion to uroepithelial cells is unfavorable since cranberry disrupts the binding between ligand-uroepithelial cell receptor pairs.

We also evaluated the effect of a cranberry extract coated catheter for the prevention of UTIs[87]. Cranberry extract in solutions of 0 to 100% was applied to silicon rubber and attachment of *E. coli* JR1 was quantified under flow conditions, using artificial urine. Coating of catheter material with cranberry extract resulted in a significantly reduced number of attached bacteria. We also observed that the cranberry extract coating was not degraded by urine.

2.5.3 *In vivo* Studies of Cranberries and UTIs

Different clinical studies have taken place where patients suffering from UTIs have been treated with cranberry juice for a determined period of time. Once this experimental time had elapsed several patients reported that the UTI symptoms had decreased significantly[68,72,88].

The molecular-level approach as well as a detailed investigation of the changes that *E. coli* undergoes due to culture in cranberry juice need to be explored further to understand how this fruit can be used as a prophylactic treatment for UTIs.

2.5.4 Cranberry Constituents and Other Infections

The isolated fraction NDM has been widely studied for its effects on dental bacteria and bacterial species that adhere to the gastric mucus[11,15,80,82,89-93]. Several studies have shown that NDMs inhibit the adhesion of *Helicobacter pylori* to human

erythrocytes and to gastric lumen[93,94] Three strains of *H. pylori* adhered to gastric mucus through sialic acid-specific adhesins that are fully expressed after 48 hrs incubation in broth[79]. This adhesin binds to sialic acid glycoconjugates found in human gastric mucus. Burger et al. showed that 50% inhibition of bacterial adhesion to *H. pylori* strains could be achieved by exposure of bacteria to 37, 125 and 305 μg/mL of NDMs for strains BZMC-25, EHL-65 and 17874[79,94]. They also found that NDMs do not reverse the aggregation of bacteria once it has adhered to the lumen. However, NDMs can be used as a prophylactic treatment to prevent gastritis and stomach ulcers.

Cranberry fractions can also act against oral bacteria. Bodet et al. reported that the non-dialyzable material obtained from cranberries has an anti-inflammatory effect on macrophages stimulated by LPS from periodontopathogens such as *Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia,* and *E. coli*[95]. The prevention of host inflammatory response by the use of NDMs can inhibit the local tissue destruction that is often seen in periodontitis[81]. Labrecque et al. also found that NDMs prevent the coaggregation of many oral bacteria and prevent the formation of streptococci biofilms[96]. Further, they found that cranberry NDMs inhibit the adhesion of *P. gingivalis* to type I collagen, fibrinogen and human serum. Significant inhibition was achieved when cranberry was used at a concentration of 62.5 μg/mL. Another group of researchers evaluated the anti-adhesive properties of a NDM-rich mouthwash[82]. Volunteers were asked to rinse twice a day with 15 mL of NDM-rich mouthwash for two weeks. Streptococci counts decreased dramatically after 14 days of rinsing and maximal inhibition of bacterial adhesion to oral surfaces was seen at 130 μg/mL of NDMs.

While cranberry constituents like NDMs and PACs do not inhibit the growth of bacteria, the inhibition of adhesion to different mammalian cells is an attractive target for the development of new therapies in the prevention of infections.

2.6 Microscopy Techniques

The work with bacteria relies on different microscopy techniques. To understand the effects that cranberry juice has on *E. coli* and how it can help prevent the recurrence of UTIs, we need to understand the physico-chemical changes that bacteria undergo when they are exposed to different cranberry compounds. This work relies on several microscopy techniques including light microscopy and AFM. In this section, a widely used staining method is discussed, as well as the fundamentals of AFM and how these two methods can offer valuable data in conjunction with other laboratory techniques.

2.6.1 Gram Staining

Gram staining is the most widely used taxonomic test of bacteria[97]. Decades ago, the detection of bacteria cells in human tissue was difficult since most of the staining techniques used would color both bacteria and mammalian cells equally[98]. It was not until late 1800s that Christian Gram developed the Gram-staining procedure that would later be used by almost every microbiologist to classify bacterial species[99]. Bacteria can be classified according to the colors they take during Gram-staining since they have different surface structures (Figure 2.8)[16].

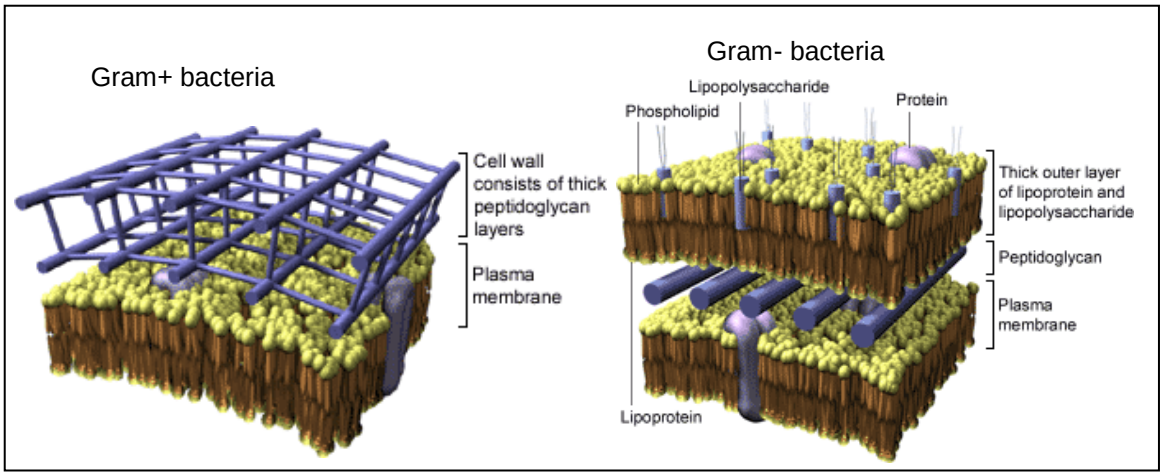

Figure 2.8: Gram classification in bacteria. Adapted from ASU[101]

Gram-positive bacteria have a very thick wall that consists primarily of several layers of peptidoglycan or murein, while the membrane of Gram-negative bacteria has only a few layers of murein, and an additional layer made of LPS [52,101]. When crystal-iodine is used to stain bacteria, an insoluble complex forms inside the cell that can be extracted by the use of organic compounds like alcohol and acetone. For Gram-positive bacteria the use of alcohol produces a dehydration of the cell where the pores of the walls close, retaining the crystal-iodine complex. In Gram-negative bacteria, the stain penetrates through the lipid as well as the peptidoglycan layer, but since this murein layer is so thin, the alcohol also goes through these layers and removes the stain [16]. Consequently, this laboratory staining technique is a very efficient method to differentiate bacterial cells. Gram-negative bacteria stain red after decolorizing and removing the crystal violet-iodine compound and using safranin as a counterstain, while Gram-positive bacteria remain purple.

While the Gram-staining method is the most reliable, user-friendly technique to classify unknown bacterial species, Gram variability can also be seen in some

bacteria[98,99]. Bacteria that are thought to be of the Gram-positive family can sometimes stain as Gram-negative bacteria (red or dark pink)[99]. This variability in Gram staining has been associated with incorrect use of the staining technique or with variability within the organism that is being studied[98]. Beveridge proposed that if Gram-positive bacteria grow in a rich environment, the organisms might not have enough time to produce a thick peptydoglycan layer that supports the fast grow and multiplication of bacterial cells[99]. This will then result in destruction of the cell wall since the bacteria cannot withstand the pressure. With a thin peptydoglycan layer, these bacterial cells would not be able to retain the crystal-violet dye and after decolorization they would stain red after safranin. One group reported the conversion of *E. coli* to the Gram-positive state by growth of bacteria in a medium containing maximum amounts of glucose, $MgSO_4$, or NaCl; although the researchers did not clarify the mechanisms by which bacteria underwent this Gram-staining change[102,103].

Variations in Gram staining can also be seen in bacteria if errors occur during the staining steps. For instance, during decolorization alcohol washes off any crystal-violet dye from Gram negative bacteria. However, if this compound is left on the slide too long, it could also decolorize Gram positive cells[98]. Bartholomew et al. also found that variations in concentration, temperature, and time of application of the reagents will influence the results of the staining method.

Although variation of Gram staining in bacteria is rarely seen, more cases have been found where Gram-positive bacteria can stain as Gram-negative cells[98]. The conversion of Gram-negative cells to the Gram-positive state is less common.

2.7 Atomic Force Microscopy

Biological, biomedical and natural sciences rely on the use of microscopes. After the introduction of light microscopy centuries ago, a more modern technology arrived with the development of the scanning electron microscope (SEM) in the 1940s, which uses electrons and electromagnetic lenses instead of light and glass lenses[104]. However, preparation of biological samples, such as bacteria for imaging using SEM requires the dehydration and coating of sample with gold, which most likely changes the conformation and physiology of the specimen. A new type of microscope arrived in 1986, when Binnig, Quate and Gerber demonstrated that an atomic force microscope (AFM) could not only explore the sample surface by mechanical scanning, but that the short range van der Waals interactions could be detected with this device[105,106]. AFM has been widely used to study the morphology of different biological specimens such as spores, fungi, mammalian cells, and bacteria, as well as the interactions between bacteria and different biomaterials[104]. AFM also provides us with high magnification and high resolution as well as the advantage of studying cells in their natural environment where minimal sample preparation is needed (without dyes, vacuum, or gold sputtering needed).

2.7.1 How AFM Works

Rather than "looking" at a surface through a lens, AFM visualizes a surface by "feeling" it with a sharp tip mounted on a long cantilever with a reflective coating. AFM has the ability to achieve atomic-level resolution of sample surfaces in their natural conditions (liquid or gaseous environments)[105]. The microscope is also capable of generating 3D images of samples ranging between 8-10 μm in height.

A sharp tip is positioned at the end of a cantilever beam (~100 μm) and acts as a probe. AFM tips are typically made from silicon or silicon-nitride where the radius of curvature measures between 1 to 10 nm. AFM tips can be rectangular or triangular (V-shaped) and are very hard and wear resistant[104]. The AFM tip is the heart of the instrument since it is what is brought into contact with the surface or specimen being studied. A sharp tip usually generates more detailed 3D images. The topography of the sample's surface is generated by recording the deflection of the cantilever as it scans the surface. Figure 2.9 shows a simplified schematic diagram of an AFM.

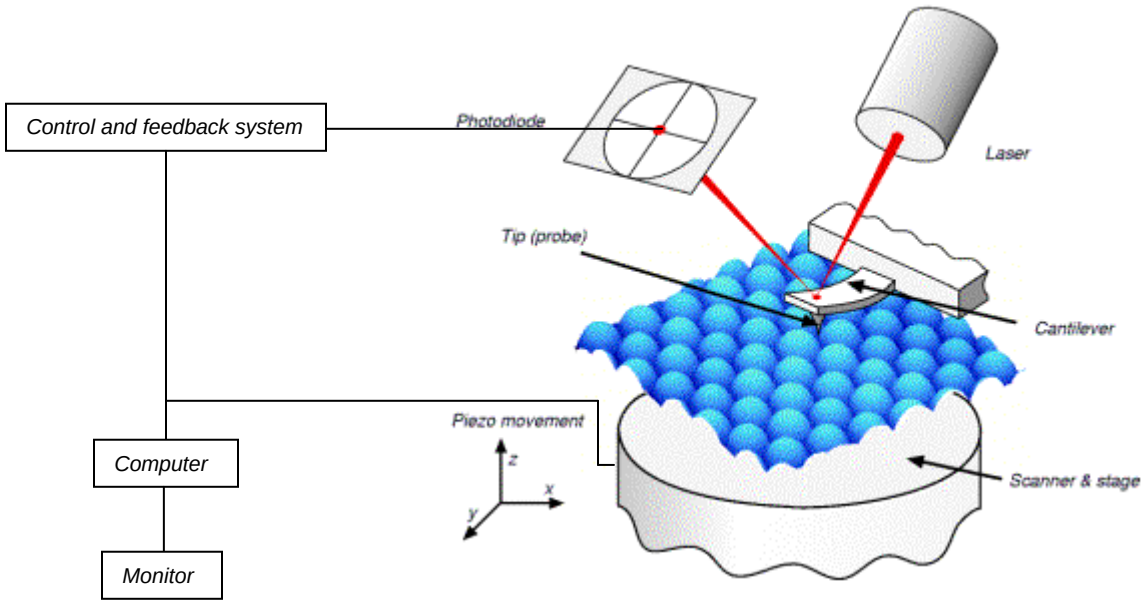

Figure 2.9: The atomic force microscope. Adapted from Uppsala U.[108]

As the sample is being scanned, the cantilever bends and the tip moves up and down, which causes a deflection of the laser that will change its position on the photodetector[104]. The computer system is equipped with a control feedback loop that

maintains the deflection of the cantilever constant. The computer then records the 3D position of the probe and uses this information to generate an image of the surface.

Cantilevers have a low spring constant, which enables the AFM to control small forces between the tip and the surface[105,108]. The spring constant is a measure of the force required to generate a given cantilever deflection (force per unit deflection, *N/m*). The spring constant value, k_z is usually specified by the manufacturer. However, several scientists have found a great discrepancy between the reported value and the actual one[109,110]. Calibration of the spring constant of cantilevers is an essential step to achieve accurate results when measuring the forces generated between the tip and the sample.

The scanner of the AFM is an important feature since it moves the tip over the sample. In modern AFMs the scanner is made from a piezoelectric tube and it is capable of extremely precise positioning[105]. The scanner sits on top of a stepper motor that is built into the base of the AFM. This motor is used by the computer to engage and withdraw the tip from the sample and it is also used by the AFM user to adjust the vertical position of the AFM tip.

There are two primary modes of operation on the AFM. The first mode known as DC mode or static mode records the static deflection of the cantilever as it scans a sample[111]. This mode is also called contact mode and it brings the tip in contact with the surface and the feedback loop keeps the cantilever deflection constant during scanning. While this is the simplest mode in the AFM, it could damage the surface of the specimen if the sample is too soft. For instance, contact mode could remove important structures on the surface of the spore *Bacillus atrophaeus* when imaging in air since considerable shear

forces were generated[112]. The second mode is the acoustic mode or AC mode and it requires setting the cantilever in oscillation by driving the cantilever with a piezoelectric motor[111]. This mode, also known as tapping or intermittent contact mode, provides higher vertical and lateral resolution of the sample since the cantilever is resonating and the tip of the AFM probe makes brief contacts with the sample[113]. The operation in tapping mode can also provide us with more detailed structures when operated in air. For instance, light tapping can offer a better resolution for imaging the surface coat of spores since the contact of the AFM probe with the sample is too brief to allow adhesive forces between the tip and the spore[112]. During tapping mode, the purpose of the feedback loop is to maintain constant cantilever oscillation amplitude.

2.7.2 Imaging and Measuring Adhesion Forces with AFM

The AFM has been widely used to obtain topographic images of a variety of biomedical materials, as well as mammalian cells, collagen fibers, bacteria, viruses, and DNA structures, among others. AFM can not only operate in air but also in liquid or fluid mode which allows scientists to study samples in their natural environment. This microscope can also obtain high resolution images (down to the nanometer range) depending on the operation mode and probe that are used. While a sample is being scanned, the AFM can capture image information on three channels simultaneously. Height data reflects the change in piezo height needed to keep the cantilever deflection constant, deflection data is obtained by the differential signal off the top and bottom photodiode segments, and amplitude data describes the surface topography by mapping changes in cantilever amplitude as the tip scans across the surface of the sample[114].

Along with images, the AFM is capable of measuring the interactions forces between the probe and the sample being studied. During force mode, the deflection of the cantilever is plotted as a function of the height of the sample (Figure 2.10)[115].

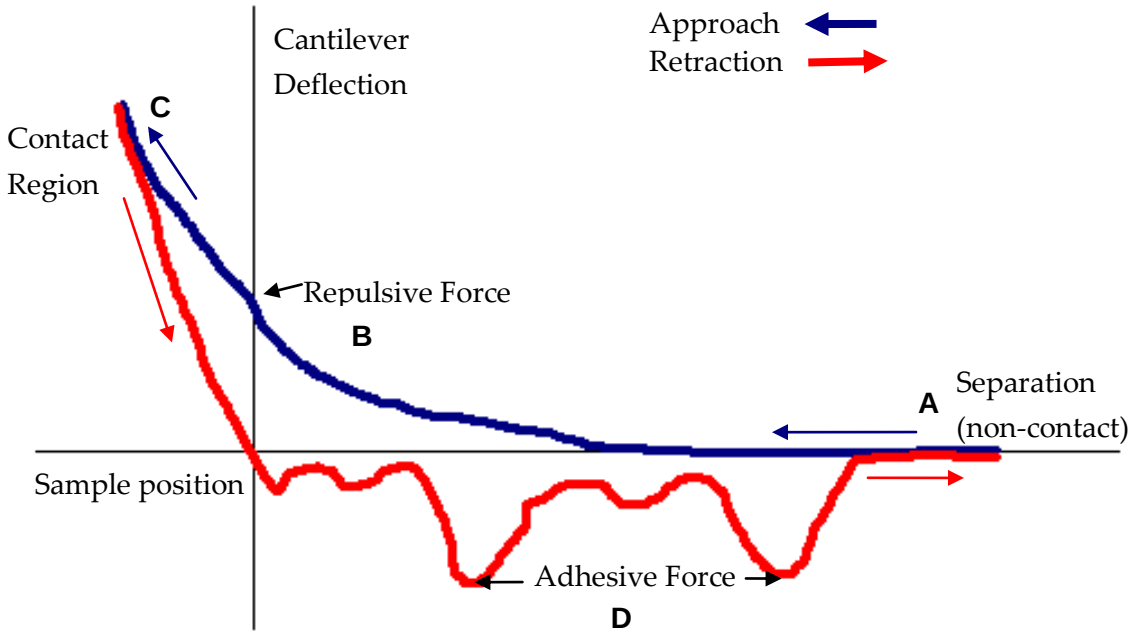

Figure 2.10: Typical force curve seen between a bare tip and bacteria in liquid

First, the tip approaches the surface (blue line at position A) and makes contact at position B. Depending on the sample being studied, attractive or repulsive forces can be seen in this region. After contact is made, the cantilever bends until it reaches the specified force limit that is to be applied (Position C). If the sample being studied is soft, the cantilever can start indenting the surface of the specimen. After contact, the tip is withdrawn until the tip is detached from the sample, and a sharp peak is seen in the retraction region (usually known as snap-off position; position D). If there is more than one polymer making contact with the AFM probe, then it is common to obtain multiple peaks in the retraction region. This indicates that multiple polymers are being detached

from the AFM probe as it retracts from the sample (position D). After detachment, the cantilever returns to resting position and the instrument is ready for another cycle (red line at position A)[111].

The instrument then plots a calibrated deflection curve for each cycle that will be later converted into a force curve using Hooke's law:

$$F = k_z x \tag{2}$$

where F is the interaction force in Newtons, k_z is the spring constant of the cantilever in Newtons/meter and x is the deflection of the cantilever (in meters). Hooke's law describes a linear relationship between force and deflection assuming thermal equilibrium.

The peaks observed in the retraction curve (during snap-off), can then be interpreted as the adhesion forces exerted by each molecule that attached to the tip during the first half of the cycle. Bacteria usually have more than one peak, since different molecules can bind to the probe.

Several biomedical tests have used the AFM to acquire valuable information on the mechanisms of adhesion of bacteria to different surfaces[1,85]. Interesting information can also be obtained by coating the tip with specific ligands or adhesins that will directly measure the adhesion forces between these molecules and receptors on different surfaces, such as mammalian cells[86].

The level of sophistication of biological AFM studies has significantly increased in the past couple of decades. A more detailed study of the biochemistry of surfaces and

the biological functioning of different cells can now be achieved through this instrument. The use of AFM can also aid in the development of new therapies for the prevention of bacterial infections. We utilize this instrument to understand how natural products such as cranberries can affect the adhesion of bacteria to uroepithelial cells and prevent UTIs.

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Chapter 3: Cell Attachment Bioassay

Effects of Cranberry Juice on *Escherichia coli* Attachment to Uroepithelial Cells and Biofilm Formation

3.1 Abstract

Cranberry juice has been used as a remedy to prevent urinary tract infections (UTIs). Development of bacterial biofilms in urethral catheters is a major concern in hospitalized patients. A bacteria-cell adhesion assay was used to investigate the effects of increasing concentrations of light cranberry juice cocktail (L-CJC) and A-type proanthocyanidins (PACs) on bacterial attachment using non-fimbriated *Escherichia coli* HB101 and the mutant, P-fimbriated *E. coli* HB101pDC1. Exposure to increasing concentrations of L-CJC resulted in a decrease of bacterial attachment to uroepithelial cells from 50.2 to 2.85 bacteria/cell for the P-fimbriated strain after L-CJC treatment (up to 27 wt.%), and from 50.2 to 10.6 bacteria/cell after PACs treatment (up to 345.8 μg/mL). We also tested the role of continuous growth in L-CJC or PACs on bacterial attachment. Following growth of HB101pDC1 in 10 wt.% L-CJC, the average number of bacteria/uroepithelial cell decreased from 50.2 to 7.9. Bacteria exposed to 10 wt.% L-CJC did not form a biofilm at any time period or culture, indicating that L-CJC can inhibit the formation of biofilms on polyvinyl chloride (PVC) substrates. Strain HB101pDC1 exposed to 128 μg/mL PACs formed a biofilm within 24 hr after the 1st culture in PACs. However, by the 12th culture, no biofilm formation was observed. L-CJC had a greater ability to prevent bacterial adhesion and biofilm formation than isolated PACs. The results of this research suggest that exposure of *E. coli* to L-CJC prevents their ability to

adhere to uroepithelial cells, and growth in L-CJC-containing media prevents their ability to form biofilms.

Keywords: Proanthocyanidins, urinary tract infections, bacterial attachment

3.2 Introduction

One of the most prevalent infections encountered by healthcare professionals is caused by the presence of bacteria in the urinary tract[1]. The Gram-negative bacteria *Escherichia coli* predominate as the most common urinary pathogen accounting for more than 80% of community-acquired UTIs[2,3]. Formation of *E. coli* biofilms in urethral and ureteral catheters is also a concern in the medical field. Once a urethral catheter is in place, the incidence of bacteriuria increases by 3-10%[4], since most bacterial species that enter the catheterized urinary tract can multiply to high concentrations in a short period of time. The rapid increase in bacterial population is a leading risk factor for blood infections[5].

Although planktonic bacteria present in the urinary tract are usually treated with common antimicrobials and analgesics[6,7], concerns about antibiotic resistance, side effects, and recurrence of UTIs in susceptible populations have led to increased interest in using other remedies to prevent bacterial infections. Bacteria present in a biofilm, such as on a urinary catheter, can be particularly difficult to treat since the bacteria are enclosed in a hydrated matrix of proteins and exopolysaccharides that act as a defense mechanism against antibiotic chemotherapies[8].

UTI prevention through the use of the North American Cranberry (*Vaccinium macrocarpon* Ait., Ericaceae) is a subject of great interest[9,10]. The main mechanism by

which cranberry can be used to prevent UTIs is believed to be by hindering the ability of pathogenic bacteria to attach to the urinary tract[10]. A similar effect has been proposed for other infections that rely on bacterial adhesion to host tissue, such as the use of cranberry to prevent *Helicobacter pylori* infections in gastric lumen[11-13], and inhibition of biofilm formation on tooth-supporting tissues and periodontal ligaments[14-19].

Uropathogenic *E. coli* contain several virulence factors that contribute to pathogenesis[20,21]. Researchers have paid special attention to P-fimbriae since these surface proteins contain class II PapG adhesins that bind to digalactoside αGal(1→4)βGal oligosaccharide receptors on the surface of uroepithelial cells[1,22-26]. This binding step is the first phase in the development of an infection.

Several *in vitro* studies have focused on the effect that cranberries have on this initial adhesion step[27,28]. Two classes of compounds have been isolated and characterized for their anti-bacterial adhesion activity including A-type proanthocyanidins (PACs[27-31]) and a high molecular weight non-dialyzable material (NDM[13,32,33]). For instance, cranberry juice at various concentrations inhibited the adherence of more than 60% of *E. coli* clinical strains from patients with UTIs[34]. While preincubation of bacteria with cranberry juice showed signs of anti-adherence activity *in vitro*, this inhibitory action was lost after two washes in buffer solutions[34]. This was well correlated with clinical studies, where the withdrawal of cranberry juice from patients resulted in the recurrence of UTIs[35].

Relatively few studies have addressed the effects of cranberry juice products on the growth of *E. coli*[34,36-38]. Changes in *E. coli* morphology due to cranberry juice have

been studied[36]. *E. coli* JR1 and DS17 grown in agar plates supplemented with 25% cranberry juice revealed cellular elongation and loss of fimbriae, which caused a decrease in bacterial agglutination to P-receptor specific latex beads. Continuous plating of *E. coli* JR1 on cranberry rich agar plates increased the inability of bacteria to agglutinate to latex beads, and washing of bacteria with buffer solutions did not return agglutination ability. Cranberry juice also affects the growth rate of *E. coli.* After growing bacteria in the presence of 33% freshly prepared cranberry juice with adjusted pH, the number of colony forming units (CFUs) decreased ~50% after 24 hrs of incubation[34]. All these studies were done by incubating bacteria in cranberry products for a short period of time (less than 48 hrs).

While many studies have attempted to understand the mechanisms of cranberry juice and its inhibition on bacterial adhesion to prevent UTIs, no studies have investigated the effect that cranberry products, such as L-CJC and PACs have on prolonged and continuous growth of *E. coli.* For patients with UTIs, long term consumption of CJC will result in the continuous growth of bacteria in a CJC or PACs rich environment. *In vitro* experiments will help us simulate the physiological conditions of these patients when they are consuming cranberry products. Further, no other research has evaluated how the culture of bacteria in the presence of L-CJC and PACs affects the adhesion of bacteria to uroepithelial cells and formation of biofilms in catheter biomaterials, such as polyvinyl chloride (PVC).

In the present study, we evaluated the efficacy of prolonged exposure and growth of *E. coli* HB101 and P-fimbriated *E. coli* HB101pDC1 to cranberry products on bacterial adhesion to human kidney cells and formation of biofilms on PVC as the substrate. Our

findings suggest that low concentrations of PACs and L-CJC block adhesin-receptor sites, thus inhibiting bacterial attachment. In addition, growth of bacteria in cranberry products inhibits biofilm formation in catheter materials, such as PVC.

3.3 Materials and Methods

3.3.1 Cranberry Juice Treatment and Proanthocyanidins

Ocean Spray light cranberry juice™ cocktail (referred to hereafter as "L-CJC") was purchased (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA). The pH of cranberry juice was adjusted to 7.0 using sodium hydroxide (Sigma-Aldrich, St. Louis, MO) and the solution was passed through a 0.2 μm Acrodisc® syringe filter for further purification. L-CJC concentrations of 0, 5, 10 and 27 wt.% were used based on the fact that consumer-grade cranberry juice contains no more than 27% juice.

Proanthocyanidins (PACs) were kindly provided by Ms. Robin Roderick (Ocean Spray Cranberries, Inc; Lakeville-Middleboro, MA). PACs stock solution was prepared by diluting 83 mg of PAC fractions in 240 mL of ultrapure water (Milli-Q water, Millipore Corp.; Bedford, MA), in accordance to Howell et al[28]. The mixture was shaken overnight or until all particles were completely dissolved. In commercially available CJC, there are 345.8 μg/mL of PACs with at least one A-type linkage, corresponding to 27% cranberry juice[28]. According to this concentration, 5, and 10 wt.% cranberry juice contains 64 μg/mL and 128 μg/mL PACs. The pH of the different PACs solutions was not adjusted since the values were very close to neutral conditions.

3.3.2 Bacterial Cultures

E. coli HB101 was obtained from the American Tissue Culture Collection (ATCC 33694). *E. coli* HB101 is a plasmidless bacterium lacking fimbriae[39]. *E. coli* HB101pDC1 is a mutant of the HB101 strain that expresses only P-fimbriae due to insertion of a chloramphenicol resistant plasmid[40]. The mutant strain was kindly provided by Dr. M. Svensson from the Department of Medical Microbiology, Lund University.

Bacterial cultures were grown at 37 ºC in 30 g/L tryptic soy broth (TSB; Sigma, St. Louis, MO); and supplemented with 0 wt.% or 10 wt. % neutralized L-CJC or 0, 128 μg/mL PACs. The mutant *E. coli* HB101pDC1 was grown in the presence of a final concentration of 20 μg/mL chloramphenicol (Sigma, St. Louis, MO). Growth rates and doubling times were analyzed by measuring the optical density of the solution at 600 nm (Thermo Spectronic, Rochester, NY). In accordance with Svanborg-Eden et al., we harvested *E. coli* at the end of the exponential growth phase (A_{600nm} = 0.9) for all of our experiments, since maximal adhesive activity is seen at this stage of growth[41], presumably because it is the phase where *E. coli* are at their healthiest state and the PapG adhesins are fully expressed.

After harvesting, 1 mL of bacteria solution was transferred to a new flask with fresh media to start the second culture in the cranberry rich environment. Twelve successive cultures of bacteria in TSB and L-CJC or PACs were done per strain. The 1st, 4th, 8th, and 12th bacterial cultures grown in L-CJC or PACs, or bacteria grown in pure TSB were centrifuged for 10 min at 4000 RPM and washed three times with 0.01 M PBS (pH 7.4, 0.138 M NaCl, 0.0027 M KCl; Sigma, St. Louis, MO) to remove all the components of the growth media[42].

3.3.3 Uroepithelial Cell Culture

Human kidney uroepithelial cells were purchased from ATCC (CRL 9520 VA) and maintained in liquid nitrogen vapor phase. The cells were grown in Kaighn's modification of Ham's F12 medium and supplemented with 10% fetal bovine serum (FBS; ATCC Manassas, VA). Culture flasks were placed in a 5% CO_2 in air atmosphere incubator at 37ºC for 7 days and the media was replaced every two days. Uroepithelial cells were harvested by adding 0.25% (w/v) Trypsin- 0.03% (w/v) EDTA (Sigma-Aldrich Co., MO) to detach the cells from the culture flasks. The cells were kept in Trypsin-EDTA for 10 min at 37 ºC and centrifuged at 800 RPM. After centrifugation the cells were resuspended in 0, 5, 10 and 27 wt. % L-CJC or 0, 64, 128 and 345.8 µg/mL PACs to determine the effect of pretreatment of uroepithelial cells with cranberry constituents on bacterial adherence.

3.3.4 Bacterial Attachment to Uroepithelial Cells: Increasing Concentration Assay

The effect of L-CJC and PACs on bacterial attachment was determined through phase contrast microscopy. Quantitative assessment of bacterial adhesion to uroepithelial cells was determined as described previously[25,41,43,44]. Briefly, bacteria cultured in pure TSB (no cranberry in the media) were suspended in aqueous solutions, diluted in PBS, containing 0, 5, 10 and 27 wt. % L-CJC or 0, 64, 128 and 345.8 µg/mL PACs for 3 hours at 37ºC with slow shaking. Uroepithelial cells were incubated with equal volumes of the different concentrations of L-CJC or PACs for 3 hours at 37ºC. After cranberry treatment, bacteria suspensions of 1 x 10^9 CFU/mL and uroepithelial cells suspensions of 1 x 10^6 cells/mL were placed in tissue culture flasks and incubated for 90 min at 37ºC with

rotation at 18 RPM. After incubation, loosely attached bacteria were removed by gentle centrifugation (100xg) for 10 min and resuspended in 0.01 M PBS. Previous research has shown that through this attachment assay and epithelial cell preparation the viability of the cells remains and is not affected by incubation in PBS[41,44]. Researchers have used stains such as trypan blue, ethidium bromide and acridine orange to assess if uroepithelial cells are still viable after incubation and it was determined that more than 80% of the epithelial cells remain viable[41,44].

Assessment of the concentration of bacteria and uroepithelial cells were accomplished using a counting chamber (Zander Medical Supplies, Vero Beach, FL). Following incubation and washing, wet mounts were prepared by placing 50 μL of cellular solution onto cleaned glass slides covered with coverslips. The number of bacteria attached to a single uroepithelial cell was quantified at a magnification of 1000x under oil immersion using a Nikon Eclipse E400 microscope operating in phase contrast mode (Tokyo, Japan) All images were collected using a camera and stored with SPOT 4.6 advance software (Diagnostic Instruments, MI). Firmly attached bacteria or bacteria that were found on the surface of mammalian cells (after washing step) were counted for 20 uroepithelial cells per sample condition.

Bacteria were only counted for isolated uroepithelial cells. If there was an aggregation of mammalian cells with a confluent layer of bacteria in between, then these cells were ignored and single uroepithelial cells were located to avoid any errors during counting.

3.3.5 Bacterial Attachment to Uroepithelial Cells: Increasing Number of Cultures in Cranberry Constituents

In order to study the effects of continuous and prolonged exposure of cranberry juice and PACs on bacterial attachment, both strains of *E. coli* were cultured in the presence of 10 wt. % L-CJC or PACs. Twelve consecutive cultures were obtained for bacteria incubated in cranberry rich media for ~10 days. The 1st, 4th, 8th, and 12th cultures of both strains in cranberry rich media were harvested in the high exponential growth phase and washed three times in PBS. Uroepithelial cells were not exposed to any cranberry product before attachment assays. Following washing, uroepithelial cells and bacteria were placed in warm PBS and incubated for 90 min at 37ºC with rotation at 18 RPM. After incubation, both uroepithelial cells and bacteria were washed with 0.01 M PBS to remove loosely attached bacteria. The number of bacteria attached to single uroepithelial cells was quantified through phase contrast microscopy, as described in the previous section. A total of 20 uroepithelial cells were studied for each condition.

3.3.6 Microtiter Plate Biofilm Production Assay

To study the effects that cranberry products have on formation of biofilms, we used a simple procedure developed by O'Toole et al[45]. Briefly, *E. coli* HB101 and HB101pDC1 were grown at 37ºC in 25g/L Luria Bertani broth (LB; Sigma, St. Louis, MO); and supplemented with 10 wt.% L-CJC or 128μg/mL PACs solution. After growth, bacteria were diluted in a 1:1 ratio with fresh LB and cranberry rich media, and 150 μL of cultured bacteria were dispensed into wells of a 96 well microtiter plate made of polyvinyl chloride (PVC; BD Biosciences, San Jose, CA), previously rinsed with 70% ethanol and air dried. Each well was inoculated with approximately 60 million bacterial

cells. The plates were then incubated at 37ºC without agitation for 0, 3, 6, 24, 30, and 48 hours. At each time interval, the formation of biofilms was detected by staining the wells with 20 μL of a 0.3% (w/v) crystal-violet solution in water for 15 minutes. After staining, wells were washed with ultrapure water three times to wash off loosely attached bacteria and residual dye. At this point, biofilms could be visible as dark purple rings formed on the side of the PVC well.

For direct observation of the biofilms by phase contrast microscopy, a small tab of PVC plastic was cut from a PVC microtiter dish (~4 mm^2) and sterilized with 100% ethanol solution and exposure to UV light. The plastic tab was placed in a well with 150 μL of freshly inoculated bacterial solution and incubated for 0 or 30 hrs at 37ºC. After incubation, the tab was stained with crystal-violet and rinsed with ultrapure water to remove all planktonic cells. The PVC plastic was then mounted on a microscope slide and observed under oil immersion at a magnification of 1000x.

To quantify the formation of biofilms, 200 μL of a 4:1 ethanol and acetone solution was dispensed to each well to detach the bacteria and destain the wells. The ethanol-acetone solution was left in the well for 1 minute and the solution was placed in a polystyrene disposable cuvette (BrandTech Scientific, Essex, CT). The destaining step was repeated and 600 μL of ultrapure water was added to the cuvette. The stage of biofilm formation was then quantified by monitoring the cell turbidity using a spectrophotometer (Thermo Spectronic, Rochester, NY) at an optical density of 600 nm. A mature biofilm was characterized by a crystal-violet-bacteria absorbance of 0.7-0.9 (A_{600nm}). Formation of biofilms was investigated for the 1^{st}, 4^{th}, 8^{th} and 12^{th} cultures of

both bacterial strains grown in L-CJC or PACs, with bacteria cultured in pure LB (no cranberry treatment) serving as the negative control.

3.3.7 Statistical Analysis

Data are expressed as the means ± the standard deviation (SD). Data were analyzed using SAS® and SigmaStat 2.03 statistical software. Statistical analysis was performed by two-way analysis of variance (ANOVA) for repeated measurements. Tukey's test was used for multiple comparisons among each treatment group, while Dunnett and Duncan's test were used for comparisons between treatment and control groups. A difference was considered significant if $P < 0.05$.

3.4 Results

3.4.1 Effects of Increasing Concentrations of Cranberry Constituents on Bacterial attachment to Uroepithelial Cells

E. coli HB101pDC1 had a high affinity to uroepithelial cells in the absence of cranberry treatment (Figure 3.1A). Bacteria attached to uroepithelial cells had a tendency to aggregate. After bacteria were exposed to increasing concentrations of cranberry juice, the number of attached bacteria decreased significantly (Figure 3.1B and 3.1C).

The number of bacteria that adhered to each uroepithelial cell was obtained for all treatment conditions after exposure to different concentrations of L-CJC and PACs (Figure 3.2A and 3.2B). Exposure of *E. coli* HB101pDC1 to increasing concentrations of L-CJC resulted in a statistically significant decrease in the number of attached bacteria/cell when compared to our control sample, according to Dunnett's and Duncan's tests ($P<0.001$). The average number of *E. coli* HB101pDC1 attached to a single

uroepithelial cell was 50.2 ± 22.9 when the cells were not exposed to any cranberry product. After exposure of bacteria and uroepithelial cells to 5 wt.% L-CJC, the number of attached bacteria per uroepithelial cell decreased to 13.6 ± 5.7. Treatment with L-CJC at 10 and 27 wt.% resulted in further decreases in attachment, to 9.3 ± 4.1 and 2.9 ± 1.5 bacteria/cell, respectively.

Exposure to isolated PACs also caused a decrease in bacterial attachment to uroepithelial cells (Figure 3.2A). For P-fimbriated *E. coli* HB101pDC1, there was a decrease in attachment from 50.2 ± 22.9 bacteria/cell, for our control condition, to 32.5 ± 13.9 bacteria/cell after exposure to 64 μg/mL PACs. *E. coli* HB101pDC1 exposed to higher concentrations of PACs (128 μg/mL and 345.8 μg/mL) resulted in a further decrease in attachment, with 28.9 ± 12.2 and 10.6 ± 2.4 attached bacteria/cell, respectively.

Using Tukey's test to compare the statistical significance of the different concentrations of L-CJC used to treat bacteria and uroepithelial cells, we found a significant difference between *E. coli* HB101pDC1 treated with 5 wt.% and 27 wt. % L-CJC (P=0.001) but no statistical difference was found between the mean numbers of bacteria treated with 5 wt.% and 10 wt.%, or between 10 wt.% and 27 wt.% cranberry juice (P=0.436 and P=0.109, respectively).

Different concentrations of PACs to treat bacteria were also compared using Tukey's test. All concentrations resulted in statistically significant differences among the mean numbers of bacteria attached, with the exception of 5 wt. % versus 10 wt. % PACs (P=0.734). Dunnett's and Duncan's tests showed significant differences between our

control group (pure TSB) and each PAC concentration for attachment of strain HB101pDC1 ($P<0.001$).

The attachment of *E. coli* HB101 to uroepithelial cells was not significantly different for any of the L-CJC or PAC treatments or for the control ($P>0.2$; Figure 3.2B). A first-order exponential decay function was used to correlate L-CJC or PACs concentration and the number of bacteria attached/uroepithelial cell, since a nonlinear relationship was observed. From fitting the data to an exponential relationship, Figure 3.2A, we found that the minimum number of *E. coli* HB101pDC1 that can attach to a single uroepithelial cell are 3 bacteria, regardless of the concentration of L-CJC used.

3.4.2 Bacterial attachment to Uroepithelial Cells After Continuous Incubation of Bacteria in Cranberry Products

E. coli HB101 and HB101pDC1 were grown in the presence of 10 wt.% L-CJC or 128 μg/mL PACs for up to 12 times (~10 days). The concentration of 128 μg/mL PACs is the equivalent amount of A-type PACs in 10 wt.% L-CJC.

The role of continuous growth of *E. coli* in L-CJC or PACs on bacterial attachment was evaluated. In the absence of cranberry in growth media, large numbers of *E. coli* HB101pDC1 bacteria attached to uroepithelial cells. The number of attached bacteria decreased after one time growth in L-CJC or PACs (Figure 3.3A).

E. coli HB101pDC1 cultured in pure TSB attached readily to uroepithelial cells. Culturing of the same strain in the presence of L-CJC or isolated PACs resulted in a significant decrease in attachment ($P<0.001$). There were no significant differences among the means of the different L-CJC or PAC treatments according to Tukey's test

(P>0.9). As expected, culturing the non-fimbriated *E. coli* HB101 in L-CJC or PACs did not result in significant differences in bacterial attachment (Figure 3.3B).

3.4.3 Effects of Cranberry Products on *E. coli* Biofilm Formation

E. coli HB101 and HB101pDC1 had the ability to form biofilms on a PVC substrate. Formation of biofilms was determined using a standard method[45,46], adapted to evaluate the effects of continuous exposure to cranberry products on the formation of *E. coli* biofilms after 0, 3, 6, 24, 30, and 48 hrs of incubation at 37ºC. Planktonic cells could be observed at 0 hrs of incubation through light microscopy (Figure 3.7A; see Supplementary Information). A thick biofilm of *E. coli* HB101pDC1 formed on the abiotic PVC substrate at 24 hrs of incubation. This monolayer of bacteria became very dense with increasing times of incubation; by 30 hrs microcolonies could be observed and complete maturation of the biofilm was achieved (Figure 3.7B; see Supplementary Information).

The formation of biofilms on PVC 96-well plates were also easily visible after staining with a crystal-violet dye (Figure 3.4). A dark purple ring was seen at the interface between the air and the medium after 24 hrs of incubation for strains with no L-CJC treatment. Biofilms only formed where oxygen levels were highest; therefore, little to no biofilm formation was seen at the bottom of the well. For all the biofilm forming bacteria, we noticed that after 48 hrs of incubation the ring of stained bacteria was lighter than at 30 hrs (Figure 3.4). O'Toole et al. have suggested that this change in staining intensity might be due to bacteria transitioning from biofilm back to planktonic state[46], which could be caused by changes in nutrition in the surrounding environment.

Biofilm formation was completely inhibited after culturing of bacteria in the presence of 10 wt.% L-CJC for our P-fimbriated strain as well as strain HB101 (Figure 3.5). According to Tukey's test, no significant relationship was found between the mean absorbance values and time of culture and incubation in L-CJC ($P>0.8$). However, there was a statistically significant difference between the absorbance values of *E. coli* HB101pDC1 cranberry treated bacteria and our control group after 24 and 30 hrs of incubation as evaluated using Dunnett's and Duncan's test ($P<0.001$).

3.4.4 A-type Proanthocyanidins and Biofilm Formation

Development of *E. coli* HB101pDC1 biofilms varied between the different culture times in the presence of 128 μg/mL PACs (Figure 3.6A). There were significant differences in absorbance values between our control group and all the cultures of bacteria in PACs for times 0, 3, and 6 hrs of incubation, according to Dunnett's and Duncan's test ($P<0.001$). Biofilms started developing after 24 hrs of incubation for most of the PAC treated bacteria (Figure 3.6A). From Dunnett's and Duncan's test, we observed that there was a significant difference in biofilm formation between our control group and cultures 4, 8 and 12 after 30 hrs of incubation, but not between our control group and culture 1. This suggests that *E. coli* HB101pDC1 have to be grown in the presence of 128 μg/mL PACs for more than four cultures to be able to reduce the ability to form biofilms. Complete inhibition of biofilm formation was observed after 12 cultures of *E. coli* HB101pDC1 in the presence of PACs.

E. coli HB101 cultured in the presence of PACs did not form a biofilm at any time or culture (Figure 3.6B). However, as with our P-fimbriated strain, absorbance

values were higher for our PACs treated bacteria than for our control group for the 0, 3, and 6 hr incubation times.

Formation of *E. coli* HB101 and HB101pDC1 biofilms was also tested by growth of bacteria in a 46.3 μg/mL PACs-rich environment. *E. coli* HB101pDC1 formed biofilms after growth in PACs (data not shown). However, this ability went away after 12 consecutive cultures in PACs. While both concentration of PACs (46.3 μg/mL and 128 μg/mL) resulted in similar results and abilities to form biofilms, we observed that higher absorbance values and denser biofilms were obtained with bacteria grown in 46.3 μg/mL PACs.

3.5 Discussion

3.5.1 Use of NaOH to Neutralize Cranberry Juice

The human body is constantly neutralizing acids that enter through the digestive system. Several sources are used to accomplish this, such as mineral ions that act as buffering agents to neutralize any acidic compound such as the benzoic acid found in cranberries. Some of these minerals include sodium, potassium and calcium, which are also present in this fruit[47].

Because L-CJC has a low pH of ~2.5, and thus could partially inhibit the growth of the test bacteria, it was decided to adjust the pH of L-CJC to 7.0 for optimal growth of *E. coli* in the new media. Using NaOH to increase the pH of cranberry juice can decrease the concentration of free benzoic acid in cranberries[48]. However, changes in pH do not affect other fruit components, such as PACs[49]. Andry et al. has studied the effects of pH on PACs, and he determined that increasing the pH of grape PACs resulted in an

intensified acylation of OH groups, which gave strong PAC microcapsule membranes with high stability[49]. Furthermore, infrared spectroscopy revealed that changes in pH did not affect the spectra of PACs since formation of esters from hydroxyl groups were seen for different pH values. These results also indicated that the antioxidant activities of proanthocyanidins are not damaged by changes in pH.

3.5.2 Limitations of Attachment Assay

The epithelium lining of the urinary tract consists of multiple cell layers including a basal layer, an intermediate layer, and a highly differentiated superficial layer of cells, also known as "umbrella" cells[50]. The umbrella cell layer is covered by a layer of glycosaminoglycans (GAGs) that acts as a defense mechanism against bacterial adherence and development of UTIs[51]. The presence of uropathogens in the urinary tract could result in damage to this protective layer since bacteria cause an inflammatory response that can affect the GAG layer[51]. The assemblage of these multiple layers provide uroepithelium with high transepithelial resistance, low permeability to small solutes, sodium transport and a stretch response[52]. Current research is taking place to develop an *in vitro* model of the epithelium lining of the urinary tract that has the same tissue characteristics as bladder epithelium *in vivo*[50,52].

Bacterial attachment to epithelial cells is often studied when cells are in suspension[27,44,53]. While these conditions do not simulate the native physiology of uroepithelium, it can offer valuable information and facilitate the observation of individual uroepithelial cells and the interaction of single bacteria with each cell under an optical microscope. Viable cells in suspension are often collected from urine[41]. While

these cells do not have the same structural properties as epithelial cells in a confluent layer, they still have their receptors present as previously seen in different studies[28,41].

Throughout our attachment studies we found that even though the epithelial cells used were in suspension, there is a change in attachment after cranberry treatment. Furthermore, for our control condition of P-fimbriated bacteria grown in pure TSB, we observed that many bacteria were able to strongly attach to epithelial cells and that this attachment process was not affected by cell washing. This helped us corroborate that the receptors on uroepithelial cells are still present when these cells were in suspension; however, complete functionality of epithelial cells may be affected and needs to be addressed, as well as viability of uroepithelial cells after cranberry treatment.

Research needs to take place in order to evaluate bacterial attachment to uroepithelial cells when they are in a confluent layer that could simulate the physiology of uroepithelium. It is essential to develop a model where all the multiple layers of epithelial cells are present that have the same functionality as *in vivo* conditions.

3.5.3 Correlating Concentration of Cranberry Products and Attachment of Bacteria to Uroepithelial cells

We investigated the ability of two cranberry products, including light cranberry juice cocktail and isolated A-type proanthocynidin compounds, to inhibit the attachment of non-fimbriated and P-fimbriated *E. coli* to uroepithelial cells. Recent studies have identified compounds in cranberries, such as fructose, that are sensitive to different types of fimbriae[38]. *E. coli* expressing type-1 fimbriae have long been recognized as sensitive organisms in the presence of D-mannose or fructose[53,54]. Recent studies have also

identified PACs as a component in cranberries that inhibit the adhesion of P-fimbriated bacteria to mannose-resistant human red blood cells[28]. Our research is the first to demonstrate the anti-adherence property of PACs with respect to living human uroepithelial cells, rather than red blood cells or epithelial cells that are usually collected from urine.

Exposure of bacteria to increasing concentrations of L-CJC resulted in a decrease of bacterial attachment. Exposing bacteria and uroepithelial cells to a 5 wt.% cranberry juice concentration resulted in a ~75% decrease of bacterial attachment. The molecular mechanism for this effect was developed in a previous study in our laboratory, where Liu et al. found through atomic force microscopy, that exposing bacteria to the same concentration of CJC resulted in an ~80% decrease in adhesion forces between P-fimbriated *E. coli* and uroepithelial cells, as well as a decrease in the length of P-fimbriae[10].

Bacterial attachment also decreases with increasing concentrations of isolated PACs. Our findings indicated that exposing bacteria and epithelial cells to 64 μg/mL PACs resulted in a significant decrease in bacterial attachment. This correlated well with previous studies. For example, Howell et al. reported a bioactivity detection threshold of 60 μg/mL PACs to inhibit the agglutination of P-fimbriated bacteria to human red blood cells[28]. It also appears that our study was different for detecting inhibition of bacterial attachment since there was a 78% decrease in bacterial adhesion after exposure of *E. coli* to 345.8 μg/mL PACs for 3 hrs compared to 4-6 hrs of exposure of PACs to epithelial cells in urine[28]. Our study suggests that higher concentration of PACs results in less time to inhibit bacterial attachment.

There was a higher response of inhibition of bacterial attachment when the uroepithelial cells and the P-fimbriated bacteria were exposed to cranberry juice compared to PACs. While exposure of bacteria to a low dose of 64 μg/mL PACs resulted in a 35% decrease in bacterial attachment, exposure to a 5 wt.% cranberry juice resulted in a ~75% decrease in adhesion. Similar results were obtained with higher concentrations of L-CJC and PACs.

Isolated PACs might need other compounds in CJC to have a more inhibitory effect on bacterial adhesion. In our laboratory we measured the zeta potentials of *E. coli* HB101 and HB101pDC1 that were exposed to different concentration of CJC or isolated PACs[55]. We found that compounds in CJC protect the PACs more than the isolated fractions since bacteria exposed to increasing concentrations of CJC did not show significant changes in their zeta potentials. On the other hand, exposure of bacteria to isolated PACs resulted in a significant decrease of the bacterial zeta potentials. However, the physical or chemical adsorption of PACs onto bacterial surfaces was found to be reversible since washing of PACs-treated bacteria resulted in a restoration of cell surface charge.

The difference in bacterial attachment when comparing L-CJC and PACs also suggests that there may be other active compounds in cranberry juice that inhibit the adhesion of bacteria to uroepithelial cells. A high molecular weight non-dializable material (NDM) has also been isolated from CJC and demonstrated bacterial anti-adhesive activity[13,38]. While most of the research has focused on the effects of NDMs on the inhibition of bacteria to gastric mucus[11,12], and to oral surfaces[15,19], a few studies have investigated the effects of NDMs on uropathogenic bacteria[56,38,57]. Our study supports the

hypothesis that a combination of different active compounds, such as NDMs and PACs help reduce the attachment of bacteria to uroepithelial cells, since lower numbers of bacteria were attached to uroepithelial cells when they were exposed to L-CJC than to PACs.

3.5.4 Correlating Growth in Cranberry Products and Attachment of Bacteria to Uroepithelial cells

Most studies in this field evaluate the effects of short term exposure of bacteria to cranberry constituents on attachment to epithelial cells or human red blood cells[27,37,58]. To our knowledge, this is the first study that investigated the effects that long-term growth of *E. coli* in the presence of L-CJC and PACs had on adhesive activity to uroepithelial cells. These *in vitro* experiments were aimed to simulate the physiological conditions of patients that have been involved in clinical trials where they have consumed cranberry products for long periods of time[9,59]. Avorn et al. conducted a placebo-controlled, double blinded study in which patients with recurrent UTIs consumed 300 mL of cranberry juice for 6 months[59]. During this long term exposure to cranberry juice, bacteria in the urinary tract can grow and multiply in a cranberry or PACs-rich environment. During *in vivo* experiments there are many unknown and uncontrollable factors that can hamper us from understanding the mechanisms of cranberry juice in inhibiting bacterial adhesion. Thus we designed these *in vitro* experiments to allow bacteria to grow in the culture medium with L-CJC or PACs to obtain better understanding of changes in bacterial attachment ability after undergoing repeated culture in cranberry.

Our studies showed that growing *E. coli* HB101pDC1 for 6 hrs in the presence of 10 wt.% CJC (1st culture), resulted in an 84% decrease in attachment of bacteria to

uroepithelial cells. These results correlated well with Ahuja et al.'s findings, since they found that plating and growth of *E. coli* JR1 in cranberry rich agar resulted in an inability to agglutinate P-receptor specific beads[36], which was attributed to ~90% of bacteria having absent or non-functional P-fimbriae. Growth of *E. coli* in the presence of 128 μg/mL PACs resulted in similar inhibition of bacterial adherence. The results of increasing concentrations of L-CJC or PACs and increasing number of cultures in the presence of these products revealed nonlinear relationships. While Gupta et al. described the effects of PACs on bacterial adhesion as a linear dose-dependence function[27], we found a exponential decay relation for all our results. This exponential decay function has been seen in our prior studies[60,61]. While we do not fully understand why this exponential relationship is so strong, we believe that there is a dose threshold needed to inhibit bacterial attachment to uroepithelial cells. However, after this threshold is achieved, the effects of inhibition of bacterial adherence are reduced with further increments of concentration of cranberry products, or increments in the number of cultures.

The results of our research then suggest that continuous consumption of commercial cranberry juice will result in an inability of bacteria to adhere to uroepithelial cells since bacteria would be growing in a CJC or PACs-rich environment. We can also hypothesize based on our results that consuming increasing concentrations of CJC will also hinder bacterial attachment; therefore, reducing the chances of developing a UTI.

3.5.5 Correlating Growth in Cranberry Products and Development of Biofilms

To our knowledge, this is also the first study that investigated the effects of prolonged bacterial growth in cranberry products on biofilm formation. Since it has been previously reported that biofilm formation is a response to different environmental

signals, such as starvation[62] and that biofilm development can be hindered by growth of bacteria in rich media[45], we utilized LB which contains only one carbon source. PVC was chosen as the test substrate since this is a biomaterial that is widely used in the manufacturing of urinary catheters; however, the development of biofilms and UTIs due to insertion of this device is almost inevitable where bacteriuria often occurs[63]. PVC catheters are often used since they are cheaper than other coated catheters and can be hydrophilic or non-hydrophilic, disposable or used for several times with the option of a prelubricating coating, offering the patient and physician the possibility of adjusting the characteristics of the catheter according to individual needs[64].

Within milliseconds of implantation, the catheter is covered by adsorbed proteins that are available in the body fluids[65]. For instance, growth related, bactericidal and chemotactic factors are quickly adsorbed on the biomaterial and change the biological properties of the catheter. Bacteria interact with some of these proteins which may inhibit or enhance the attachment of microbes and the development of biofilms[65].

E. coli HB101pDC1 and HB101 formed biofilms on PVC substrates. The formation of biofilms on a substrate, such as PVC can be explained by non-specific interactions between bacteria and the substrate. The *in vitro* development of biofilms on PVC can be a result of bacteria undergoing starvation. It is also possible that proteins found in LB media and that were not consumed by *E. coli* were adsorbed onto the substrate which enhanced the attachment of bacteria to the abiotic surface. Regardless of the process of biofilm development, researchers have shown that the attachment of bacteria to an inert substrate is inevitable, as this is a strategy of survival for many bacterial species[62].

Growth of bacteria in the presence of 10 wt.% L-CJC resulted in complete inhibition of biofilm formation to PVC. This was expected, since exposure and growth of bacteria in the presence of L-CJC resulted in ~85% decrease of bacterial attachment. The presence of other compounds in L-CJC, such as fructose, offers a richer environment for bacteria. Since it has been suggested that biofilm formation can be caused due to starvation[62], the presence of L-CJC in LB can hinder the ability of bacteria to form biofilms since they have more carbon sources available.

Effects on biofilm formation were different in PACs than L-CJC since there was development of biofilms of *E. coli* HB101pDC1 for most cultures in PACs. However, the ability of bacteria to form a biofilm on PVC decreased with increasing number of cultures or time of incubation of bacteria in PACs. These results again suggest that PACs might not be the only active compounds in cranberry juice that can inhibit biofilm formation. For instance, the presence of other carbon sources in cranberry juice, such as carbohydrates, might affect the ability of *E. coli* to form biofilms on a PVC substrate. Other active compounds such as NDMs can also play an important role in preventing biofilm development. This can be correlated with studies on the effects of NDMs on the formation of biofilms on dental surfaces[17-19]. NDMs exhibit tannin-like properties, are devoid of proteins, carbohydrates, and fatty acids, and contain 0.35% anthocyanidins and 65.1% proanthocyanidins[19,66].

While use of PACs show a decrease in biofilm formation, this effect takes a long time to develop. This condition was never seen by using whole L-CJC, which suggests that a combination of different active compounds in cranberry juice have a better ability to inhibit the formation of biofilms on PVC substrates than just isolated PACs, although

more studies are needed to understand why isolated PACs do not have an equal effect on bacterial adhesion and biofilm formation than CJC. Further, more research is needed to recognize what other compounds in L-CJC affect bacterial adhesion and biofilm formation.

The mechanism of action of CJC clearly seems to be associated with an interference of bacterial attachment to uroepithelial cells by modification of surface components of *E. coli*[21,67]. It is not clear yet whether the inhibitory effect of L-CJC or PACs on biofilm formation or bacterial attachment might be due to blocking of signals in the biofilm formation genetic pathway. More studies have to be made to investigate the effects of cranberry products on the production of extracellular signals that are responsible for the development of biofilms.

While the mechanisms of action of cranberry products on bacterial adhesion and biofilm formation are not yet well understood, this study indicates that beneficial effects of cranberry can be achieved by long-term exposure or growth of bacteria in cranberry constituents. While we showed that cranberry juice or PACs do not inhibit the growth of bacteria, either can be used as a prophylactic treatment to prevent the formation of biofilms in uroepithelium or in urethral and ureteral catheters. This study also suggests that more benefits can appear from cranberry juice than isolated A-type PACs, although the reasons for this are not yet clear.

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Fig 3.1 Phase contrast images of bacteria exposed to increasing concentrations of cranberry juice cocktail

A) *E. coli* HB101pDC1 no cranberry treatment

B) *E. coli* HB101pDC1 after 10 wt. % cranberry juice treatment

C) *E. coli* HB101pDC1 after 27 wt. % cranberry juice treatment

Bacteria cultured to exponential growth phase (A_{600nm} = 0.9), exposed for 3 hours to different concentrations of cranberry juice and incubated with uroepithelial cells for 90 minutes. Magnification: 1000x

Fig 3.2 Effects of concentration of L-CJC and PACs on bacterial attachment for A) *E. coli* HB101pDC1 and B) HB101. Average number of adhered bacteria per uroepithelial cell with standard deviation (Tukey's test $p<0.001$)

Fig 3.3 Effects of continuous incubation of bacteria in 10 wt.% L-CJC or 128 μg/mL PACs on bacterial attachment (mean and standard deviation; $p<0.001$), for A) *E. coli* HB101pDC1 and B) *E. coli* HB101.

Fig 3.4 Representative biofilm formation of P-fimbriated *Escherichia coli* on PVC microtiter plate wells, arranged from low to high formation of biofilms. Incubation time for wells from left to right: 0 hr (A); 3 hrs (B); 6 hrs (C); 24 hrs (D); 30 hrs (E); 48 hrs (F) stained with 0.3% crystal violet solution after incubation at 37ºC. The star symbol represents the transition of bacteria from biofilm back to planktonic state since the intensity of the dye decreased.

Fig 3.5 Biofilm formation of bacteria grown up to 12 times in the presence of 10 wt.% L-CJC, for A) *E. coli* HB101pDC1 and B) *E. coli* HB101. Data points represent the average reading from six replicates and standard deviation.

Fig 3.6 Biofilm formation of bacteria grown up to 12 times in the presence of 128 μg/mL PACs, for A) *E. coli* HB101pDC1 and B) *E. coli* HB101. Data points represent the average reading from six replicates and standard deviation.

Fig 3.1

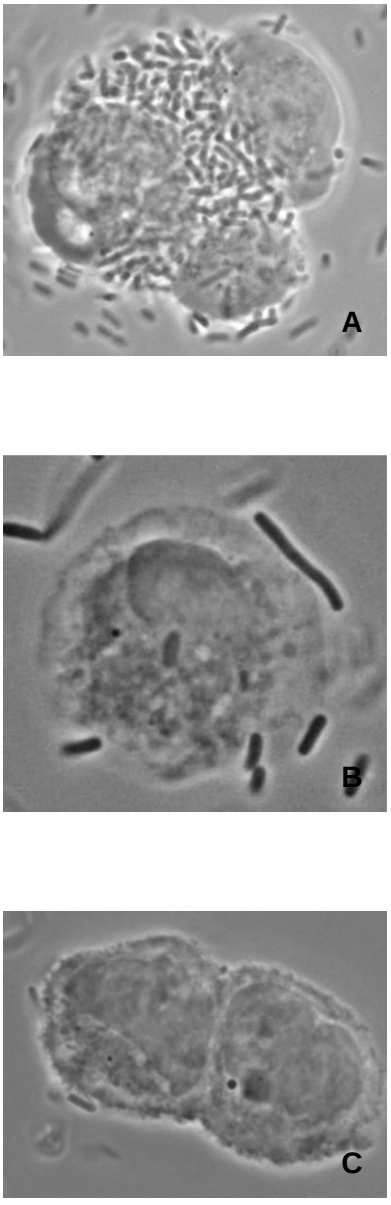
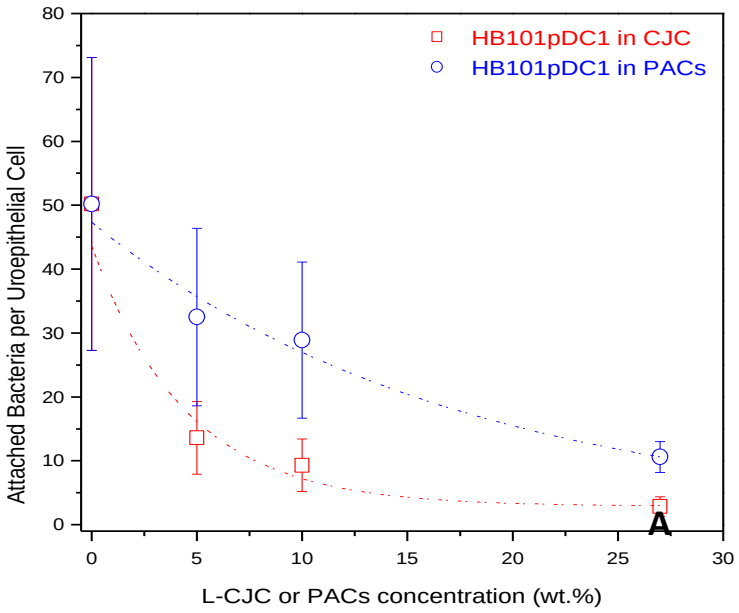


Fig 3.2


HB101pDC1 in CJC
HB101pDC1 in PACs
Attached Bacteria per Uroepithelial Cell
L-CJC or PACs concentration (wt.%)
A

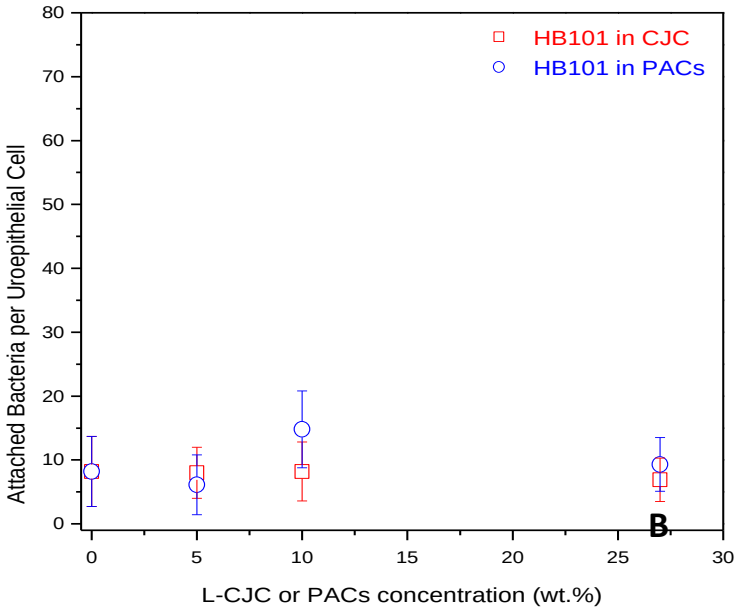
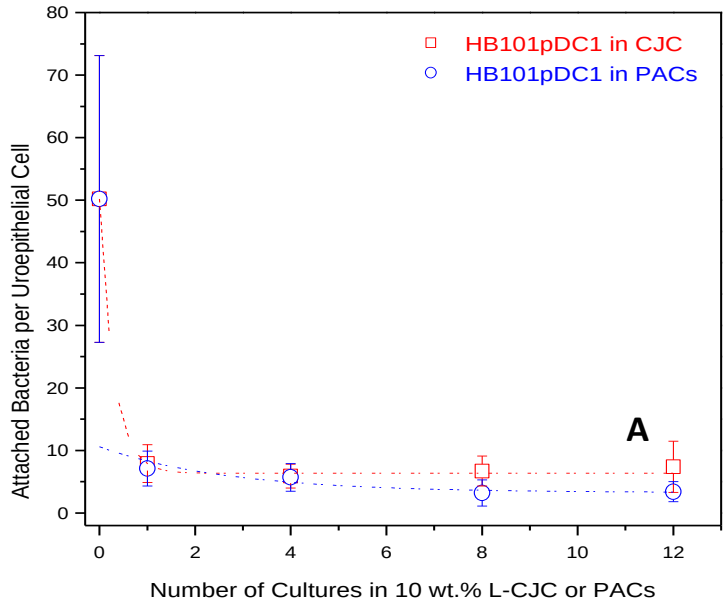

HB101 in CJC
HB101 in PACs
Attached Bacteria per Uroepithelial Cell
L-CJC or PACs concentration (wt.%)
B

Fig 3.3


HB101pDC1 in CJC
HB101pDC1 in PACs
Attached Bacteria per Uroepithelial Cell
Number of Cultures in 10 wt.% L-CJC or PACs
A
0
10
20
30
40
50
60
70
80
0
2
4
6
8
10
12

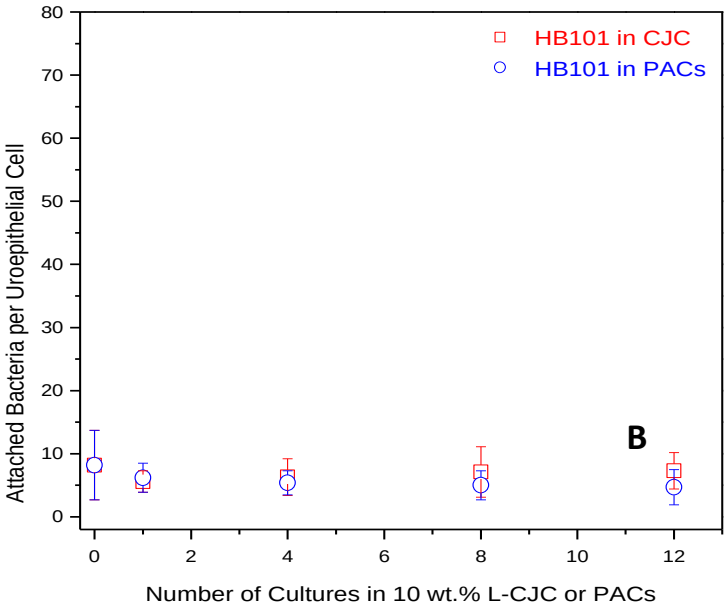

HB101 in CJC
HB101 in PACs
Attached Bacteria per Uroepithelial Cell
Number of Cultures in 10 wt.% L-CJC or PACs
B
0
10
20
30
40
50
60
70
80
0
2
4
6
8
10
12

Fig 3.4

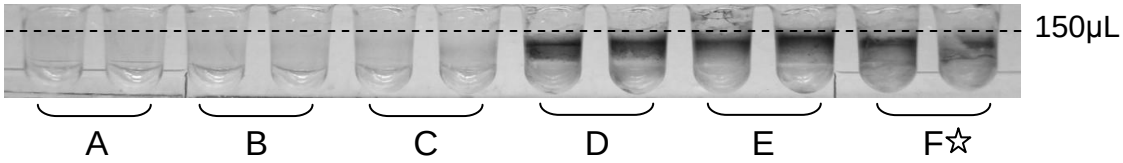
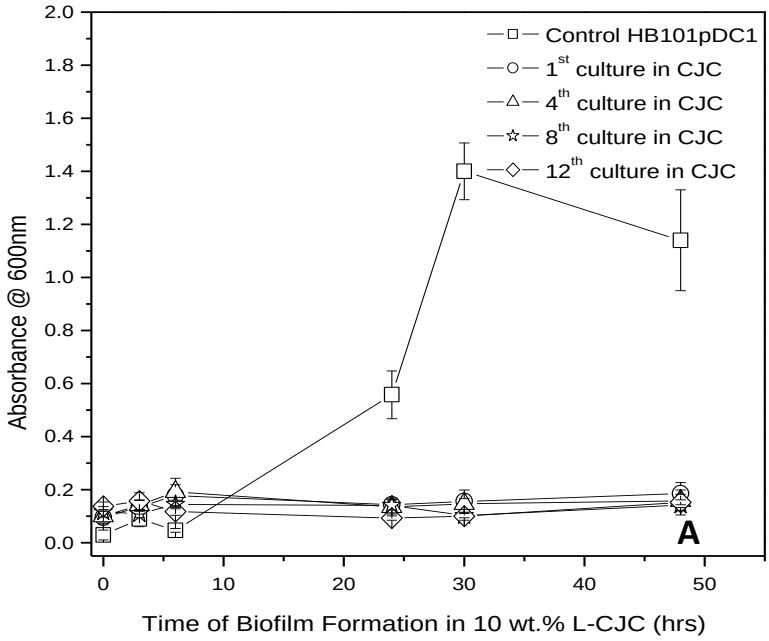


Fig 3.5


Control HB101pDC1
1st culture in CJC
4th culture in CJC
8th culture in CJC
12th culture in CJC
Absorbance @ 600nm
Time of Biofilm Formation in 10 wt.% L-CJC (hrs)
A

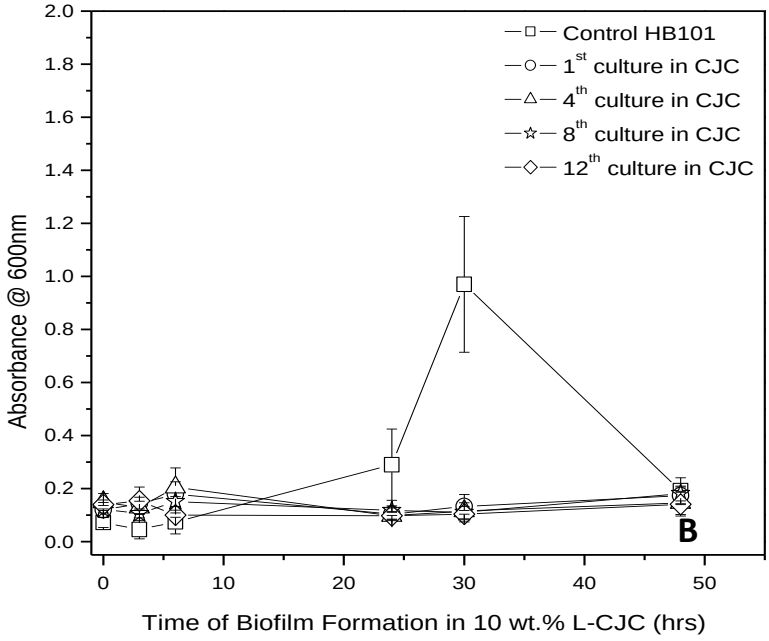
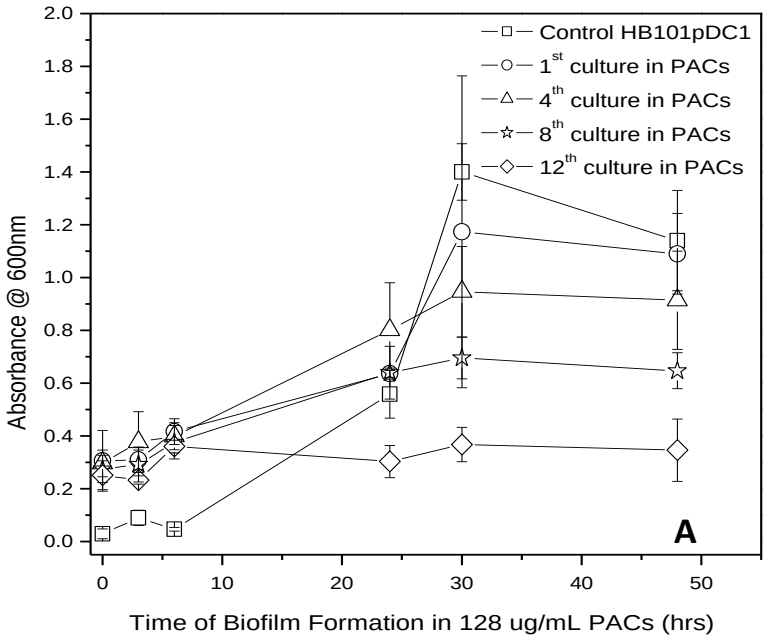
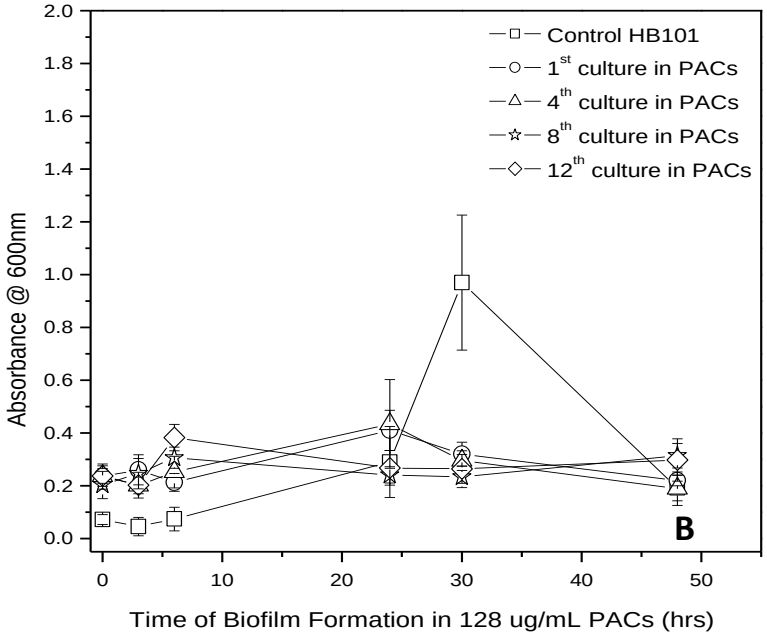

Control HB101
1st culture in CJC
4th culture in CJC
8th culture in CJC
12th culture in CJC
Absorbance @ 600nm
Time of Biofilm Formation in 10 wt.% L-CJC (hrs)
B

Fig 3.6

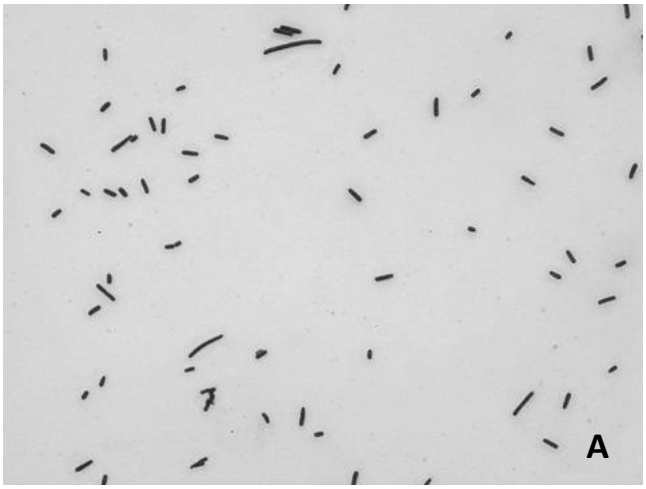

Control HB101pDC1
1st culture in PACs
4th culture in PACs
8th culture in PACs
12th culture in PACs
Absorbance @ 600nm
Time of Biofilm Formation in 128 ug/mL PACs (hrs)
A

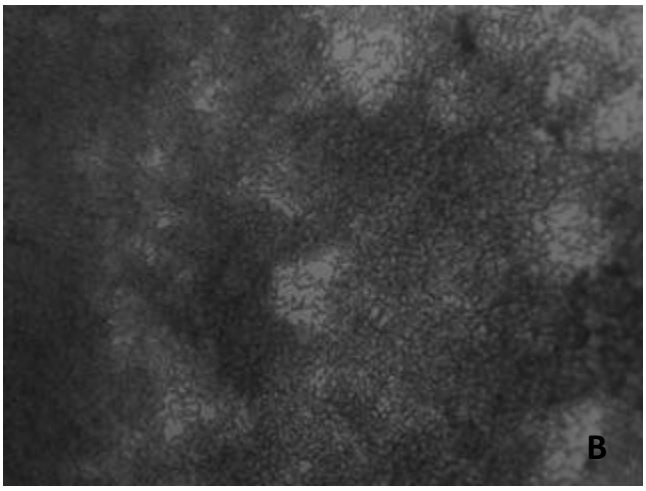

Control HB101
1st culture in PACs
4th culture in PACs
8th culture in PACs
12th culture in PACs
Absorbance @ 600nm
Time of Biofilm Formation in 128 ug/mL PACs (hrs)
B

Supplementary Information

Figure 3.7 Light microscopy images of the formation of an *E. coli* HB101pDC1 biofilm on a poly-vinyl chloride substrate for A) Biofilm formation at 0 hrs and B) Biofilm formation at 30 hrs after incubation. Biofilms grown in PVC microtiter plate wells and stained with 0.3% crystal violet solution, magnification: 1000x

Fig 3.7





Chapter 4: Adhesion Force Assay

Detection of Interactive Forces of *Escherichia coli* and Changes in Surface Charge Due to Cranberry Juice

4.1 Abstract

Bacterial attachment to uroepithelial cells is the first step in the development of a urinary tract infection (UTI). Previous research has suggested that the consumption of cranberry products prevents the adhesion of *E. coli* to uroepithelial cells since compounds in cranberry juice change conformation and physicochemical properties of fimbriae. Experiments were designed to understand how adhesion between *E. coli* and a bare silicon-nitride probe is affected by light cranberry juice (L-CJC) and isolated cranberry proanthocyanidins (PACs). The P-fimbriated *E. coli* strain HB101pDC1 and the non-fimbriated strain HB101 were grown in the presence of 10 wt. % L-CJC or 128 μg/mL PACs for 12 consecutive cultures. Force measurements were taken in 0.01 M phosphate buffer saline (PBS) solution + 0.138 M NaCl + 0.0027 KCl using an atomic force microscope (AFM). *E. coli* HB101pDC1 and HB101 that were grown in 10 wt. % L-CJC and 128 μg/mL PACs showed less adhesion forces with the silicon nitride probe than bacteria grown in media that did not contain cranberry products. Further, adhesion forces decreased as the number of bacterial cultures in cranberry constituents increased. A change in zeta potential was also observed for both L-CJC and PACs treated bacteria. PACs treated bacteria expressed a more negative surface charge than bacteria grown in pure media, which was correlated with a decrease in adhesion forces. The results of our studies indicated that the presence of cranberry products in the growth media affects surface characteristics of *E. coli* and bacterial adhesion to silicon nitride.

Keywords: Atomic force microscopy, proanthocyanidins, urinary tract infections, bacterial adhesion, zeta potential

4.2 Introduction

The adhesion of bacteria to uroepithelial cells and urinary catheters is the first step in the development of a urinary tract infection (UTI)[1,2]. The treatment to eradicate UTIs results in medical expenditures exceeding $2 billion each year[3]. Bacterial adhesion to biotic and abiotic surfaces is governed by long range forces including electrostatic and steric interactions and by short range forces, such as hydrophobicity, van der Waals forces and surface charge. The presence of specific proteins on the bacterial surface also plays a role in bacterial adhesion[4]. The expression of extrapolymeric substances (EPS) including flagella, hemolysins and fimbriae allow bacteria to adhere to surfaces, which results in the development of biofilms[5,6].

Infections in the urinary tract are mostly caused by Gram-negative organisms[7]. *Escherichia coli* is the cause of ~85% of UTIs where ~90% of acute pyelonephritis cases are caused by *E. coli* expressing type P fimbriae[8]. The presence of adhesins at the distal end of fimbriae has been the main focus of researchers since these adhesins can bind to receptors found on the surface of uroepithelial cells[9,10]. Several classes of G adhesins have been identified that play a role on UTIs[8]. The Class I, II, and III G adhesins encoded by *pap* or *prs* gene clusters have been closely related to development of acute pyelonephritis and acute cystitis, since they bind to receptors of the αGal(1→4)βGal glycolipid family on uroepithelial cells[10].

Due to increasing antibiotic resistance, the use of natural products for the prevention and treatment of infections are being sought by researchers. Cranberries (*Vaccinium macrocarpon* Ait., Ericaceae) have long been recognized for their potential health benefits against UTIs, although the mechanism of action is not well understood[11]. Decades ago, it was believed that the acidity of cranberries played a role on bacterial adhesion[12]. The increase of the excretion of hippuric acid in urine due to the consumption of cranberries was believed to have an effect on bacteria since uropathogens would not be able to adhere to uroepithelium in an acidic environment. However, more recent studies have shown that the ingestion of cranberry products does not alter the pH of urine significantly and is not bacteriostatic[13]. Cranberry products may inhibit the attachment of bacteria to epithelial cells interfering with molecules on the surface of *E. coli*[13].

The presence of certain phytochemicals in cranberries, such as A-type proanthocyanidins (PACs), have been linked to the ability of this fruit to inhibit the adhesion of *E. coli* to uroepithelial cells[14]. Howell et al. reported that exposure of bacteria to a concentration of 60 μg/mL PACs was sufficient to inhibit the agglutination of P-fimbriated *E. coli* to human red blood cells[15]. Liu et al. found that increasing concentrations of cranberry juice cocktail resulted in a decrease in adhesion forces between a silicon nitride probe and P-fimbriated *E. coli*[16]. Further, through steric modeling, it was reported that the length of bacterial fimbriae decreased with exposure to increasing concentrations of cranberry juice[16].

Few studies have addressed the effects of cranberry products on the growth of uropathogenic bacteria. Loss of fimbriae due to growth in cranberry juice has been reported[17]. This loss of fimbriae resulted in the inability of bacteria to agglutinate P-

receptor beads and an elongation of *E. coli* JR1 and DS17 cells was also observed after growth of bacteria in 25% cranberry-rich agar[17]. Changes in growth rate due to long time incubation of bacteria in cranberry juice have also been reported[13].

Traditional methods to study the adhesive properties of bacteria are by enumeration of bacteria adhered or attached to receptor specific beads, epithelial cells or erythrocytes through light microscopy[10,18,19]. While these methods can provide important information, they are very imprecise and time consuming. Atomic force microscopy (AFM) has long been used to examine properties of microbial surfaces at a molecular resolution[20,21]. Interaction forces between bacteria and different substrates can also be obtained through AFM in their natural environments where minimum sample preparation is required[16,22]. To our knowledge, our laboratory was the first to investigate the effects of cranberry juice on the adhesion forces of *E. coli* using AFM[16]. No previous research has investigated how culturing of *E. coli* HB101 and HB101pDC1 in the presence of light cranberry juice cocktail (L-CJC) and PACs affects bacterial adhesion through direct force measurements. These *in vitro* experiments will simulate physiological conditions of patients that have been advised to consume cranberry juice to prevent recurrent UTIs[11], where bacteria start growing in a cranberry-rich environment. Our findings suggest that growth of *E. coli* in L-CJC or PACs results in a decrease of adhesion forces with increasing number of cultures. Further, our findings were correlated with changes in the surface potentials of *E. coli* strains after growth in L-CJC and PACs.

4.3 Materials and Methods

4.3.1 Cranberry Juice and Isolated Proanthocyanidins

Ocean Spray light cranberry juice™ cocktail (L-CJC) was purchased (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA). The pH of the juice was adjusted to 7.0 using sodium hydroxide (Sigma-Aldrich, St. Louis, MO) and the solution was passed through a 0.2 μm syringe filter (Acrodisc® Pall Life Sciences, East Hills, NY). A concentration of 10 wt.% L-CJC was used for all experiments. This concentration was obtained based on reported values on the amounts of PACs in commercial CJC[15].

A 345.8 μg/mL stock solution of isolated cranberry proanthocyanidins (PACs; Ocean Spray Cranberries, Inc; Lakeville-Middleboro, MA) was prepared in ultrapure water (Milli-Q water, Millipore Corp.; Bedford, MA). A PACs concentration of 128 μg/mL was used for all experiments, which is the amount of PACs in 10 wt.% L-CJC[15]. The mixture was shaken overnight at room temperature away from any light source. The pH of the PACs solutions was not adjusted since the values were very close to neutral conditions.

4.3.2 Bacteria and Growth Conditions

E. coli HB101 was purchased from the American Tissue Culture Collection (ATCC 33694). *E. coli* HB101 was used as our negative control since these cells are plasmid-less and lack fimbriae[23]. The P-fimbriated *E. coli* mutant HB101pDC1 was kindly provided by Dr. M. Svensson from the Department of Medical Microbiology, Lund University. *E. coli* HB101pDC1 is a mutant of the HB101 strain expressing P-fimbriae only due to the insertion of a chloramphenicol resistant plasmid[24].

Bacteria were grown at 37 ºC in 30 g/L tryptic soy broth (TSB; Sigma, St. Louis, MO); and supplemented with 0, 10 wt.% neutralized L-CJC or 128 μg/mL isolated PACs. *E. coli* HB101pDC1 was grown in the presence of 20 μg/mL chloramphenicol (Sigma, St. Louis, MO). Growth rates and doubling times were analyzed by measuring the optical density of the solution at 600 nm (Thermo Spectronic, Rochester, NY) and bacteria were harvested at the end of the exponential growth phase (A_{600nm} = 0.9), since maximal adhesive activity is seen at this period of growth[1].

After harvesting, 1 mL of bacteria solution was transferred to a new flask with fresh media to start the second culture in the cranberry-rich media. Twelve successive transfers of bacteria in TSB and L-CJC or PACs were done per strain. The 1st, 4th, 8th, and 12th bacterial cultures grown in L-CJC or PACs, and bacteria grown in pure TSB were centrifuged for 10 min at 4000 RPM and washed three times with ultrapure water (Milli-Q water, Millipore Corp.; Bedford, MA) to remove all the components of the growth media[21]. Bacteria were resuspended in ultrapure water for bacterial bonding to glass experiments.

4.3.3 Glass Slide Preparation

Glass slides were soaked in a 3:1 (vol/vol) HCl/HNO_3 solution (Fisher Chemical; Fair Lawn, NJ) for 45 minutes and rinsed with ultrapure water (Milli-Q water, Millipore Corp.; Bedford, MA). Following rinsing, slides were then immersed in a 7:3 (vol/vol) H_2SO_4/H_2O_2 solution (Fisher Chemical; Fair Lawn, NJ) and rinsed with at least 50 mL of water. The cleaned slides were stored at room temperature in a beaker of ultrapure water.

Before AFM experiments, amino groups were attached to clean glass slides by treating slides with a 35% aminosilane solution (3-aminopropyltrimethoxysilane; Sigma, St. Louis, MO) in distillation quality methanol (Sigma, St. Louis, MO). The slides were kept in the aminosilane solution for 15 minutes and rinsed with methanol followed by 25 mL of ultrapure water.

4.3.4 Covalent Bonding of *E. coli* to Glass Slides

E. coli from cultures 1, 4, 8, 12 and control (no cranberry treatment) were immobilized on acid clean glass slides using a standard procedure[25]. Briefly, *E. coli* grown in the presence of L-CJC or PACs were coupled to amino groups with a protein-protein cross-linking reaction. A 300 µL volume of a 100 mM EDC solution (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl; Pierce, Rockford, IL) was added to previously washed bacteria with rotation at 18 RPM for 10 minutes at room temperature. A 600 µL volume of a 40 mM NHS solution (N-Hydroxysulfosuccinimide; Pierce, Rockford, IL) was then added to the bacterial solution with rotation at 18 RPM for 20 minutes. The combined *E. coli* bacteria solution, EDC and NHS were poured into previously treated aminosilane glass slides and allowed to shake with rotation at 70 RPM for 6 hrs. EDC/NHS treated bacteria remain viable and no cell damage occurs during this covalent bonding process[25]. Viability of the cells was assessed by placing the coated glass slide back into growth media and evaluating growth of bacteria after the AFM experiment.

4.3.5 AFM Force Measurements

During AFM experiments, bacteria-coated slides were kept hydrated using a 0.01 M phosphate buffer saline (PBS) solution + 0.138 M NaCl + 0.0027 KCl to simulate physiological conditions. AFM measurements were carried out by using a Digital Instruments 3100 with Nanoscope III Controller (Santa Barbara, CA). Silicon nitride AFM tips (DNPS, Veeco Instruments, Inc, CA) were used to acquire images in fluid in tapping mode, which was performed at a resonant frequency of ~19 kHz. Images were obtained with 512 x 512 points at a scanning rate of 1 Hz. Silicon nitride AFM tips have been widely used by researchers to understand the interaction forces between the bare AFM probe and bacterial cells, and how this model system can provide fundamental understanding of the properties of bacterial surfaces[4,16].

Before force measurements were made, the spring constant of the cantilever was measured using the thermal calibration method[26]. The average spring constant was 0.06 ± 0.01 N/m. Before using the cantilevers, they were cleaned with ethanol and exposed to UV light to remove potential contaminants.

Force measurements were carried out in fluid and 5 individual cells were probed, where 6 force cycles were recorded per bacterium per condition. Data was converted to ASCII format and exported to a spreadsheet where the information was converted from deflection data to forces as discussed previously[27].

4.3.6 AFM Retraction Curve Analysis

Peaks observed in the retraction curve were identified as independent and random events that occur when the AFM tip retracts from the bacterial surface, and fimbriae or

other molecules are released from the probe (also known as pull-off events). Each peak represents the adhesion force exerted between each *E. coli* surface molecule and the bare silicon nitride AFM probe.

While there were a number of multiple peaks in each retraction curve, certain criteria were used to identify what peaks corresponded to the release of a molecule from the AFM probe. The first criterion to investigate was to determine the noise level exerted by the microscope for a single set of conditions. This depended in the degree of humidity in the environment as well as the noise level surrounding the microscope. To minimize noise, all experiments were carried out during the night and a dehumidifier was used to adjust the humidity of the environment to ~20%. It was determined that most experiments had a noise magnitude of 0.05 nN, which meant that if there was a peak displaying an adhesion force of 0.05 nN or less, it was discarded and assumed to be background noise.

The second set of criteria is based in the force pattern of each of the retraction curves. If there were multiple peaks in the retraction region two characteristics were taken into account. First, the pull-off distance between peaks was carefully studied since this would determine if the AFM probe is fully releasing a molecule from the tip or just stretching a previously coiled fimbriae or LPS molecule. Liu et al. has determined that the average size of P-fimbriae is 148 ± 125 nm[16]. We have used this data to determine the minimum distance that can exist between two peaks. Taking into account the large standard deviation of the length of P-fimbriae we determined that if the pull-off distance between two peaks was less than 25 nm, then we would analyze the peak with the greatest adhesion force. Other criteria taken into account are the magnitude of the adhesion force between two adjacent peaks. It the difference between two adjacent peaks

is less than 0.05 nN then we assumed that the tip had not fully released the molecule from the probe and the peak with greatest magnitude was analyzed. Distance and magnitude of the forces had to been investigated simultaneously to decrease any chances of data misinterpretation. These set of criteria depend on the surface of the bacteria being studied as well as the conditions of bacterial growth.

Once all the retraction peaks were analyzed, a histogram for all observations within a given condition was plotted. The horizontal axis, adhesion force or pull-off distance, was divided into intervals of equal length. The number of adhesion forces or pull-off distances within each range or interval was determined for all treatment conditions and plotted. Since retraction curves can result in different number of adhesion peaks, the sample size of every condition is different. Data were analyzed using SigmaStat 2.03 software and a Kruskal-Wallis one way ANOVA on ranks was used to analyze all conditions with different sample sizes. Dunn's test was used to compare among treatment groups and to compare cranberry treated groups against the control condition (pure TSB).

4.3.7 Zeta Potential Measurements

The electrophoretic mobilities and surface potentials of both *E. coli* strains were measured using a zeta potential analyzer (Zetasizer Nano ZS; Malvern Instruments, MA). *E. coli* at a concentration of $1x10^7$ cells/mL were washed three times after growth in cranberry products. The bacterial cells were resuspended in PBS and 1 mL of bacterial solution was placed in folded capillary cells (DTS1060; Malvern Instruments, MA). Zeta potentials are calculated according to the Smoluchowski equation[28]. Measurements were repeated three times for every condition.

Data are expressed as the means ± the standard deviation (SD). Data were analyzed using SAS® and SigmaStat 2.03 statistical software. Statistical analysis was performed by two-way analysis of variance (ANOVA) for repeated measurements. Tukey's test was used for multiple comparisons among each treatment group, while Dunnett and Duncan's test were used for comparisons between treatment and control groups. A difference was considered significant if $P < 0.05$.

4.4 Results

4.4.1 Analysis of Retraction Force Data after Growth of *E. coli* in L-CJC

The interaction forces between biomolecules on the surface of bacteria and a silicon nitride AFM tip were obtained for all treatment conditions. Using a single set of conditions, force measurements of a single bacterium were reproducible even though the heterogeneous nature of the surface of bacteria varies within the same species (Figure 4.1A). After probing bacteria with the silicon nitride probe, the system showed multiple peaks in the retraction section of the cycle, indicating that the tip is making contact with more than one surface polymer or more than one location of the polymer.

The growth of bacteria in the presence of 10 wt.% L-CJC resulted in a decrease of adhesion forces. For the mutant strain *E. coli* HB101pDC1, the adhesion forces decreased with increasing number of cultures (Figure 4.1B). There was a statistically significant difference between the control group (bacteria grown in pure TSB) and *E. coli* HB101pDC1 grown in the presence of L-CJC for all cultures periods ($P<0.001$). However, Dunn's test showed that no significant difference was seen between the adhesion forces of cultures 4, 8 and 12 ($P>0.05$). Bacteria cultured in pure TSB showed

strong adhesion forces with an absolute average of 1.77 ± 0.72 nN. After the first culture of *E. coli* HB101pDC1 in 10 wt.% L-CJC the adhesion forces decreased to 1.13 ± 0.6 nN.

The distribution of the pull-off forces showed that while culturing of *E. coli* HB101pDC1 in pure TSB (no cranberry treatment) resulted in strong adhesion forces of up to 3.5 nN, the continuous culturing of the same strain in L-CJC resulted in weak adhesion forces of 1.5 nN or less (after 4 cultures, ~48 hours). The histogram also shows that 100% of adhesion forces were lower than 1.0 nN for cultures 8 and 12 (Figure 4.2A).

The pull-off distances increased with increasing number of cultures (Figure 4.2B). According to Dunn's test, there was no significant difference among the means of the different L-CJC treatments, with the exception of culture 4 and culture 8 ($P>0.05$). Dunn's method also showed that there was a statistical significant difference between the pull-off distances of our control group and *E. coli* HB101pDC1 cultured in L-CJC 1, 4, and 12 times ($P<0.05$).

The adhesion forces of non-fimbriated *E. coli* HB101 were generally lower compared to our P-fimbriated bacteria. There was a decrease in adhesion forces with increasing number of cultures in L-CJC (Figure 4.3A). By the 8^{th} culture, 100% of the adhesion forces had decreased to <1.0 nN. According to Dunn's test, there was a statistically significant difference between the adhesive forces of bacteria grown in pure TSB and all the cranberry treatments with the exception of the 1^{st} culture. The absolute value of adhesion forces of the non-fimbriated *E. coli* HB101 decreased from 1.47 ± 0.75, for the control, to 0.46 ± 0.15 for bacteria grown 12 times in L-CJC.

Similarly to the *E. coli* HB101pDC1 data, the pull-off distance values for strain HB101 increased with increasing culture times in L-CJC (Figure 4.3B). However, these values were typically lower than distances in the P-fimbriated strain. Dunn's test showed that there was a statistically significant difference between the values of pull-off distances for our control bacteria and the *E. coli* HB101 grown in the presence of L-CJC ($P<0.05$). There was no significant differences among the cranberry treated bacteria groups.

4.4.2 Analysis of Retraction Force Data after Growth of *E. coli* in PACs

The interaction forces of *E. coli* HB101pDC1 after growth in the presence of 128 μg/mL PACs were obtained for all treatment conditions (Figure 4.4A). After one culture of the bacteria grown in PACs, the adhesion forces increased from an absolute force value of 1.77 ± 0.72 nN to 2.2 ± 1.1 nN; however, this increase in adhesion force was not significant according to Dunn's test ($P>0.05$). A statistically significant decrease in adhesion forces was seen with increasing number of cultures in PACs after the 4th culture with average force values of 0.94 ± 0.57 nN, 0.56 ± 0.25 nN and 0.49 ± 0.2 nN for cultures 4, 8 and 12, respectively ($P<0.05$). Multiple comparisons among the adhesion force values of all PACs treated bacteria revealed that there was a significant difference among treatments with the exception of cultures 4 vs. 8 and cultures 8 vs. 12. After 8 cultures in the presence of PACs 100% of adhesion forces had decreased to <1.0 nN, and ~80% of adhesion forces were <0.5 after 12 cultures in PACs.

No clear trends were seen on the effects of PACs on pull-off distances for the P-fimbriated bacteria (Figure 4.4B). The pull-off distances for any culture in PACs were somewhat larger than expected. Dunn's test showed that the difference in the values of pull-off distances were significantly different between the control group and cultures 1

and 4 (P<0.05). However, no difference was detected between bacteria grown in pure TSB and other PACs treated bacteria. According to Dunn's multiple comparisons test, there was no significant difference in pull-off distance values among PACs treated bacteria with the exception of culture 1 vs. 12 and 4 vs. 12 (P<0.05), suggesting that the pull-off distances were not very sensitive to PACs treatment.

The adhesion force values were also obtained for the non-fimbriated *E. coli* strain (Figure 4.5A). Even though this strain does not express fimbriae , we observed that there was a decrease in adhesion forces with increasing number of cultures in PACs. The force of adhesion for *E. coli* HB101 decreased from an absolute value of 1.49 ± 0.7 nN to 0.37 ± 0.29 nN after the cells were cultured in the presence of PACs 12 times. More than 90% of all adhesion values were <0.5 nN after the cells were grown in the presence of PACs. Dunn's test also confirmed that the adhesion values of all PACs treated bacteria were significantly different when compared to the control group (bacteria grown in 100% TSB; P<0.05). This finding also suggests that PACs also affects the non-specific adhesion forces.

Similarly to our P-fimbriated bacteria, the pull-off distances of *E. coli* HB101 were obtained for all PACs conditions (Figure 4.5B). There was a decrease in pull-off distances with increasing cultures in PACs. After culturing the non-fimbriated strain in PACs 12 times, almost 70% of the pull-off distances had decreased to <100 nm. According to Dunn's test, there was a significant difference of pull-off distance values between the PACs treated bacteria and the control group (P<0.05).

4.4.3 Effects of Cranberry Constituents on Bacterial Surface Potentials

The surface potentials of the control *E. coli* HB101 and HB101pDC1 were -22.2 ± 0.3 mV and -19.3 ± 0.4 mV in 0.01 M PBS, respectively (Table 4.1). The P-fimbriated strain was always less negatively charged than *E. coli* HB101 for all treatment conditions. After culturing of *E. coli* HB101pDC1 in 10 wt.% L-CJC, the zeta potentials of bacteria became less negative where a statistically significant difference was seen between the control group and each one of the cultures in L-CJC ($P<0.05$). Tukey's test also showed that there was no significant difference among the zeta potential values of our cranberry treated bacteria, regardless of the culture number ($P>0.2$).

There was also a change in zeta potential when the non-fimbriated *E. coli* HB101 strain was cultured in L-CJC (Table 4.1). There were statistically significant difference between the zeta potential values of *E. coli* HB101 grown in pure TSB and potentials of bacteria grown in the presence of L-CJC ($P<0.04$), but we could not detect an effect of time of culturing in L-CJC on the zeta potential.

The zeta potentials of bacteria became more negative after culturing of bacteria in the presence of 128 μg/mL PACs (Table 4.2). For *E. coli* HB101, the surface potentials decreased from -22.2 ± 0.3 mV (bacteria cultured in pure TSB) to -35.8 ± 0.7 mV after 12 cultures in PACs. There was a significant decrease in zeta potentials between PACs treated bacteria and the control *E. coli* HB101 group with the exception of 1st culture. Tukey's test also revealed that comparison among all PACs treatment groups resulted in significantly different zeta potential values except for culture 8 vs. 12 ($P=0.185$).

Zeta potentials of *E. coli* HB101pDC1 became more negative with increasing number of cultures in PACs. However, surface charge values were less negative than the non-fimbrieated *E. coli* strain. According to Dunnett's test there was a significant difference among the mean zeta potentials of the control HB101pDC1 strain and cultures 8 and 12 in PACs ($P<0.05$). However, no significant difference was seen between the control strain and cultures 1 and 4. Comparisons among PACs treated groups resulted in a significant difference of zeta potential values for all groups except culture 1 vs. 12 and 8 vs. 12 ($P=0.996$ and $P=0.909$, respectively).

Washing of PACs treated bacteria in pure PBS resulted in a return to original surface potential values, indicating that the adsorption of PACs onto bacterial surfaces is reversible.

4.5 Discussion

4.5.1 Bacteria Preparation

The integrity of the bacterial surface was not damaged by the EDC/NHS protocol and operation in tapping mode ensured that light forces were applied to the cells. Bacteria bonded to silanized glass remained viable during the duration of the AFM experiment (~3 hours per slide). Bacteria attached to glass slides resumed growth after placement of the slide into fresh growth media. Furthermore, Camesano et al. evaluated the viability of the cells by the use of a live/dead staining technique and concluded that bacterial cells kept all their surface properties after undergoing the EDC/NHS chemical reaction and remained viable at all times[25].

The chemical treatment targets the carboxyl groups found on the cell surface forming an unstable, intermediate complex that later comes into contact with the amino groups on the modified glass slide[25,29]. Our results showed that treatment of bacteria with cranberry products did not affect the EDC/NHC bonding protocol, since more than 20 bacterial cells were found per 10 μm^2 for all treatment conditions. In addition, while Liu et al. reported that stock solutions of 0.5 M EDC and 0.1 M NHS were sufficient to provide strong immobilization of *E. coli* HB101 and *S. epidermidis*[29], we showed that much lower stock solutions of 100 mM EDC and 40 mM NHS were high enough to immobilize both strains of *E. coli* where repeated scanning over the same area did not result in any removal of bacterial cells.

4.5.2 Correlating Growth in Cranberry Products and Adhesive Forces of *E. coli*

We investigated the ability of PACs and L-CJC to reduce the adhesion forces between a silicon nitride AFM probe and *E. coli* HB101 and HB101pDC1. Recent studies have recognized that A-type proanthocyanidins inhibit the adhesion of P-fimbriated *E. coli* to red blood cells[15], and that exposure of bacteria to increasing concentrations of cranberry juice decrease bacterial attachment to uroepithelial cells[30]. Most studies evaluate short term exposure of bacteria to cranberry products on bacterial adhesion. Our research is the first to demonstrate a change in adhesion forces after growth of *E. coli* HB101 and HB101pDC1 in the presence of L-CJC or PACs. Growth of bacteria in a cranberry-rich environment helps simulate physiological conditions of people that are continuously drinking cranberry juice to prevent recurrent UTIs.

The adhesion forces between bacteria and a bare silicon nitride AFM probe can be affected by cranberry products. After growth of our P-fimbriated strain in a 10 wt.% L-

CJC concentration, the adhesion forces decreased with increasing number of cultures. Growth of the same strain in pure TSB (no cranberry treatment) results in absolute adhesion forces of up to 3.4 nN, where only ~20% of adhesion forces were less than 1.0 nN. Growing HB101pDC1 in L-CJC 8 consecutive times resulted in 100% of force values in the 0 to 1.0 nN range. This is supported by previous studies where short-term exposure of the same strain to the same concentration of juice resulted in adhesion forces ranging from 0 to 1.2 nN, even though the researcher did not grow bacteria in the presence of L-CJC[16].

This study is also supported by previous research done in our laboratory where bacteria receiving the same cranberry treatment were incubated with uroepithelial cells and the number of bacteria attached were quantified[31]. During this study there was also a decrease in bacteria-uroepithelial cell attachment with increasing number of cultures in cranberry media. Ahuja et al, also reported a loss of bacterial attachment to P-specific receptor beads after growth of *E. coli* JR1 and DS17 in cranberry-rich agar[17].

There are several proposed mechanisms to explain the interactions between cranberry and bacteria[16]. The decrease in equilibrium length of P-fimbriae indicates that proteins on the bacterial surface are becoming compressed[16,32]. Also components from cranberry juice can bind to P-fimbriae hindering the adhesion to receptors on mammalian cells or to other surfaces.

While these hypotheses explain the interactions between cranberry and P-fimbriated bacteria, these mechanisms cannot explain our results for the non-fimbriated *E. coli* strain. During our study, the non-fimbriated *E. coli* strain exhibited adhesion

forces of up to 4.5 nN when grown in pure TSB (no cranberry treatment). However, growth of the same strain in cranberry juice decreased the interactions between the AFM probe and bacterial cells to forces that were <1.0 nN for all bacteria grown in L-CJC eight and twelve consecutive times. The decrease in adhesion forces for this strain indicated that cranberry juice does not only affect the expression of fimbriae on our P-fimbriated bacteria, but also affects other molecules found on the surface of *E. coli*. *E. coli* HB101 lacks an EPS layer and the LPS layer contains short molecules, since there is no O-antigen[23,33]. While we do not fully understand why *E. coli* HB101 is affected by L-CJC we believe that cranberry juice also affects small molecules in the LPS layer of non-fimbriated bacteria. We also showed that cranberry affects specific and non-specific adhesion, since HB101 can only attach non-specifically to surfaces.

Growth of *E. coli* in PACs resulted in a decrease in adhesion forces with increasing number of cultures in PACs. This can be supported by experiments done in our laboratory where bacterial attachment to uroepithelial cells was monitored after bacterial growth in PACs[31]. The number of bacteria attached to uroepithelial cells decreased with increasing exposure of *E. coli* to PACs. Our results also indicated that *E. coli* is more sensitive to L-CJC than PACs. Growth of both strains of *E. coli* in PACs resulted in a slower decrease in adhesion than bacteria cultured in L-CJC. This expanded biofilm studies done in our laboratory where there was an initial detection of biofilm formation for the first few cultures of bacteria in PACs, but culturing of the same strain of *E. coli* in L-CJC resulted in complete inhibition of biofilm development[31]. Longer exposure of bacteria to PACs was necessary to achieve the same effects as L-CJC. This could be explained by the presence of other active compounds in cranberry juice that aid

in the inhibition of bacterial adhesion and biofilm formation[34]; however, more studies need to be done to fully understand how these other compounds work in conjunction with PACs.

The analysis of pull-off distances of bacteria grown in PACs resulted in similar observations than bacteria grown in L-CJC. For our P-fimbriated strain, there were no clear trends observed for the effects of PACs or L-CJC on pull-off distances. However, we observed a decrease in pull-off distances for our non-fimbriated *E. coli* strain with increasing number of cultures in cranberry products. We also noticed that the pull-off distances were of similar magnitude to the L-CJC treated bacteria. While we expected to have short pull-off distances for both of our strains, we believe that L-CJC and PACs not only affect the expression of fimbriae, but the overall strength of the cell surface. Mendez-Villas et al. have observed rupture or indentation of bacterial peptidoglycan by local stress caused by the AFM probe[35]. Our results indicate that growth of bacteria in cranberry juice results in weakening of the surface layer of *E. coli* since long magnitudes of pull-off distances were observed, possibly by indentation of the cells. However, more testing needs to be done to understand this phenomenon more thoroughly.

4.5.3 Correlating Growth in Cranberry Products and Changes in Surface Potentials

We investigated the effects of L-CJC and PACs on the surface potentials of both of our *E. coli* strains. The zeta potential values of *E. coli* are negative at neutral pH due to carboxylic groups located on the surface of bacteria[28]. Non-fimbriated *E. coli* HB101 were more negatively charged than HB101pDC1 (-22.2 ± 0.3 mV, -19.3 ± 0.4 mV, respectively). *E. coli* HB101pDC1 is a mutant strain of the non-fimbriated HB101, where

a plasmid encoding P-fimbriae has been inserted into HB101[24]. The expression of P-fimbriae proteins results in bacteria being more positively charged than HB101 and the electrostatic repulsion between bacteria and uroepithelial cells is then reduced, explaining why these proteins can bind to receptors on uroepithelial cells.

E. coli HB101 and HB101pDC1 grown in L-CJC expressed similar zeta potential values for all cranberry treatments. Compared to the control group, the surface potentials became more positive after culturing in L-CJC. Our results indicated that long-term exposure or growth of bacteria in L-CJC is required to change the electrostatic properties of both strains of *E. coli*. This was supported by previous work done in our laboratory where bacteria were exposed to the same concentration of L-CJC for a short period of time, resulting in no changes of surface charge[36].

Growth of bacteria in PACs resulted in an increase in negative surface charge for both *E. coli* strains. The zeta potentials became more negative with increasing number of cultures in PACs. A similar phenomenon has been observed where the surface potentials of *E. coli* became more negative at higher PACs concentrations[36]. Our results indicated that PAC molecules are adsorbed onto the surface of bacteria. When the surface potentials of bacteria become more negative, we hypothesize that the electrostatic repulsion between bacteria and uroepithelial cells could increase; therefore, inhibiting bacteria-uroepithelial cell interaction.

Our results also indicated that this change in surface potential is reversible, since washing of bacteria with PBS resulted in a return to original surface charge values for both of our *E. coli* strains (Table 4.2). This is well supported by *in vivo* studies, since the

withdrawal of cranberry juice from patients with recurrent UTIs resulted in development of UTI-related symptoms and re-growth of bacteria in the urinary tract[37].

The results of this study indicated that growth of bacteria in cranberry products modifies the properties of bacterial surfaces since a decrease in adhesion was seen for both of our *E. coli* strains. Furthermore, we demonstrated that cranberry compounds not only alter P-fimbriated bacteria but can affect the non-fimbriated *E. coli,* as well. L-CJC and PACs may affect other small molecules on the surface of *E. coli* HB101. We also demonstrated that cranberry products change the surface potentials of both strains of bacteria and that PACs could increase the electrostatic repulsion between bacteria and mammalian cells, although more testing needs to be done to verify this hypothesis. Future research should also be aimed at the analysis of bacteria-mammalian cells interactions, by coating AFM probes with single bacterial cells or adhesins, such as PapG, to study the effects of cranberry juice and PACs on bacterial adhesion.

4.6 Acknowledgments

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Table 4.1 Effects of L-CJC on bacterial zeta potentials. For each experimental condition, the mean ± S.D. zeta potential are presented. A total of 3 runs were made for each condition.

Table 4.2 Effects of continuous growth of bacteria in PACs and washing on zeta potentials. For each experimental condition, the mean ± S.D. zeta potential are presented. A total of 3 runs were made for each condition.

Table 4.1: Effects of continuous growth of bacteria in cranberry juice on zeta potentials

Cranberry culture (#)	Zeta Potentials (mV)	
	E. coli HB101	*E. coli* HB101pDC1
Control (no L-CJC)	-22.2 ± 0.3	-19.3 ± 0.4
1st	-17.9 ± 1.3	-15.2 ± 0.9
4th	-18.5 ± 1.3	-16.2 ± 0.6
8th	-17.5 ± 1.1	-15.6 ± 0.3
12th	-17.1 ± 2.0	-15.1 ± 0.8

Table 4.2: Effects of continuous growth of bacteria in PACs on zeta potentials

Cranberry culture (#)	Zeta Potentials (mV)		Zeta Potentials (mV) after Washing	
	E. coli HB101	*E. coli* HB101pDC1	*E. coli* HB101	*E. coli* HB101pDC1
Control	-22.2 ± 0.3	-19.3 ± 0.4	-22.2 ± 0.3	-19.3 ± 0.4
1st	-23.8 ± 1.2	-23.2 ± 2.9	-19.4 ± 1.4	-18.1 ± 1.6
4th	-26.9 ± 1.1	-22.7 ± 2.2	-18.5 ± 1.0	-18.5 ± 0.9
8th	-33.8 ± 1.5	-28.1 ± 0.8	-17.0 ± 0.7	-20.8 ± 2.0
12th	-35.8 ± 0.7	-29.2 ± 0.6	-18.5 ± 1.3	-18.7 ± 0.1

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Fig 4.1 Representative retraction curves of *E. coli* HB101pDC1 cultured in 10 wt.% L-CJC, probed by a clean silicon nitride AFM tip in 0.01 M phosphate buffer saline (PBS) solution + 0.138 M NaCl + 0.0027 KCl (A). Representative retraction curves for each set of conditions on the effect of bacterial growth in cranberry products on bacterial adhesion (B).

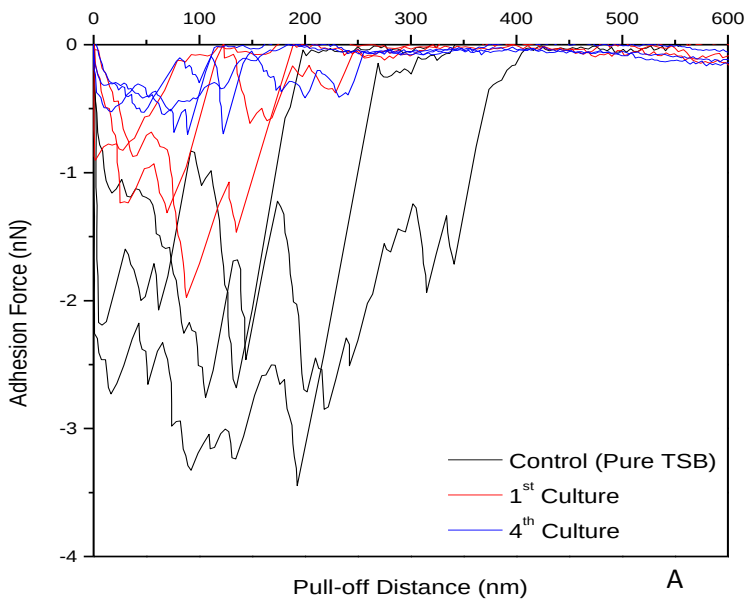
Fig 4.2 Distribution parameters from AFM retraction curves. A) Adhesion forces for *E. coli* HB101pDC1 as a function of culturing time in 10 wt.% L-CJC, n=30; B) Pull-off distance as a function of culturing time in 10 wt% L-CJC, n=30.

Fig 4.3 Distribution parameters from AFM retraction curves. A) Adhesion forces for *E. coli* HB101 as a function of culturing time in 10 wt.% L-CJC, n=30; B) Pull-off distance as a function of culturing time in 10 wt% L-CJC, n=30.

Fig 4.4 Distribution parameters from AFM retraction curves. A) Adhesion forces for *E. coli* HB101pDC1 as a function of culturing time in 128 μg/mL PACs, n=30; B) Pull-off distance as a function of culturing time in 128 μg/mL PACs, n=30.

Fig 4.5 Distribution parameters from AFM retraction curves. A) Adhesion forces for *E. coli* HB101 as a function of culturing time in 128 μg/mL PACs, n=30; B) Pull-off distance as a function of culturing time in 128 μg/mL PACs, n=30.

Fig 4.1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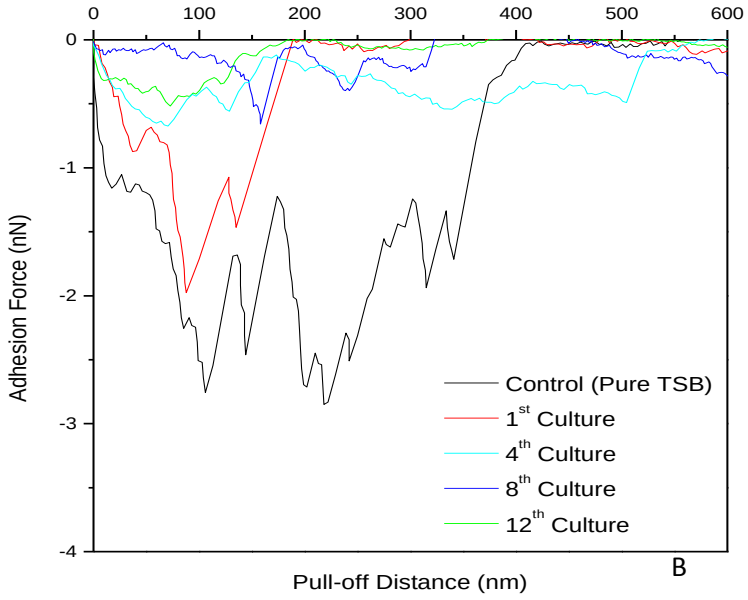
Pull-off Distance (nm)
Control (Pure TSB)
1st Culture
4th Culture
A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-2
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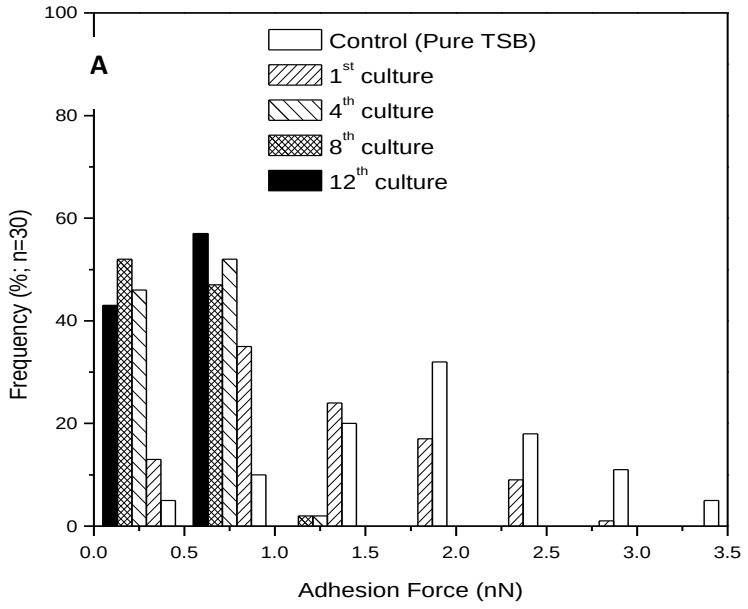
Pull-off Distance (nm)
Control (Pure TSB)
1st Culture
4th Culture
8th Culture
12th Culture
B

Fig 4.2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1st culture
4th culture
8th culture
12th culture
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80
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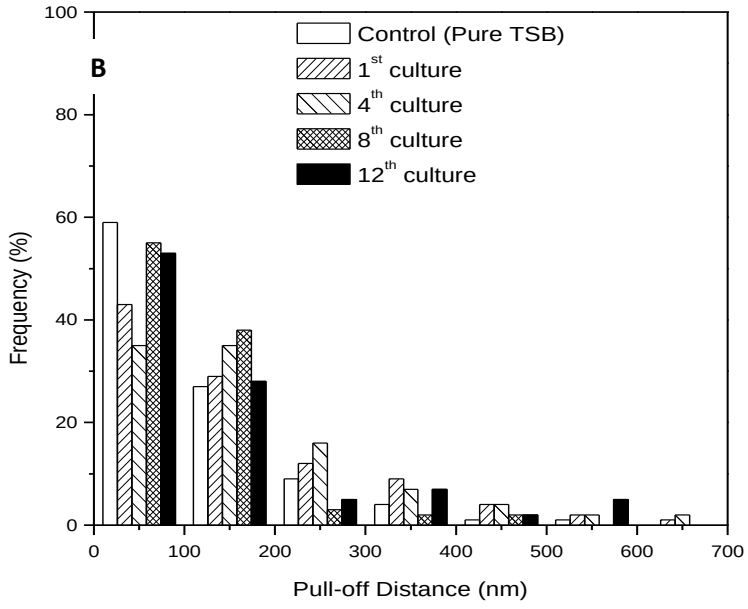
3.5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Control (Pure TSB)
1st culture
4th culture
8th culture
12th culture
Frequency (%)
Pull-off Distance (nm)
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200
300
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500
600
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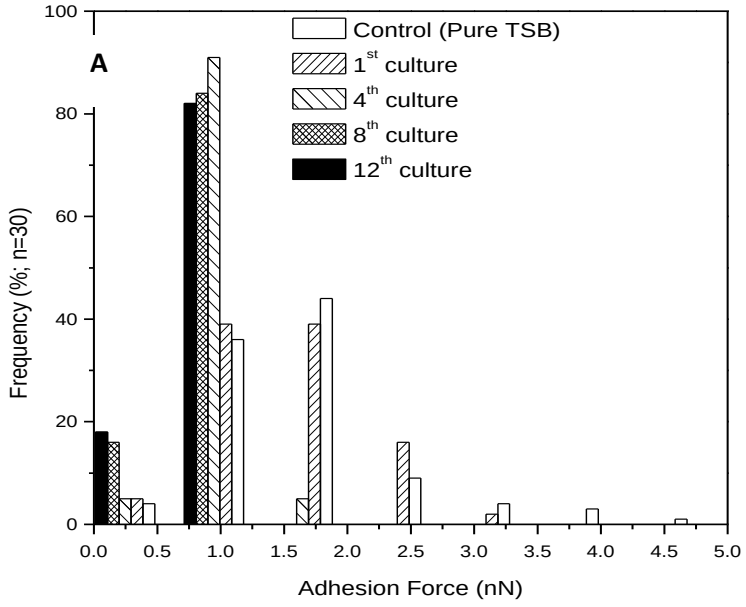
Fig 4.3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Adhesion Force (nN)
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60
80
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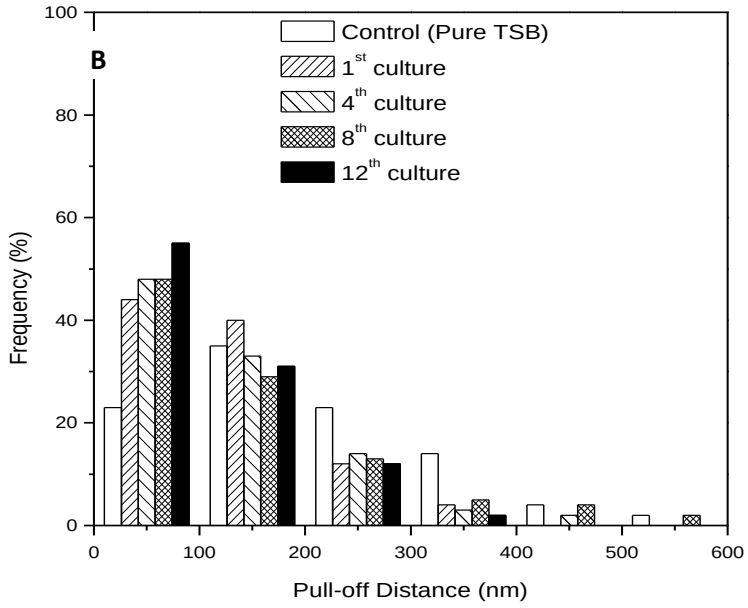
B
Control (Pure TSB)
1st culture
4th culture
8th culture
12th culture
Frequency (%)
Pull-off Distance (nm)
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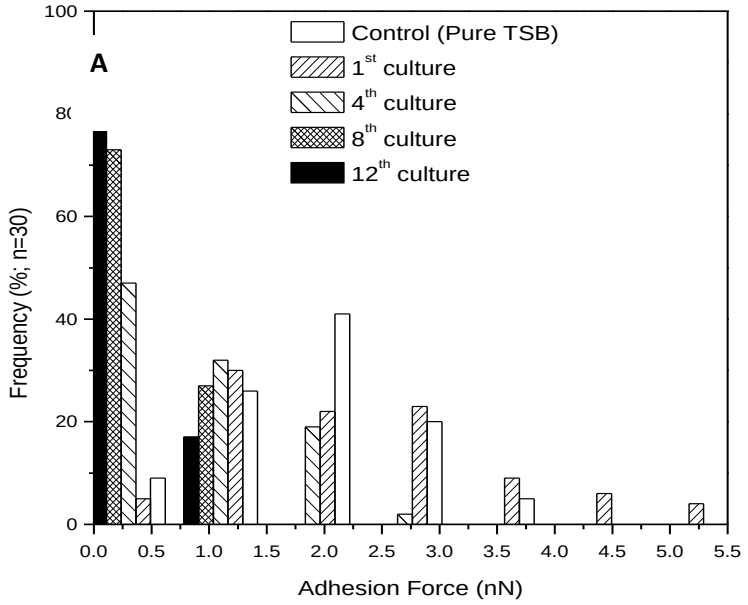
40
60
80
100
0
100
200
300
400
500
600

Fig 4.4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1st culture
4th culture
8th culture
12th culture
Frequency (%; n=30)
Adhesion Force (nN)
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40
60
80
100
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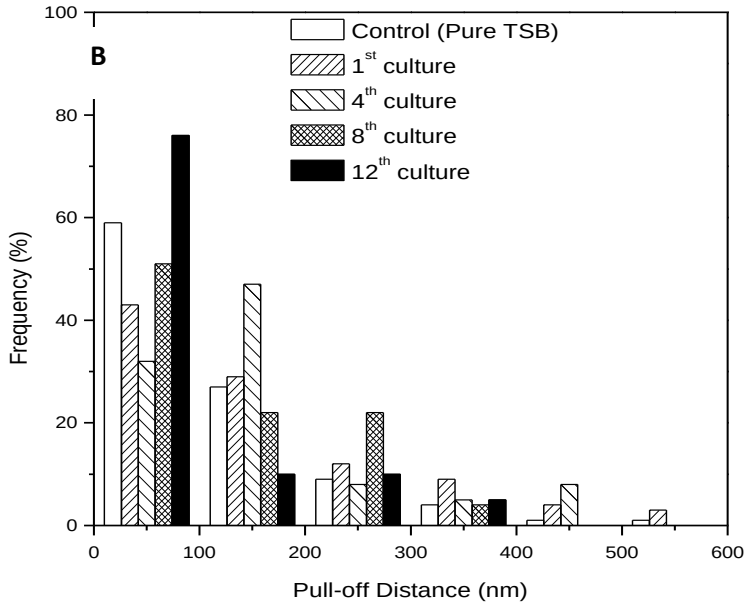
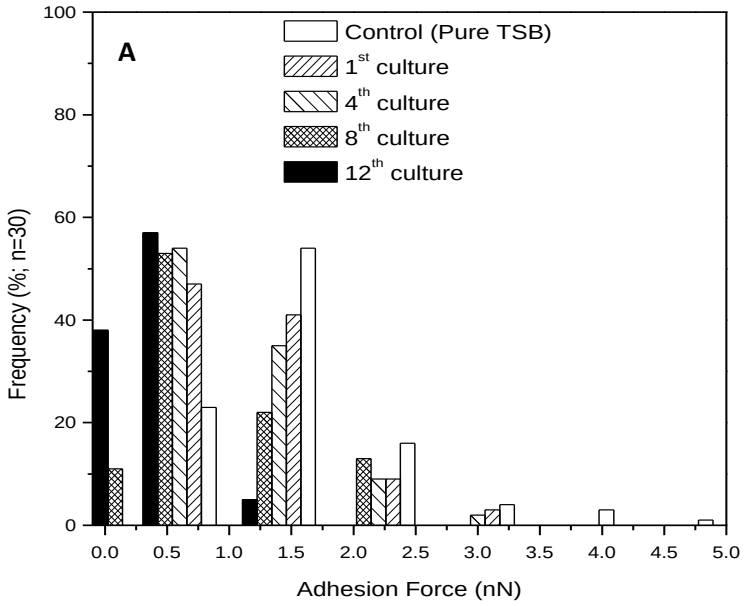
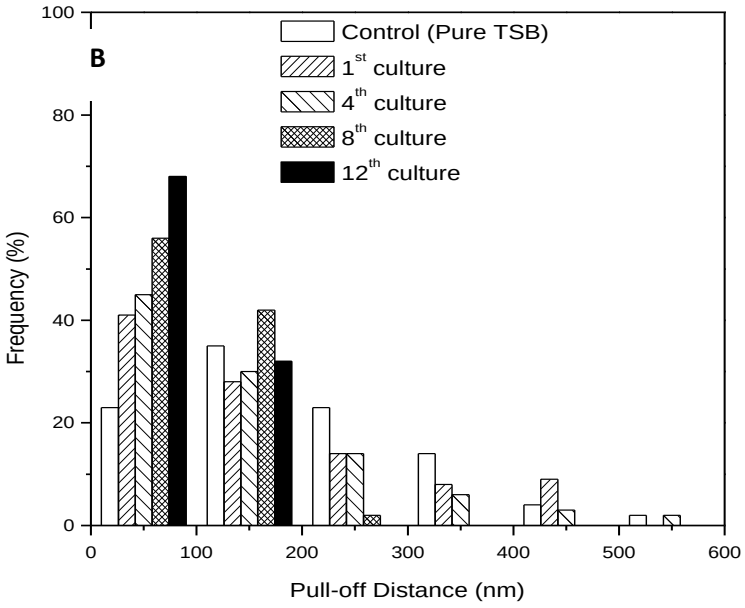

B
Control (Pure TSB)
1st culture
4th culture
8th culture
12th culture
Frequency (%)
Pull-off Distance (nm)
0
20
40
60
80
100
0
100
200
300
400
500
600

Fig 4.5


A
Control (Pure TSB)
1st culture
4th culture
8th culture
12th culture
Frequency (%; n=30)
Adhesion Force (nN)
0
20
40
60
80
100
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0


B
Control (Pure TSB)
1st culture
4th culture
8th culture
12th culture
Frequency (%)
Pull-off Distance (nm)
0
20
40
60
80
100
0
100
200
300
400
500
600

Chapter 5: Cell Surface Assay

Surface and Growth Rate Changes of *Escherichia coli* HB101 caused by Cranberries

5.1 Abstract

The effects of cranberry products on *E. coli* morphology and production of extracellular signals have not yet been addressed. To fully understand the benefits of cranberry juice on inhibiting bacterial adhesion and development of urinary tract infections, it is essential to understand first the changes that cranberries impart on *E. coli* morphology. Experiments were designed to evaluate the effects of light cranberry juice cocktail (L-CJC) and proanthocyanidins (PACs) on *Escherichia coli* HB101; a plasmidless, non-fimbriated bacterium. *E. coli* was continuously cultured in tryptic soy broth supplemented with 10 wt.% L-CJC or 128 μg/mL PACs. The media was successively replaced twelve times. Bacteria from different culture periods were tested for changes in doubling time, bacterial surface morphology and Gram-staining properties, and production of indole. Cells that were repeatedly cultured in L-CJC or PACs resulted in delayed growth. Through Gram staining, changes in cell membrane were demonstrated for bacteria grown in L-CJC, since some of the rod shaped bacteria were able to retain crystal violet after decoloration with alcohol and acetone; this normally being a property of Gram-positive bacteria. We also found that bacteria staining as Gram-positive cells lost their ability to produce the extracellular signaling molecule indole. This investigation suggests that cranberry juice causes changes in the cell surface structure of *E. coli* and the production of essential extracellular signals.

Keywords: Indole, doubling time, Gram variability, proanthocyanidins

5.2 Introduction

Cranberries have long been recognized for their beneficial properties against urinary tract infections (UTIs)[1,2]. The mechanisms of action of cranberries have been linked to inhibition of bacterial adhesion to epithelial cells due to changes in the expression of adhesins found on fimbriae[3-5]. A high molecular weight polymer isolated from cranberries, also known as A-type proanthocyanidins (PACs), has been found to be an active component in cranberry juice that inhibits bacterial adhesion[5,6] While most cranberry research relies on the effects that cranberry juice and PACs impart on bacterial adhesion, no studies have addressed the effects that these products have on bacterial growth, surface changes and production of extracellular signals.

Changes in the physicochemical properties of bacteria as a response of environmental factors have been observed for decades[7,8]. For instance, temperature, pH, and composition of growth media are important factors that can affect bacterial growth, enzymatic reactions, excretion of byproducts and cell membrane functionality[9-12]. Morphological changes in *E. coli* were observed by Ahuja et al., when bacteria were grown in the presence of cranberry juice cocktail[13]. When P-fimbriated *E. coli* were grown in 25% cranberry juice, the cells became elongated and lost their fimbriae. While this elongation of bacteria is rather unusual, it has also been observed in a small percentage of the population of bacteria grown in pure broth as a stress response[8]. When *Bacillus typhosus* and *Bacillus mallei* are far along the stationary growth phase and are starving, the organisms reach their maximum size. *E. coli* and other bacteria usually decrease in size during starvation[8].

Nutritional, chemical and environmental stresses are also associated with changes in membrane composition[14]. The permeability of the cell membrane could also be changed due to the use of organic contents that might be present in growth media. Adaptation to the new cell membrane can result in changes in the overall shape of bacteria as seen in *Pseudomonas putida* CP1[14].

Changes in membrane composition associated with growth conditions can also result in variability of Gram staining[15]. Bacteria are characterized according to their cell wall composition[10]. Gram-positive bacteria possess a thick peptidoglycan layer while Gram negative bacteria are associated with a thin murein layer and a lipid bilayer[16]. Gram variability can be seen due to environmental stressors such as changes in nutrients, temperatures, pH, or electrolytes[17]. For instance, when *E. coli* is grown in excess amounts of glucose, $MgSO_4$, or NaCl, the cells start changing to the Gram-positive state, since they start retaining the crystal violet-iodine complex in their surface[18,19]. Beveridge reported that changes in Gram staining can be associated with different stages of growth phase[17].

Changes of environmental conditions can also affect chemicals that are released by different types of bacteria. The use of chemical signals as a mean of communication among bacteria species is a widespread phenomenon[20,21]. Extracellular signals can have an impact in the development of biofilms, as well as the regulation of functions including DNA transfer, differentiation and virulence[21,22]. For instance, indole is an extracellular signal secreted by *E. coli* in stationary growth phase, that regulates the expression of different genes[21,23]. Changes in growth media can repress the production of this signal, which can result in a decrease of biofilm formation in flow cells[22].

In this study, we investigated the effects that growth in a cranberry-rich media has on *Escherichia coli* HB101. The choice of growing bacteria in cranberry juice or PACs was made on the basis that people are advised to consume cranberry juice to prevent UTIs, which results in bacteria growing in a cranberry-rich environment. We demonstrated that growth of *E. coli* in cranberry juice or proanthocyanidins affects bacterial growth and modifies their membrane structure. Further, we evaluated the expression of indole after cranberry juice treatment and hypothesize how these physico-chemical changes affect the formation of biofilms and potential development of UTIs.

5.3 Materials and Methods

5.3.1 Isolated Proanthocyanidins and Cranberry Juice Preparation

Cranberry PACs were obtained from Ms. Robin Roderick (Ocean Spray Cranberries, Inc; Lakeville-Middleboro, MA). Stock solutions of 345.8 μg/mL PACs in ultrapure water (Milli-Q water, Millipore Corp.; Bedford, MA) were kept refrigerated at 5 ºC until use and away from any light source. A concentration of 128 μg/mL PACs was used for all experiments. The solutions of PACs used were not passed through any filters since we did not want to lose any essential components that might have affected the results of the study.

Light cranberry juice cocktail (L-CJC) was purchased (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA). Sodium hydroxide (Sigma-Aldrich, St. Louis, MO) was used to neutralize the pH of the juice and the solution was passed through a 0.2 μm Acrodisc® syringe filter to reduce all possible contaminants. A concentration of 10 wt.% L-CJC was used for all experiments.

5.3.2 Bacteria and Growth Conditions

E. coli HB101 from the American Tissue Culture Collection (ATCC 33694) was grown as previously described[24]. Briefly, bacteria were grown to middle exponential phase in 30 g/L tryptic soy broth (TSB; Sigma, St. Louis, MO) in the presence of 10 wt.% L-CJC or 128 μg/mL PACs. Growth rates and doubling time were analyzed by taking samples every 30 minutes and measuring the optical density of the solution at 600 nm (Thermo Spectronic, Rochester, NY). After harvesting, a 1 mL volume of bacterial solution was transferred to a new flask of fresh media and left shaking at 37 ºC to middle exponential growth phase. This was considered the second culture in the cranberry-rich environment. Successive transfer and continued culture of bacteria in TSB, and cranberry products was done a total of twelve times. *E. coli* HB101 grown in pure TSB served as control for all experiments.

5.3.3 Gram-Staining Procedure

Cultures 2, 4, 6, 8, 10, and 12 were Gram-stained after harvesting. After growth, 100 μL of bacterial solution was placed on a clean glass slide and heat fixed. Crystal violet, stabilized iodine and safranin (BD Biosciences; San Jose, CA) were used to stain the cells and all excess dyes were washed off with ultrapure water. The slides were air dried. Bacteria were observed with an oil immersion 100x, 1.33 numerical aperture objective, with a DIA ILL (A) filter block, on a Nikon Eclipse E400 microscope (Tokyo, Japan).

Changes in Gram staining were characterized by plating bacteria from different culture times onto agar that was selective for the growth of Gram-positive and Gram-

negative bacteria. For the growth of Gram-negative bacteria, Levine eosin-methylene blue rich agar media was used, while colistin and nalidixic acid rich media was used for the plating of Gram-positive organisms (Becton Dickinson Microbiology Systems; Sparks, MD). Plates were incubated at 37ºC for 24 hours.

5.3.4 Indole and Glucuronidase Characterization

The production of glucuronidase and indole were tested by using the ColiScreen screening test for the identification of *E. coli* (Hardy Diagnostics; Santa Maria, CA). Briefly, from an 18-24 hour old culture, a heavy and visible inoculum of bacteria was collected and stabbed into a tryptophan and nitrophenyl-beta-glucopyranosiduronic acid rich media. The caps of the tubes containing the media were loosened and the tubes were placed in an incubator at 35ºC for 5 hours. A change in color in the media, from clear to yellow, indicated that bacteria produced glucuronidase. Indole production was tested by placing 5 drops of Kovacs indole reagent (Hardy Diagnostics, Santa Maria, CA) into the media once the production of glucuronidase had been confirmed. The appearance of a pink or red color in the top alcohol layer indicated a positive reaction.

In accordance with the manufacturer, different strains of bacteria were used to check for signs of contamination or deterioration of the product. For the characterization of the production of indole, *Pseudomonas aeruginosa* AK1401 and *Staphylococcus epidermidis* were used as negative controls[25-27].

5.4 Results

5.4.1 Changes in Doubling Time Due to Cranberry Constituents

The time it takes *E. coli* bacteria to undergo one binary fission step was obtained for all treatment conditions (Table 5.1). *E. coli* HB101 grown in pure TSB (no cranberry treatment) had a doubling time of 58.1 min. After incorporating L-CJC in the media, there was an increase in doubling time with increasing number of cultures. After culturing *E. coli* in 10 wt.% L-CJC six times, the doubling time almost tripled (58.1 min vs. 152.4 min). Continuous culture of *E. coli* HB101 in L-CJC resulted in a decrease in binary fission time after the 6^{th} culture where a doubling time of 50.5 min was obtained for bacteria cultured 12 times in L-CJC.

Growth of bacteria in PACs resulted in a similar effect. PACs-treated bacteria resulted in increasing doubling times reaching a maximum of 198.4 min during the 6^{th} culture. After this time, doubling times started decreasing again and growth rates returned to baseline values after the 12^{th} culture.

Replicates of this experiment were done several times and qualitatively, the same pattern was observed for all experiments, an initial increase in doubling time followed by a decrease after reaching a maximum value. However, the increase and decrease of doubling times were seen at different time cultures, preventing us from doing any statistical analysis since it would yield very large standard deviations. A representative experiment was chosen to illustrate the effects of cranberry products on *E. coli* binary fission.

5.4.2 Variations in Gram Staining Due to Cranberry Products

Bacteria grown in the presence of L-CJC and PACs were Gram-stained. *E. coli* HB101 cultured in pure TSB (no L-CJC) strained red or pink since all the crystal violet was washed off the cells after the decolorization step (Figure 5.1A). Changes in staining were seen in cultures 6 and 8 of *E. coli* HB101 grown in L-CJC (Figure 5.1B). A significant percentage of bacterial cells retained the crystal violet-iodine complex and resulted in a blue/purple color when observed under the microscope. Further growth in L-CJC did not return all bacterial cells to the Gram-negative state, and a mixture of colors was seen in subsequent cultures.

The presence of PACs in growth media did not result in Gram-staining changes. *E. coli* HB101 from all cultures in PACs lost the crystal violet dye during the staining process and retained the safranin color, resulting in 100% of cells staining red.

5.4.3 Effects of Cranberry Products on Indole and Glucuronidase Production

E. coli HB101 was tested for the production of indole and glucuronidase for all treatment conditions (Table 5.2). The production of indole alone cannot be tested for the identification of *E. coli* strains, since there are other Gram-negative bacteria that produce this organic compound. Therefore, the test used relies on the production of both indole and glucuronidase.

E. coli HB101 grown in pure TSB tested positive for the production of indole and glucuronidase since the agar turned bright yellow with a bright pink alcohol layer on top. The incubation time required for the production of both compounds was 1.5 hrs for the control strain. Growth of the same strain in 10 wt.% L-CJC resulted in the production of

glucuronidase after 5 hrs of incubation, and it was denoted by a change in agar color. However, no development of pink was seen for this condition after addition of the Kovacs reagent, indicating that this strain was not producing indole. The growth of *E. coli* HB101 in PACs resulted in the production of both compounds after 2 hrs of incubation.

To test for contamination of the agar or deterioration of the chemicals used, a Gram-negative and a Gram-positive bacterium with well known production of enzymes were tested and used as our negative controls. After a 2 hrs incubation of *P. aeruginosa* AK1401 at 35 ºC, there was a development of a light yellow in the agar, which indicated the production of glucuronidase. The indole test was negative since there was no development of a pink color. The Gram-positive organism *S. epidermidis* tested positive for both compounds. The results were as expected, according to manufacturer's quality control data.

5.5 Discussion

There has been extended research on the effects of cranberry on bacterial adhesion and prevention of UTIs[4,5,28]. The scope of this study was to explore how cranberry juice and cranberry proanthocyanidins affect physico-chemical properties of *E. coli* and how these changes might relate to inhibition of bacterial adhesion. The choice of growing bacteria in cranberry products was made on the basis that patients that are prone to UTIs are advised to consume cranberry juice for long periods of time, which will result in the growth of bacteria in a cranberry-rich environment. Growth of bacteria in cranberry

products offers the advantage of simulating physiological conditions through *in vitro* experimentation.

The growth of *E. coli* in cranberry juice resulted in changes in bacterial growth rate since there was an increase in doubling time. After six continuous cultures of *E. coli* HB101 in L-CJC or PACs, binary fission reached a maximum doubling time that was three times larger than that of bacteria grown in pure TSB. The increase in doubling time can be assumed to delay development of UTIs since this could result in retardation of biofilm development. We also observed that after six cultures in cranberry media, the doubling times started decreasing and returned to normal growth rates. Perhaps the change in doubling time is related to bacteria undergoing an acclimation process since they are faced with a new environment.

Changes in Gram-staining were also seen after bacteria were grown in L-CJC. After growth of *E. coli* HB101 in L-CJC for six times, ~25 percent of the planktonic cells stained as Gram-positive. After associating these results to the increments in doubling times, we find it likely that the slow growth of bacteria in L-CJC results in more synthesis of peptidoglycan layers within the cell wall, which results in the retention of crystal violet-iodine complex that is characteristic of Gram-positive cells. A similar, but converse phenomenon was observed by Beveridge, where an increase in growth rate in Gram-positive bacteria resulted in less production of peptidoglycan and thinner cell walls[17,29]. These Gram-positive cells became sensitive to the Gram staining procedure and lysed, which resulted in loss of the crystal violet-iodine complex.

E. coli HB101 that stained as Gram-positive cells were isolated using selective growth media and tested for their production of indole. The production of glucuronidase and indole have been widely used as specific markers for the identification of *E. coli* species[21]. *E. coli* grown in L-CJC did not produce indole after being cultured in cranberry-rich media six times. Since expression of indole has been tightly linked to formation of biofilms in *E. coli* species, it is likely that these bacteria will be impaired in biofilm formation, since the extracellular signal is not being produced[30]. This was well supported by previous studies in our laboratory where growth of *E. coli* HB101 and the P-fimbriated strain HB101pDC1 in 10 wt.% L-CJC resulted in a complete inhibition of biofilm formation on a polyvinyl-chloride abiotic surface[28].

E. coli HB101 grown in PACs produced indole and retained their Gram-negative characteristics at all times. This suggested that PACs alone cannot inhibit the development of biofilms. This can be supported by previous research in which biofilm development was observed for bacteria grown in PACs[28].

The results of this study indicated that growing *E. coli* in cranberry juice modifies the cellular wall; as was observed through Gram staining and alterations of doubling times. Furthermore, we demonstrated that following modification of the cellular wall, *E. coli* grown in L-CJC stops producing the extracellular signal indole, which has been associated with inhibition of biofilm formation[28]. While we do not understand how the biofilm genetic pathway is being affected by cranberry juice, we hypothesize that a gene encoding the tryptophanase enzyme is being deactivated, preventing the cells from transmitting signals to develop biofilms. Our study is the first to address how cranberry juice affects the expression of extracellular signals and changes in the cellular wall. It

would be advantageous for future studies to focus on identifying the specific genes that are being influenced by cranberry juice. Hence, this could lead to synthesizing better prophylactic treatments for the prevention of urinary tract infections.

5.6 Acknowledgments

Thanks to Ms. Robin Roderick (Ocean Spray Cranberries, Inc.) for providing us with cranberry proanthocyanidins. We also thank Dr. Reeta Prusty Rao, Yatao Liu and Joshua Strauss for their assistance in this project. This work was supported by the National Science Foundation (BES 0238627 and EEC 0452809) and the Cranberry Institute and Wisconsin Cranberry Board. The first author received additional fellowship support from the American Association of University Women.

Table 5.1: Changes in doubling time for *E. coli* HB101 cultured in the presence of L-CJC and PACs

Culture (#)	Doubling time (min) in cranberry products	
	10 wt.% L-CJC	128 μg/mL PACs
Control[a]	58.1	58.1
2nd	81.1	76.3
4th	78.2	89.2
6th	152.4	198.4
8th	137.1	112.0
10th	72.6	77.3
12th	50.5	63.8

[a] Bacteria grown in pure TSB (no cranberry treatment)

Table 5.2: Effects of cranberry products on the production of glucuronidase and indole in bacteria.

Organism	Incubation Time (hr)	Chemical Production[b]	
		Glucuronidase	Indole
E. coli HB101 (control[a])	1.5	+ +	+ +
E. coli HB101 (10 wt. % CJC) 6th culture	5	+ +	–
E. coli HB101 (128 μg/mL PACs) 6th culture	2	+ +	+ +
P. Aeruginosa (AK1401)	2	+	–
S. epidermidis (clinical isolate)	5	+ +	+

[a] Bacteria cultured in pure TSB

[b] Production of glucuronidase and indole. + + positive reaction (bright yellow or pink); + positive reaction (light yellow or pink); – negative reaction (no color change)

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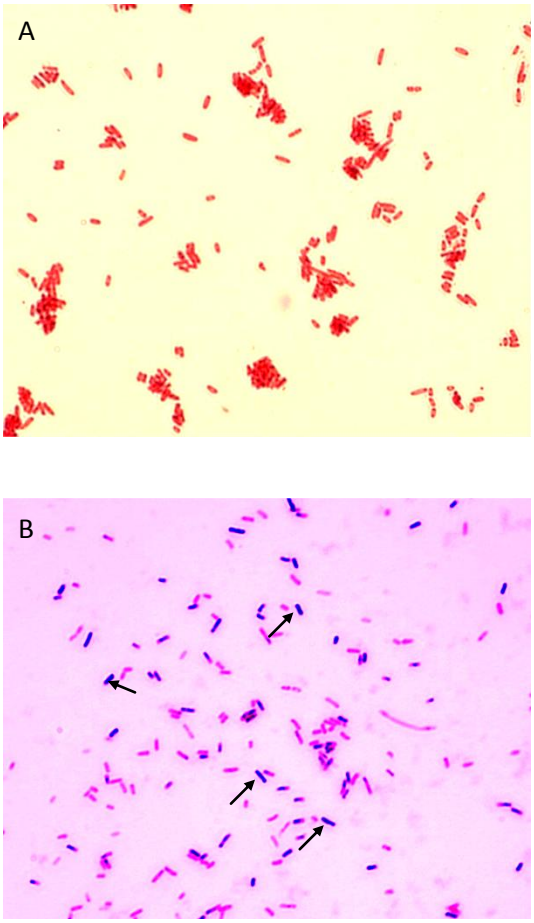
Fig 5.1 Gram staining of *Escherichia coli* HB101. **A,** *E. coli* HB101 cultured in pure TSB (0wt. % CJC). **B,** *E. coli* HB101 cultured 6 times in 10 wt.% CJC. A significant percentage of bacteria show signs of conversion of cells normally Gram-negative to the Gram-positive state (black arrows). Cells stained at OD_{600}~0.9, magnification 1000x.

Fig 5.1



Chapter 6: Research Summary

Throughout this project, we have sought to better understand the mechanisms by which cranberry juice prevents the recurrence of UTIs. In particular, we have made emphasis on evaluating how light cranberry juice cocktail (L-CJC) and isolated cranberry proanthocyanidins (PACs) affect individual bacterial cells through the use of atomic force microscopy (AFM) and light microscopy. We have continuously grown *E. coli* bacteria in the presence of PACs and L-CJC to simulate what happens in the human body once people start consuming cranberry products for long periods of time. Long-term consumption of cranberry juice will result in growth of bacteria in a cranberry-rich environment. We were the first group to evaluate how cranberry affects the attachment of *E. coli* through these conditions.

In Chapter 3, we investigated the effects of both L-CJC and PACs on the attachment of *E. coli* HB101 and HB101pDC1 to human uroepithelial cells. We evaluated the effects of short-term exposure of bacteria to increasing concentrations of L-CJC and PACs to determine what threshold concentration of cranberry constituents is needed to significantly reduce the number of bacteria attached per uroepithelial cells after incubation. We also tested long term growth of bacteria in a cranberry-rich environment and determined that attachment of bacteria to uroepithelial cells decreases with increasing concentrations or number of cultures in L-CJC and PACs for our P-fimbriated *E. coli* strain. We also evaluated the effects of cranberry products on formation of biofilms on a model surface (polyvinyl-chloride), and concluded that growth of bacteria in L-CJC

resulted in total inhibition of development of biofilms. Growth of *E. coli* in PACs resulted in initial biofilm formation; however, this ability decreased with increasing number of cultures in PACs.

In Chapter 4, we evaluated the effects of growth of bacteria in L-CJC and PACs on the adhesion forces between single *E. coli* bacteria and a silicon nitride AFM tip. We determined that growth of bacteria in L-CJC or PACs reduce the adhesion forces significantly for both of our *E. coli* strains. This indicated that cranberry constituents not only affects the proteinaceous fimbriae on the surface of *E. coli* HB101pDC1 but also affects small molecules on our non-fimbriated strain. We also investigated the impact of L-CJC and PACs on the zeta potentials of both *E. coli* strains and determined that growth in PACs results in more negative surface charges with increasing number of cultures, which may increase the repulsion forces between bacteria and epithelial cells, therefore inhibiting bacterial attachment.

In Chapter 5, we attempted to understand the effects of growth in cranberry juice on doubling time, morphology and changes in production of extracellular signals in *E. coli.* Our results indicated that growth of *E. coli* in L-CJC or PACs delays bacterial growth rates for some time, although bacteria were eventually able to adapt to the cranberry environment. Bacteria were also Gram stained for studies of cellular structure and we found that growth of bacteria in L-CJC resulted in development of Gram-variability within our *E. coli* strain. Cells staining as Gram-positive started appearing after 6 continuous cultures in L-CJC. These cells were tested for production of extracellular signal indole, which has been linked to development of biofilms. "Gram-

positive" *E. coli* cells did not produce indole which helped explain the inability to form biofilms on a PVC substrate that we observed previously.

This research has provided valuable information for the mechanism of action of cranberries in the inhibition of bacterial adhesion. We have attempted to simulate physiological conditions through the design of *in vitro* experiments and the results have provided a number of tools and techniques by which the prevention of UTIs through the consumption of cranberry juice can be further investigated. This information can be used in future experiments to continue the study of cranberries and other natural products for the prevention of bacterial adhesion and biofilm formation.

Chapter 7: Future Work

It is important to understand what happens to cranberry juice during digestion. At the moment, little is known about the amount of PACs that get into the urinary system and what dosage of cranberry juice is effective to prevent biofilm formation and UTIs. In a previous study, isolated *E. coli* bacteria from the urine of volunteers who consumed 240 mL of cranberry juice showed an anti-adhesive activity utilizing a mannose-resistant human red blood cell hemagglutination assay that was specific for P-fimbriated bacteria[1]. The fact that bacteria collected from urine were not able to agglutinate to red blood cells indicated that at least some PACs reach the urinary system and that are not altered during digestion.

We have started collaborating with Dr. Amy Howell from Rutgers University in NJ, in an attempt to characterize the amount of PACs that are found in urine after consumption of different concentrations of cranberry juice cocktail. Dr. Howell will be collecting urine from volunteers who have consumed cranberry juice where urine collection will be made over a 6-hour period. The metabolites found in urine will be freeze dried and resuspended in physiological PBS. We will measure the adhesion forces of a clinical *E. coli* strain using the resuspended metabolites as our AFM fluid, and assess whether the presence of PACs in the media alters the adhesion forces between *E. coli* and silicon nitride.

Future experiments can also investigate the possibility of developing a cranberry or PACs-rich material for the coating of urinary catheters. This would allow the catheter

to stay in place without any risks of development of biofilms and UTIs. Issues such as biocompatibility and bioactivity of the biomaterial need to be addressed during the design of the material. Also it is imperative for the coating of the catheter to retain its structure and properties after implantation and for the duration of catheterization.

REFERENCES

1. Howell, A. B., Reed, J. D., Krueger, C. G., Winterbottom, R., Cunningham, D. G. & Leahy, M. A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity. *Phytochemistry* **66**, 2281-91 (2005).