

**INVESTIGATING THE INHIBITORY EFFECTS OF
CRANBERRY JUICE METABOLITES ON UROPATHOGENIC
ESCHERICHIA COLI FOR THE PREVENTION OF
URINARY TRACT INFECTIONS**

A Thesis

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Life is an adventure. I am ready and excited to start next phase of life.

Tomorrow is another day! Cheers!

Abstract

Regular ingestion of American cranberry (*Vaccinium macrocarpon*) has been traditionally utilized for its health benefits against urinary tract infections. The proanthocyanidins (PACs), in particular, the unique A-type double linkages of PACs present in cranberry, have been identified as the active components. However, A-type PACs and any other active agents have not yet been detected or identified in urine. Additional experiments are required to investigate the inhibitory effects and persistence of cranberry metabolites present in urine collected following CJC consumption, and to determine how these compounds act against uropathogenic *Escherichia coli* for the prevention of urinary tract infections.

Two separate bioassays (a biofilm formation assay and a bacterial cell viability assay) were used to determine the *in vitro* effect of cranberry juice cocktail (CJC) oral consumption on bacterial anti-adhesion activity in a double-blind, placebo-controlled pilot clinical trial. A single dose of 16 oz. of CJC or a placebo beverage was given to ten healthy women, ages ranging from 18 to 27, and urine samples were collected in the following 48 hours. A washout period of seven days was allowed. Bacteria (*Escherichia coli* B37, CFT073, BF1023, HB101, and *Staphylococcus aureus* ATCC43866) were cultured in the urine samples, supplemented with media, and the amount of biofilm formed was measured using a crystal violet absorbance assay in a 96-well plate. In the urine of volunteers who had consumed CJC, biofilm formation was inhibited within 24 hours after CJC consumption, and started to increase after 48 hours by 49-67%. *S. aureus* showed the least biofilm formation after incubation with post-CJC urine. The results indicated that drinking CJC can be an effective preventive measure for bacterial adhesion and biofilm formation in healthy women. The anti-biofilm activity peaks between 24 and 48 hours after drinking CJC. The viability assay showed that the colony count after culturing in

urine collected following consumption of CJC or placebo were not significantly different, implying that CJC works as an inhibitor by blocking bacterial adhesion instead of killing the bacteria or restraining its growth.

Another randomized, placebo-controlled, double-blind, crossover study was conducted to further investigate the molecular-scale effect of cranberry juice metabolites on two P-fimbriated *E. coli* strains: B37 and CFT 073, as assessed by atomic force microscopy (AFM). Three female subjects were asked to consume 8 oz. CJC or water. The washout period was 7 days. The urine samples were collected at 2, 4 and 6 hours post-ingestion of CJC or water. Urine collected before consumption of CJC was used as a control. For this control urine, the average adhesion force between *E. coli* and uroepithelial cells was 13.09 ± 11.60 nN for CFT073 and 10.30 ± 5.50 nN for B37. For post-CJC urine treatment, the adhesion forces decreased to 2.94 ± 1.82 nN at 2 hours after consumption then increased slightly to 5.51 ± 2.78 nN at 6 hours after ingestion for CFT073, while they decreased to 4.77 ± 3.33 nN after consuming for 2 hours and seemed to be stable in the next 4 hours following consumption (5.52 ± 4.04 nN after drinking for 4 hours; 5.05 ± 4.42 nN after drinking for 6 hours) for B37. The adhesion forces in post-water consumption urine were similar to those of the background for *E. coli* B37; meanwhile a downward trend for the adhesion forces in post-water consumption urine compared to the background was observed for *E. coli* CFT073. However, these adhesion forces in post-water consumption urine were still higher than those measured after CJC consumption at the same time intervals. The mean differences between the cranberry and placebo groups were statistically different according to the two way ANOVA procedure followed by Holm-Sidak test. Our results suggest a significant inhibitory interaction between the daily consumption of 8 oz. cranberry juice and bacterial adhesive activity.

These results help form the mechanistic understanding of how cranberry products can be used to prevent bacterial attachment to host tissue, and may lead to new therapeutic strategies to prevent the rising problem of bacteria antibiotic resistance.

Authorship

The contents of this thesis are a representation of the work done by the main author. Contributions to this project were made by Yuanyuan “Angela” Tao, a Master of Science graduate in Chemical Engineering at Worcester Polytechnic Institute. She did part of experiments for the biofilm formation assay. Paola A. Pinzón-Arango, a Ph.D. candidate in Bimolecular Engineering at Worcester Polytechnic Institute, also performed part of the experiments for the biofilm formation assay.

Racquela Richard participated in the 2010 REU program at WPI and assisted in the experiments related to bacterial cell viability. Regina Roberto from Health Services, Worcester Polytechnic Institute, helped us with recruiting volunteers and sample collection for the clinical trial. Dr. Amy B. Howell from Rutgers University, NJ provided us urine samples for the AFM study.

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Chapter I: Research Motivation

Urinary Tract Infections (UTIs) are among one of the most common bacterial infections affecting humans. It has been estimated that one out of three women experience a UTI at least once in her lifetime [1]. The cost of direct and indirect treatment exceeds two billion dollars per year [2]. Bacteria, especially gram-negative *Escherichia coli* (*E. coli*), are the most predominant cause of UTIs [3]. Due to the lack of knowledge of effective dose for the antibiotics to treat UTIs and the increasing concern of antibiotic-resistant bacteria [4], alternative therapies to prevent and treat UTIs are needed.

American cranberry (*Vaccinium macrocarpon*), a native fruit to North America, has a long history of use to prevent UTIs [5-9] and is utilized widely to maintain urinary tract health. However, the active components and molecular mechanism of cranberry's actions are still not well documented. Its natural high acid content was thought to be responsible for its health benefits for generations [10-12]. However, this theory was disproved by later studies [13, 14]. Researchers today focus on the anti-adhesion mechanism, with the predominant theory being that cranberry juice inhibits bacteria from attaching to uroepithelial cells, interfering with the important initial step in the development of an infection [15, 16]. Recently, A-type proanthocyanidins (PACs) present in cranberry were identified as the agents that cause this anti-adhesive effect [17, 18]. However, there are few reported studies addressing whether the active compounds can survive in the digestive system. Furthermore, whether A-type PACs eliminate the colonization of uropathogenic *E. coli* known as biofilm or whether they act on uroepithelium directly remains unclear. This study was designed to address these questions. We believe that these results could help understand how cranberry products can be used to prevent bacterial

attachment to host tissue, and may lead to the development of better therapies for the prevention of UTIs.

Chapter II Literature Review

2.1 Urinary Tract Infections

The function of the urinary system, including the urethra, bladder, ureters, and kidney, is to maintain proper water and salt balance throughout the body. Healthy urine is generally sterile even though it contains a variety of fluids, salts, and waste products. When a bacterium invades one or more parts of the urinary system, grows and multiplies, the infections develop. UTI is defined as the presence of bacteria in the urinary tract system [19].

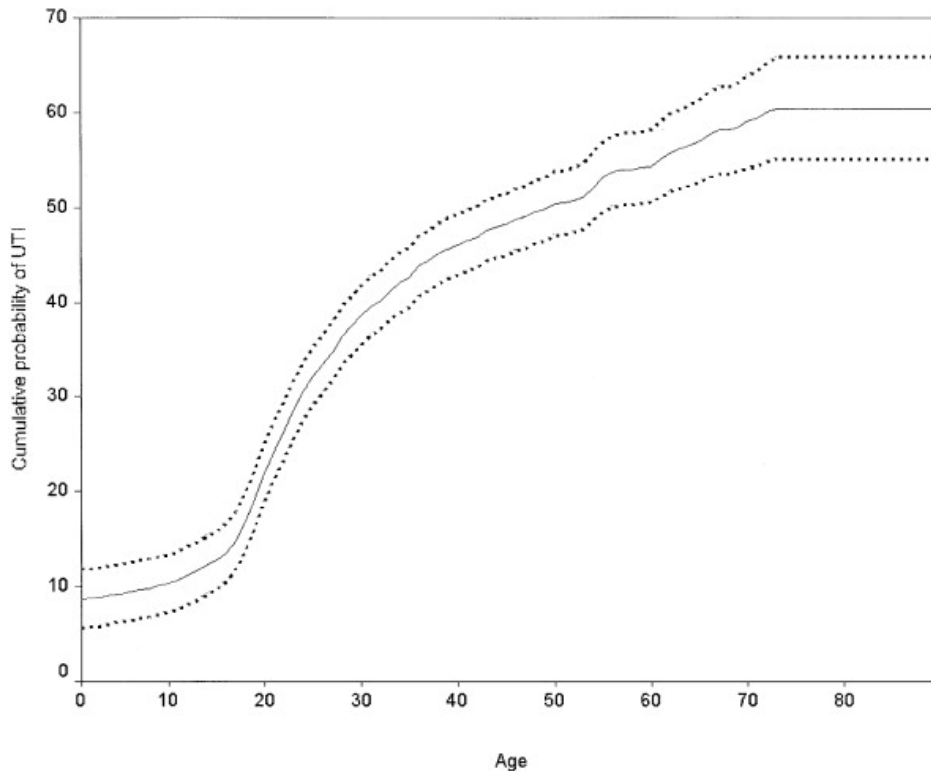


Figure 2.1 Cumulative probability of self-reported physician-diagnosed urinary tract infection by age among 2000 women in the United States participating in a random digit dialing survey [1]

UTIs account for 80% of all bacterial infections affecting humans today, and are especially prevalent in females since they have shortened urethra [8] compared to males. It is estimated that about 60% of healthy women suffer symptoms of UTI [2] at sometime in their life

and many will experience them several times, with a significant incidence increase (Figure 2.1) related to age [1, 9, 20]. The management of UTIs accounts for over 9.6 million office visits to physicians annually, with the cost per visit nearly \$100 [21].

2.1.1 Acute Cystitis and Pyelonephritis

A UTI can occur either from the bottom of the urinary tract systems or from the upper part. If bacteria multiply at the opening of the urethra and travel up to the bladder, acute cystitis (bladder infection) can develop, which is the most common type of UTI. A more serious condition is pyelonephritis (kidney infection), caused by bacteria spreading from the bloodstream to the kidney. Cystitis has a high recurrence rate [22]. Urine is generally a good culture medium for bacteria, and this can trigger a recurrent infection [23].

2.1.2 Pathogenesis and Virulent Factors

Since a healthy urinary system is generally sterile, bacterial entry and proliferation is a must for UTIs to occur [7]. They can be caused by any number of bacteria but are most often brought on gram-negative bacteria, especially *Escherichia coli* (*E. coli*). Uropathogenic strains of *E. coli* account for 85-95% of cystitis cases and 90% of acute pyelonephritis infections [3, 8], while *Staphylococcus saprophyticus* is the cause in 5-10%. *E. coli* is normally present in the intestines and can spread by, entering through the opening of the urethra where urine is excreted. Most of the time, the immune system eliminates *E. coli* that colonizes in the wrong areas of the body. However, when this mechanism fails, *E. coli* can grow, multiply, travel up the urinary system, and cause infection in the form of cystitis, and possibly reaching your kidneys.

Bacterial adherence to mucosal cells has been considered as a critical step in the development of infection. *E.coli* adhesins, the fimbrial structures, which aid bacteria to adhere to

the surface of the urinary epithelium [6] have been studied, especially type I pili [24] and P fimbriae [25]. Type I pili mediate bacteria to adhere to mannose-specific receptors on urinary epithelium and are expressed in almost every uropathogenic *E. coli* isolate [26]. Their presence at the bacterial surface between isolates causing cystitis or pyelonephritis is the same [27]. P fimbriae, which have a terminal receptor for the 'P' antigen [8] (a blood group marker), not only binds to red cells, but also helps bacterial adhesion to the disaccharide alpha-D-Gal(1-4)-beta-D-Gal specific receptors [28], resulting in a biofilm on the surface of the urinary epithelium and catheter [26].

2.1.3 Treatment and Problems

Although UTI is considered a minor illness, it may cause severe discomfort [8]. If the bacteria have inflamed the kidneys and bladder, the patient may feel pain in the lower back or pelvis. However, they are also easy to treat and prevent with a course of antibiotics which are commonly used.

Although antibiotics are used routinely for treatment of UTIs, knowledge of the dose is still not well documented. Hence, these agents are not always effective. Overtreatment can cause such side effects sometimes as nausea, diarrhea, Candidal infections or even poses a threat of complications. Certain groups, especially women, are more prone to repeated infections. Recurrences frustrate the patient and may contribute to the development of bacterial antibiotic resistance. It has been suggested that uropathogens (e.g. 15-20 % or more of *E. coli* strains) are continuously resistant to antibiotics in the United States [4] and worldwide. In the case of kidney infection, the patient may require a hospital stay and intravenous antibiotics to treat. Due to the need to supplement bactericidal therapeutic strategies, alternative therapies that inhibit bacterial adhesion processes are increasingly important. Consumption of American cranberry (*Vaccinium*

macrocarpon) has been utilized for prevention of urinary tract infections for decades [7, 8, 15, 21, 29, 30].

2.2 Biofilms

A biofilm is defined as a matrix-enclosed bacterial population adherent to a surface or interface [31]. Biofilms are common in nature and they can be regarded as a crucial survival mechanism that hinders the eradication of bacteria [32] from the environment.

2.2.1 Development of Biofilms

A biofilm is a complex polymer aggregate of microorganisms growing on surface (Figure 2.2). Initially the free-floating microorganisms attach to the surface by weak van der Waals forces, which are reversible. The attached microorganism could be easily separated from the surface and immediately washed away by fluids. If it does not, the microorganism can use its specific cell adhesion molecules such as pili to anchor themselves to the surface permanently. Bacteria living in the biofilm can exhibit significantly different properties from free-floating bacteria due to the different patterns of gene expression [33]. At a higher level of organization, the dense extracellular matrix and the outer layer of cells could help protect the bacteria inside the film to withstand phagocytic cells and host immune responses. Also, the bacteria within biofilms have increased resistance to detergents and antibiotics compared to the non-attached planktonic ones. For this reason, the antimicrobial may only kill the bacteria in the outer layers of the biofilm, allowing the healthy bacteria within it to regrow rapidly. Beyond that, repeated use of antimicrobial agents can cause bacteria to develop resistance [4].

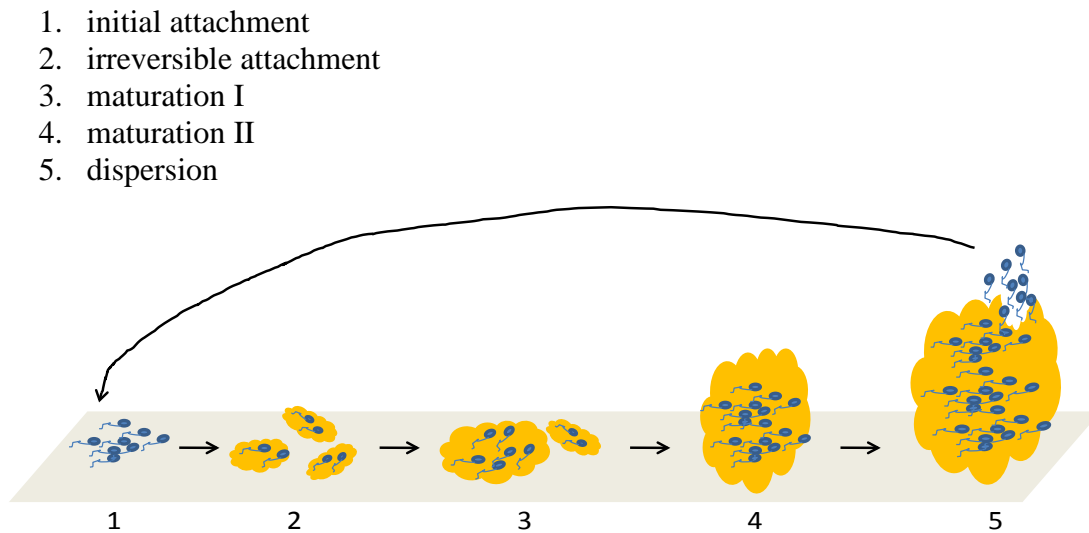


Figure 2.2 Schematic of Phase of Biofilm Formation Development

2.2.2 Biofilms and Infectious Diseases

Biofilms are very commonly found on most wet surfaces in nature and can cause severe environmental problems. Humans have suffered from acute bacterial infections for many centuries; various ways are developed to treat the microbial infections. However, infection threats due to organisms present in biofilms remain an issue.

Biofilms are associated with a wide variety of microbial infections in the body, including UTIs [23, 34], catheter infections [35], formation of dental plaque [36], gingivitis [37] and infections on contact lenses [38]. Biofilms can also be found on the inert surfaces of implanted devices such as catheters, prosthetic cardiac valves, and intrauterine devices [39].

2.2.3 Biofilms and UTIs

During cystitis, uropathogenic *E. coli* invade the healthy epithelial cells and rapidly increase in numbers to form biofilms. Under the protection of biofilm, the bacteria are more resistant to immune-system attacks and antibiotic treatments, and are more firmly anchored in infected cells. This is often the cause of chronic UTIs [23].

Protective mechanisms that interfere with bacterial adhesion in the urinary tract have not been completely elucidated. However, it has been proposed that ingestion of an adhesion-interfering substance may impair bacterial infection capability [16, 21, 40].

2.3 Impact of Natural Products on Infections

Numerous studies have indicated that dietary consumption of fruits and vegetables reduces the risk of many chronic diseases such as cardiovascular disease and cancers [41-43]. They are rich in micronutrients such as carotenoids, vitamins C and E, folic acid, flavonoids, phenols, isothiocyanates, and fiber, which might be a potential reason for the health effects. Hence, extracts of vegetables and fruits are often used as a compound of functional foods, dietary foods or dietary supplements. As a part of human diet all over the world, the beneficial effects of berries have attracted a great attention for research interests, especially cranberry (*Vaccinium* fruit).

2.3.1 Cranberry Constituents and Infections

The American cranberry (*Vaccinium macrocarpon*), a native member of the North American fruit family, is composed of water (80-88%) and carbohydrates (10%) [44]. The remaining 10% is made up of flavonoids, anthocyanins, catechin, triterpenoids, organic acids and ascorbic acid [30]. Because this fruit is rich in vitamin C, dietary fiber and the essential dietary

mineral, manganese, it has been widely used in a variety of food products including juices and sauces. Cranberry was ranked the fifth highest selling herb in the US and sales of cranberry have shown continued growth for years, exceeding \$15 million in 2005 [45]. The fresh cranberry has a high content of acid and is astringent for consuming directly. A sweetened drink, cranberry juice cocktail which contains ~27% cranberry juice, sweetener, water and added vitamin C was introduced.

The health benefits of the American cranberry (fruits and leaves) have been of interest for generations. Some researchers are exploring the cranberry's effects on heart disease [46, 47], yeast infections and other conditions, and other researchers are investigating its potential against cancer [46, 48]. No major adverse effects or interactions were reported or identified in recent studies [49, 50]. Drinking cranberry juice regularly is a promising method to reduce or eliminate UTIs.

2.3.2 Cranberry and UTIs

Clinical studies showed a significant role of cranberry in maintaining urinary tract health, but the molecular mechanism is still unclear. It has been proposed that cranberry contains two components, fructose and a specific A-type PACs [16], both of them could inhibit the pathogenic bacteria from binding to the uroepithelium cells, which is the first step of the infection. Further research is required to clarify unanswered questions regarding the role of cranberries in protecting against UTI in general.

2.3.3 Mechanism of Cranberry Juice Health Benefits on UTIs

2.3.3.1 Urinary Acidification

Factors that do not favor bacterial growth include a low pH (5.5 or less) and a high urea concentration [8]. The latter varies due to individual conditions and dietary habits. In 1923, Blatherwick and Long [10] proposed that the cranberries' naturally high acid content could enhance acidification of the urine, producing an antibacterial effect in the body. Furthermore, Kinney et al. [11] and in Jackson et al. [12] showed that cranberry juice can lower urinary pH. However, the quantities of cranberry juice required to cause a pH change were much higher than that generally consumed. Later, Nickey [13] and Avorn et al. [14] demonstrated that regular consumption of cranberry juice reduced the presence of bacteria in the urine, but this effect was not related to more acidic urine, indicating that increased urinary acidification does not appear to have a significant role in cranberry's effect in maintaining urinary tract health.

2.3.3.2 Anti-adhesion Effect on Pathogenic E. coli

Recent research [13, 14] suggested that acidification of urine was not the reason for the anti-bacterial properties of cranberry juice. In 1984, Sobota [15] proposed a more likely potential mechanism, which is that the preventative effect is achieved by inhibiting bacteria from adhering to uroepithelial cells. Later, Zafriri et al. [16] found that this benefit is due to two components in cranberry. Fructose, a constituent of many fruit juices including cranberry juice, orange juice, and pineapple juice, has been implicated as an important indicator of type I pili (mannose-sensitive) mediated adherence [16]. Proanthocyanidins (PACs), uniquely present in cranberries, have been demonstrated to inhibit type P pili (mannose-resistant) mediated adherence irreversibly [16, 51].

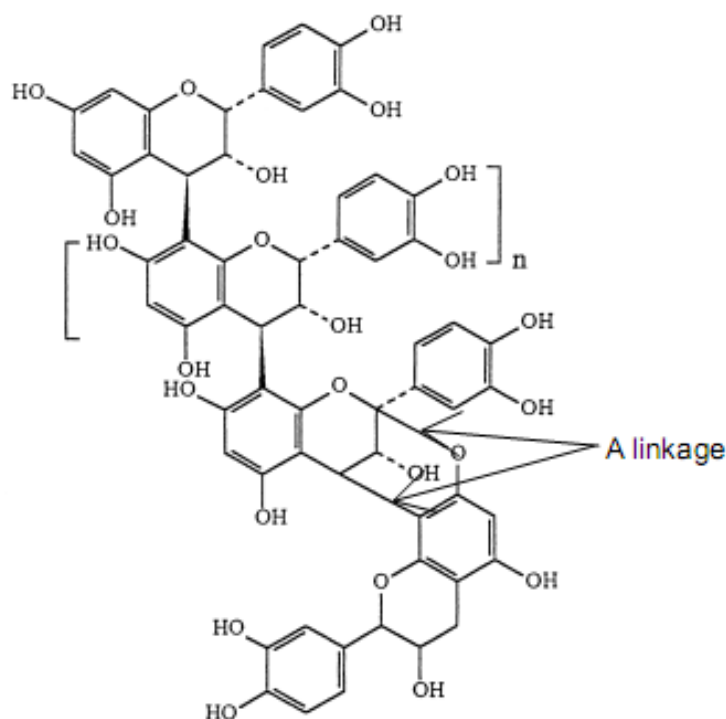


Figure 2.3 Molecular structure of A-type PACs [17]

Foo et al. and Howell et al. [17, 18] identified that the unique PACs with special A-type double linkages, which refer to the double bonds between the epicatechin/catechin units in PACs molecules (Figure 2.3), may be responsible for the anti-adhesion process. Ofek et al. [52] supported this hypothesis by showing that no anti-adhesion activities were observed after consuming other food sources of PACs that only contain common B-type linkages which are single bonds, including chocolate, grape, apple and green tea.

2.4 Atomic Force Microscope

Biofilm assay is an effective way to demonstrate bacterial adhesion activity, but other techniques are needed to directly and quantitatively detect cranberry juice cocktail's anti-adhesive ability.

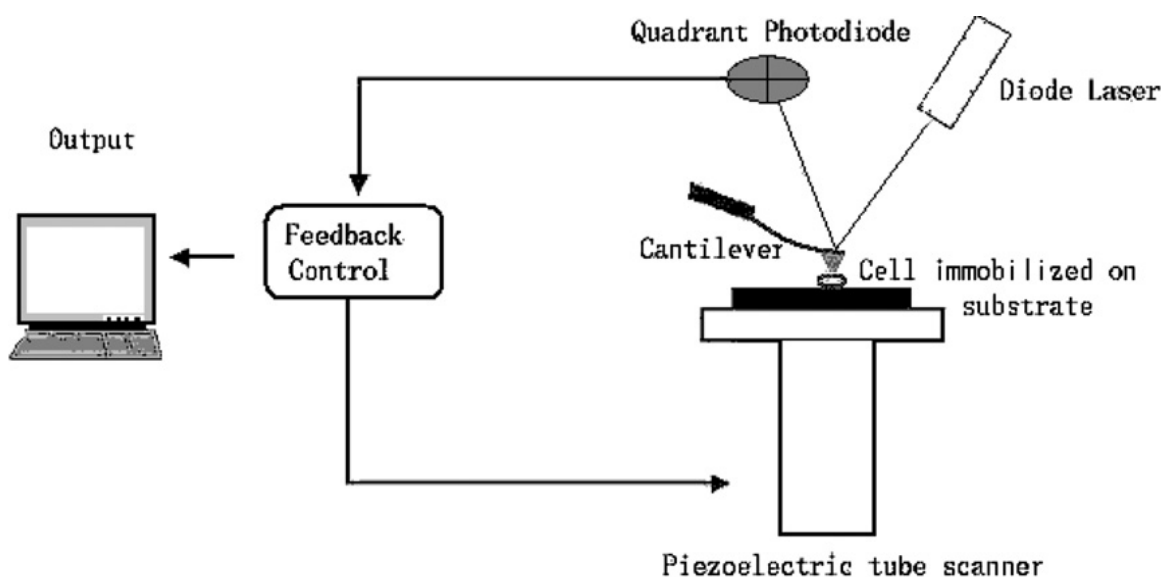


Figure 2.4 Schematic of AFM operating principles [53]

The atomic force microscopy (AFM) is a sensitive tool for measuring forces (in the pico- to nano-Newton range) as a function of separation distance. A flexible cantilever with a sharp tip, which may either be an inert material such as silicon or silicon nitride or can be functionalized with a bacterial cell, is operated as a probe of a sample surface and bends in response to surface forces. These deflections are detected by monitoring the position of a laser focused on the tip, through a four-quadrant photodiode detector (Figure 2.4). This signal is recorded and used to adjust the feedback loop. The adjusted voltage then applied to a piezo to control the movement of the tip further in order to bring it alternately into contact with the sample surface.

2.4.1 Direct Force Measurements Using Atomic Force Microscopy

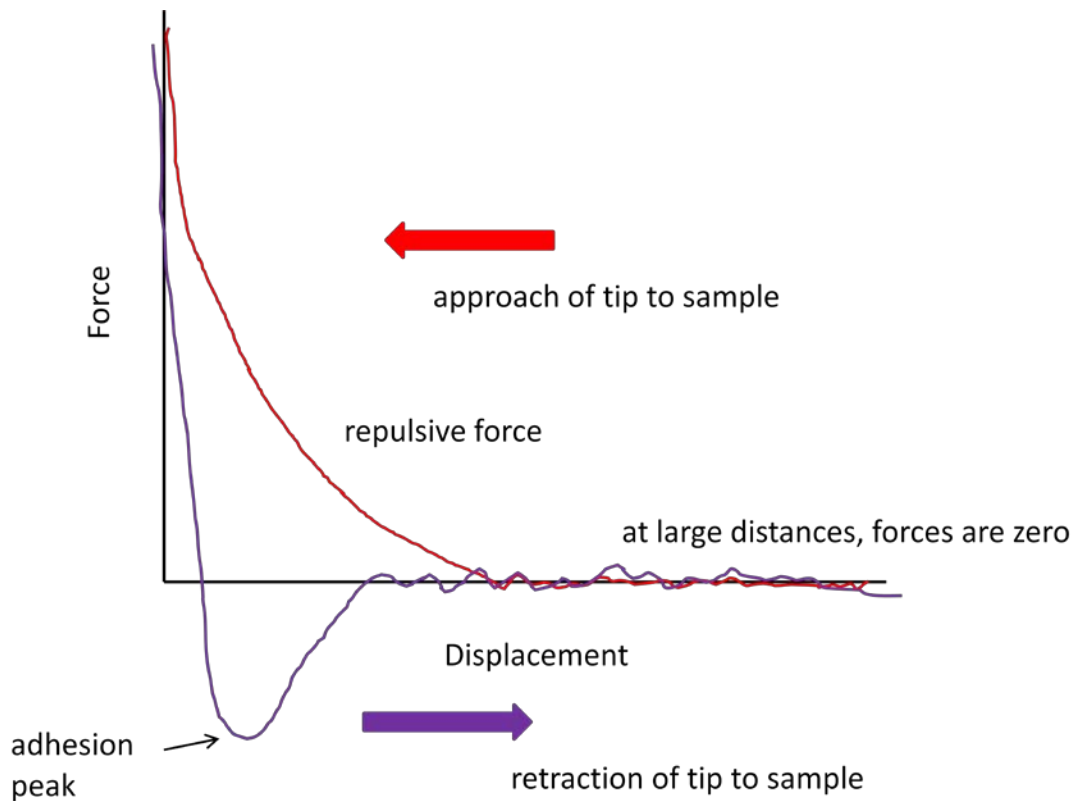


Figure 2.5 Forces are measured during the approach (upper, red line) and retraction (lower, purple line) of the probe with the sample. The “approach” curve describes the interfacial forces acting between the bacterial probe and uroepithelial cells. The “retraction” curve represents the adhesive forces that hold the two together after contact has been made.

Using AFM, forces can be easily measured both during the approach of the probe to the sample (red arrow in Figure 2.5), as well as during the retraction of the probe from the sample after contact has been made (purple arrow in Figure 2.5). The approach of the AFM profiles reflect the interaction forces between the probe and sample, which is generally repulsive (red curve showing $F > 0$ in Figure 2.5). Retraction profiles provide information on adhesion forces (purple peak showing $F < 0$ in Figure 2.5) between the probe and tip. By directly detecting such interactions, we can determine the mechanism controlling the adhesion of *E. coli* to uroepithelial cells in occurrences of UTIs. The adhesion between a biological functionalized probe, where an

E. coli bacterium is coated to the probe and uroepithelial cell surfaces will be therefore quantified in this study. This technique represents a more rapid way to quickly screen among different treatments, such as comparing the behavior of multiple solutions or various bacterial isolates.

The tip used for the force measurements is calibrated individually before use in order to obtain the cantilever spring constant (k). Once the spring constant is known, the force (F) as a function of separation distance between the sample and tip (x) can be calculated from Hooke's Law for linear and elastic springs, which states that

$$F = kx \qquad \text{Equation 2.1}$$

2.5 Preliminary Studies of Bacterial Attachment in Our Lab

Using biofilm assay and AFM technique, the preventive effects of cranberry on urinary tract infections has been clinically evaluated in many products, including cranberry juice concentrate [54], cranberry juice cocktail [14] and cranberry capsules [55]. Cranberry products that have been studied in our lab were mainly cranberry juice cocktail [56-59] or isolated proanthocyanidins (PACs) [60] in previous studies.

2.5.1 Cranberry Juice on Physicochemical Surface Characteristics and Adhesion Behavior of *Escherichia coli*

In order to better understand the mechanism associated with the molecular-scale effects of cranberry juice cocktail on physicochemical surface properties of *E. coli*, experiments [57] were conducted to investigate both bacterial surface characteristics and adhesion forces between a probe surface (silicon nitride) and the bacteria after a short exposure period (<3 h) via AFM. Two *E. coli* strains: HB101, which has no fimbriae, and the mutant HB101pDC1 which

expresses P-fimbriae were tested. The results showed that cranberry juice could affect the P-fimbriated bacteria by altering the conformation of the surface macromolecules on *E. coli* HB101pDC1, which decreased the adhesion forces between the treated bacterium and the AFM tip.

Furthermore, a thermodynamic approach was used in our lab [59] to calculate the changes in the Gibbs free energy change of adhesion changes (ΔG_{adh}) for bacteria-uroepithelial cell (UC) interactions. *E. coli* HB101pDC1 (P-fimbriated) and HB101 (non-fimbriated) were exposed to cranberry juice at different concentrations (0–27 wt %). For HB101 interacting with UC, ΔG_{adh} was always negative and the values were insensitive to cranberry juice concentration. For the HB101pDC1-UC system, which could form strong bonds with the Gal–Gal disaccharide receptor on uroepithelial cells, ΔG_{adh} became positive at 27 wt % cranberry juice, indicating that adhesion was unfavorable. The results suggested that cranberry juice may disrupt bacterial ligand-UC receptor binding.

2.5.2 Anti-adhesive Effects of Cranberry Juice Cocktail vs. Isolated PACs

In order to better answer questions regarding whether PACs are the main or the only active components in CJC, our lab measured the adhesion forces between an AFM probe (silicon nitride) and individual *E. coli* cells grown in cranberry products [60]. P-fimbriated bacterial strain *E. coli* HB101pDC1, and the non-fimbriated *E. coli* HB101, were used. Bacteria were cultured in tryptic soy broth (TSB) supplemented with neutralized, light cranberry juice cocktail (L-CJC) from Ocean Spray Cranberries, Inc. or a solution of isolated cranberry juice proanthocyanidins (PACs) provided by Ocean Spray. The results showed that growth of *E. coli* HB101pDC1 and HB101 in L-CJC or PACs resulted in a decrease in adhesion forces with an increasing number of cultures, and the behavior was dose-dependent fashion. When the bacteria

were exposed to increasing concentrations of L-CJC treatment (27% by weight) and PACs treatment (345.8 $\mu\text{g/mL}$), the adhesion forces further decreased. This effect was reversible. When bacteria were regrown in cranberry-free medium, they could regain their ability to attach to uroepithelial cells, and the adhesion forces reverted to the values of the control case.

In addition, a biofilm formation assay was used to detect *in vitro* anti-adhesion activity of cranberry products such as CJC and isolated PACs. Briefly, bacteria were cultured in Luria-Bertani media and mixed with CJC or PACs in PVC 96 well plates for 48 hrs. After incubation for 0, 6, 24, and 48 hrs at 37 °C, the amount of biofilm was measured. The results showed that CJC completely eliminates biofilm formation for all cultures. However, the isolated PACs reduced biofilm formation compared to the control, but did not eliminate biofilm formation. This may be related to the fact that PACs become unstable when they are removed from the juice environment or that PACs are not the only active components. These results revealed the need to further study non-PACs components.

2.5.3 Atomic Force Microscopy (AFM) Studies of Bacterial-Uroepithelial Cell

Interactions

Our lab was the first to measure the adhesion forces between uropathogenic P-fimbriated *E. coli* and uroepithelial cells exposed to cranberry juice (0, 2.5, 5, 10, and 27 wt%) directly by use of AFM [56]. In the presence of CJC, a decrease in adhesion forces between *E. coli* and the uroepithelial cells was observed and the adhesion forces were dose-dependent. Adhesion forces between *E. coli* HB101 (non-fimbriae bacteria served as a control) were relatively low and did not change significantly in CJC.

Further, our lab investigated the effects of cranberry metabolites in urine collected from a volunteer who had consumed CJC on several strains of uropathogenic *E. coli* [58], including P-fimbriated strains (CFT073, B37, J96, and BF1023) and non-fimbriated but mannose-resistant hemagglutination demonstrating strains (B73 and B78). AFM results showed that within 2 hours after CJC consumption, bacteria of the clinical strains treated with the corresponding urine sample demonstrated lower adhesion forces than those treated with urine collected before CJC consumption. The adhesion forces continued decreasing with time after CJC consumption over the 8-hour measurement period, while the adhesion forces of bacteria after exposure to urine collected following water consumption did not change. But this research only used a single volunteer. Since different individuals might respond to CJC in various ways, more volunteers need to be involved to elucidate CJC's effectiveness of control and treatment for UTIs.

Previous studies in our lab have showed that for some cases, PACs alone do not have as great an anti-adhesion effect against *E. coli* as CJC [60]. The weaker performance of isolated PACs lead us to shift our research approach from studying the *in vitro* effects of cranberry juice or isolated PACs in our AFM adhesion assays to studying the metabolites in urine directly. In working with the urine samples, we believe it could remove uncertainties about whether the material is stable, and about how relevant these solutions are compared to what occurs in the body when a person consumes cranberry juice. The purpose of this study is to get a more detailed picture of when the anti-adhesive activity of urine peaks, to explore how long the anti-adhesion effect persists in the urine, and to understand the differences in how individual volunteers respond to CJC consumption.

Chapter III Effects of cranberry juice metabolites on uropathogenic *Escherichia coli* *in vitro* biofilm formation

3.1 Abstract

A double-blind, placebo-controlled pilot clinical trial of the effect of cranberry juice cocktail (CJC) consumption on biofilm formation was conducted in ten healthy women between the ages of 18 and 27. A single dose of 16 oz. of CJC or a placebo beverage was given to the volunteers, and urine samples were collected in the following 48 hours. A washout period of seven days was allowed. Bacteria (*E. coli* B37, CFT073, BF1023, HB101, and *S. aureus* ATCC43866) were cultured in the urine samples supplemented with media and the amount of biofilm formed was measured using a crystal violet absorbance assay in a 96-well plate. In the urine of volunteers who had consumed CJC, biofilm formation was inhibited within 24 hours after CJC consumption, and biofilm formation started to increase after 48 hours by 49-67%. *S. aureus* showed the least biofilm formation after incubation with post-CJC urine. The results indicated that drinking CJC can be an effective preventive measure for bacterial adhesion and biofilm formation in healthy women. The anti-biofilm activity peaks between 24 and 48 hours after drinking CJC. In addition, the viability assay results showed that the colony count after culturing in urine collected following consumption of CJC or placebo were not significantly different, implying that CJC works as an inhibitor by blocking bacterial adhesion instead of killing the bacteria or restraining its growth.

3.2 Introduction

Escherichia coli (*E. coli*) strains have been considered as the most predominant cause of urinary tract infections (UTIs). As the first step of bacterial infection, biofilm formation, which hinders the eradication of bacteria [32] from the environment, is of significant interest to researchers [61]. Biofilms allow bacteria to persist a long time in the genitourinary tract and create more favorable conditions for colonization and infection. In addition, biofilm help the bacteria within it avoid immune system attacks and antibiotic treatments, which increase the difficulty of treating UTIs.

Native American cranberry (*Vaccinium macrocarpon*) has been long known for its preventive benefits on UTIs. *E. coli* adhesins, mainly fimbrial in nature, promote bacterial adhesion to uroepithelial cells or urinary catheters, which is the first step in the development of biofilm formation and UTIs. The unique A-type linkages proanthocyanidins (PACs) in cranberry have been implicated as important inhibitors of primarily P-fimbriated *E. coli* attachment to uroepithelial cells. However, the activity of the post-ingested cranberry metabolites has not been greatly studied. The mechanisms of the anti-adherence effect and the nature of the anti-adherence compounds are still poorly understood.

Although cranberry products have been consumed by many healthy women as a preventive measure, further research is needed to elucidate how oral consumption of cranberry affects the activity of uropathogenic bacteria. Previous studies demonstrated that the active compounds are not destroyed in the body by the digestive system after intaking cranberry juice [58, 62]. However, no studies have identified the molecular structure of the effective components in urine. Additional experiments are required to investigate its persistence in urine samples over a broader time period, and to determine if the urinary anti-adhesion effect following cranberry is

detected within volunteers of different origins. By using human urine, this model could represent *in vivo* conditions.

A limited number of studies have examined the role of cranberry on biofilm formation. DiMartino et al showed that cranberry juice consumption decreased biofilm development of uropathogenic *E. coli* on inert surfaces [63]. They used samples from two volunteers and tested the urine at a single time point. Another placebo-controlled clinical trial showed that UTIs were prevented in elderly women who had received cranberry juice therapy [14], but there have been few studies in younger age groups. We built upon prior research in several important ways, by examining more volunteers, testing the urine over a longer time (up to 48 hrs), and examining a wider range of uropathogenic strains. Therefore, the present study used a biofilm formation assay to determine the efficacy of cranberry juice cocktail (CJC) to reduce biofilm formation in the urine of ten healthy women, for 48 hours following CJC consumption.

3.3 Subjects and Methods

3.3.1 Healthy Volunteers

Ten healthy female volunteers with a normal diet, ranging in age from 18 to 27 years, from the student population of Worcester Polytechnic Institute (Massachusetts) were recruited. The study was a double-blind, randomized, placebo-controlled and cross-over study. Exclusion criteria included confirmation that the ten volunteers had no history of urinary tract infections and the urine had a normal urine composition by a dipstick test. During this trial, the volunteers maintained their usual unrestricted diet but were asked to not consume any other berries or juices in order to exclude the intervention of metabolites possibly similar to cranberry metabolites. All volunteers were asked to sign their informed consent.

3.3.2 Study Protocol

The double-blind study was carried out using commercially available cranberry juice cocktail (CJC; Ocean Spray Cranberries, Inc., USA) and placebo beverage. Ingredients include filtered water, cranberry juice concentrate (~27%), sucrose, aspartame, and ascorbic acid. The placebo beverage mimics the flavor and color of the cranberry beverage but has no cranberry ingredients or proanthocyanidins.

Each volunteer received 16 oz (480 ml) of CJC or placebo. A wash-out period of at least one week was allowed. Urine samples were collected at 2, 8, 24, and 48 hours after CJC or placebo consumption. Baseline urine samples (indicated as 0 hour later) were taken before ingesting cranberry juice or placebo on the day of the study. All specimens and data were confidentially coded. In order to remove epithelial cells, bacteria, or other particles from the urine, the urine samples were centrifuged at 7000 RPM for 10 min, sterilized by filtration using polyethersulfone syringe filters (VWR International TM, West Chester, PA) with 0.8 μm and 0.2 μm membranes sequentially, and stored at -20 °C for future use.

3.3.3 Bacteria Cell Preparation

Three clinical uropathogenic P-fimbriaed *E. coli* isolates (CFT073, B37, and BF1023) and a *Staphylococcus aureus* (*S. aureus*) strain (ATCC 43866) were used in this study. *E. coli* HB101, a non-pathogenic lab strain with no fimbriae that does not adhere to epithelial cells, served as a control. Pure cultures were maintained at -80 °C in colonizing factor antigen (CFA) media composed of 1% (w/v) casamino acids, 0.078% (w/v) yeast extract, 0.4 mM MgSO₄, 0.04 mM MnCl₂, in ultrapure water, at a pH of 7.4. Cultures were streaked onto Tryptic Soy Agar (40g/L, Sigma-Aldrich, St. Louis, MO) plates that were then incubated at 37 °C for 24 h. The strains were cultured in Luria-Bertani broth (20g/L, Sigma-Aldrich, St. Louis, MO) at 37 °C with

gently shaking to enhance expression of P-fimbriae and then harvested at late exponential phase (corresponding to 0.9-1.0 optical density (OD) units at 600 nm).

3.3.4 Crystal Violet Biofilm Assay

The inhibitory effect of cranberry metabolites on the biofilm formation of *E. coli* CFT073, *E. coli* B37, *E. coli* BF1023, *S. aureus* ATCC 43866, *E. coli* HB101 on the bottom of 96-well PVC microtiter plates (BD Falcon, Durham, NC) was examined. Briefly, the harvested bacteria were mixed with urine samples at a ratio of 1: 1 (v/v %). The mixed suspension was transferred into a 96-well PVC microtiter plate and incubated at 37 °C. After 0, 6, 24, 48 hours of incubation, media and unattached bacterial cells were decanted from the wells. 20 μ L of crystal violet solution (Becton, Dickinson and Company, Sparks, MD) was added to stain bacteria cells for 15 minutes. The microtitre plates were then washed three times with 200 μ L of deionized water each time to remove the remaining planktonic or loosely bound bacteria cells. Bacteria cell-bound crystal violet was released by the addition of 150 μ L of extracting agent (20% acetone in ethanol v/v) for 15 min at room temperature. The concentration of crystal violet, as the indicator of the amount of biofilm, was then quantified by measuring the absorbance of the solution at 600 nm with a microtiter plate reader. Each isolate was assayed in four replicate wells.

3.3.5 Bacterial Cell Viability Assay

Plate counts were used to verify loss of cell viability when suspended in urine collected after CJC or placebo consumption. Harvested bacterial cell suspensions were diluted in the same pre-warmed medium (Luria-Bertani broth) from 10^7 to 10^6 and mixed with the urine samples with a ratio of 1:1 (v/v %). Mixture with equal quantity of growth media was used as a control. The mixture was spread to Luria-Bertani agar (35g/L, Sigma-Aldrich, St. Louis, MO) plate and incubated at 37 °C for 24 h. The numbers of bacterial colony were counted. These measurements

were repeated using three different samples (prepared from separate cultures) of each bacterial dilution.

3.3.6 Statistical Analysis

3.3.6.1 Crystal Violet (CV) Biofilm Formation Assay

For each volunteer, comparisons of the biofilm amount after post-CJC and post-placebo urine sample treatment were evaluated using one-way analysis of variance (abbreviated one-way ANOVA) method, following the variance examination with Tukey's test. For all the ten volunteers, the difference of biofilm formation in urine following consumption of cranberry juice cocktail or placebo was also examined by one-way ANOVA procedure, following with Tukey's Test. A value of $P < 0.05$ was considered statistically significant and the time point zero samples served as a baseline control. Results were reported as means \pm standard deviation (SD) for all experiments.

3.3.6.2 Bacterial Cell Viability Assay

The statistical significance of differences was evaluated with Student's t test for paired values. P values below 0.05 were considered significant. All experiments were performed in triplicate.

3.4 Results

3.4.1 Effects of Cranberry Juice Metabolites on *E. coli* Biofilm Formation

To determine cranberry juice metabolites' anti-adhesive ability to clinical UTI isolates, biofilm formation assay (which represents a rapid screening method for bacterial adhesion abilities in 96-well microtitre plates) was conducted in this study.

The inhibitory effects of cranberry juice metabolites on the five strains after 0, 6, 24, and 48 hours incubation are presented in Figures 3.1 - 3.4, respectively. The adherence rates of the tested bacteria differed from strain to strain. For non-pathogenic *E. coli* HB101 (served as a control), the amount of biofilm formed after culturing in post-CJC urine stayed unchanged over 48 hours and did not show a difference from the biofilm amount formed with post-placebo urine treatment for all incubation time points (0, 6, 24, and 48 hours). Before they had been put into the 37 °C incubator (Figure 3.1, 0 hour), *E. coli* B37, BF1023 did not show any difference in biofilm formation between the post-CJC and post-Placebo urine treatment. Only *S. aureus* and *E. coli* CFT073 exhibited a significant lower biofilm formation after drinking CJC for 24 hours and 48 hours compared to placebo.

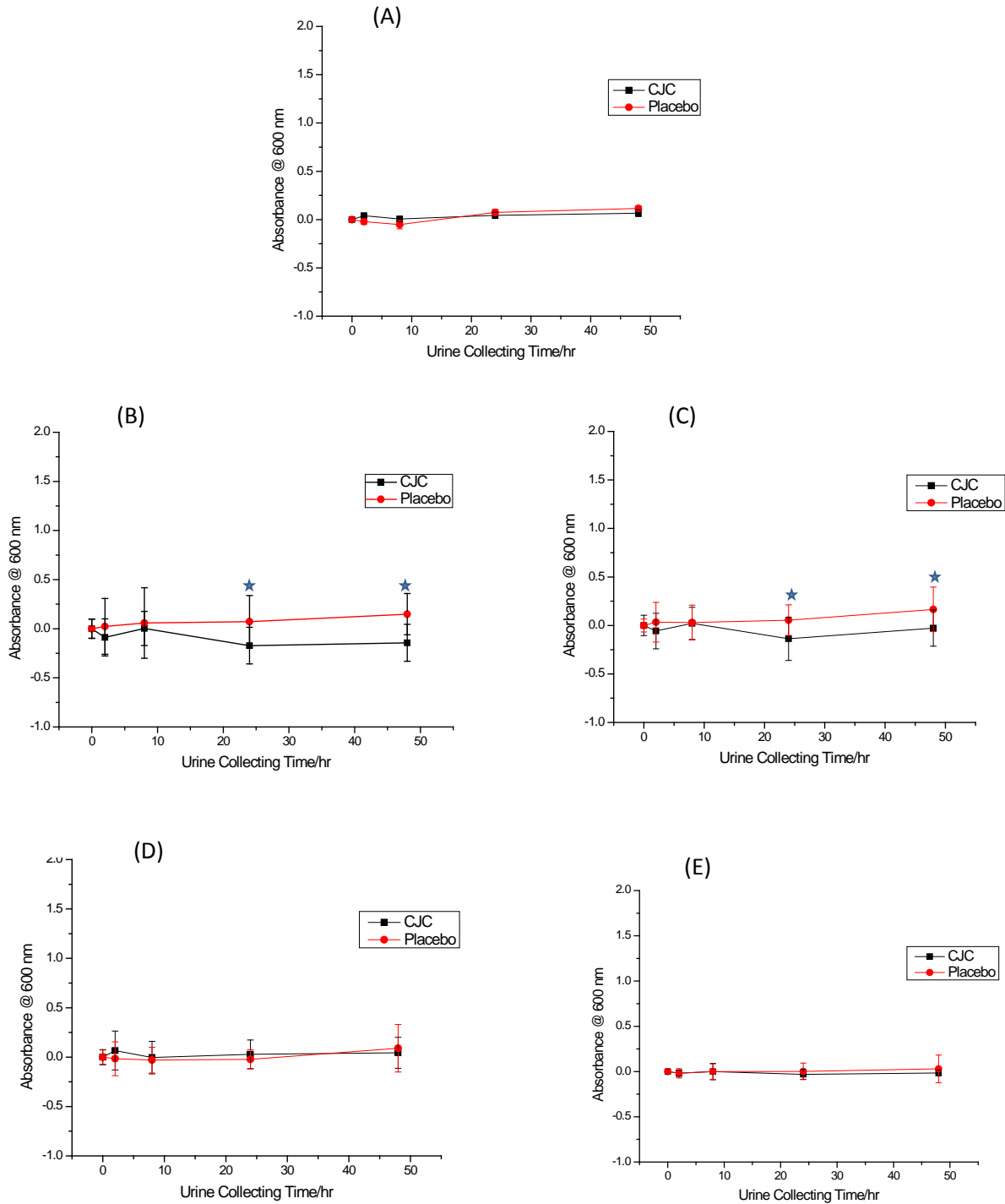


Figure 3.1 Amount of biofilm formed after incubation of 0 hour at 37 °C (A) *E. coli* HB101; (B) *S. aureus*; (C) *E. coli* CFT073; (D) *E. coli* B37; (E) *E. coli* BF1023. ★ indicates that biofilms formed in urine following ingestion of CJC or placebo were significantly different.

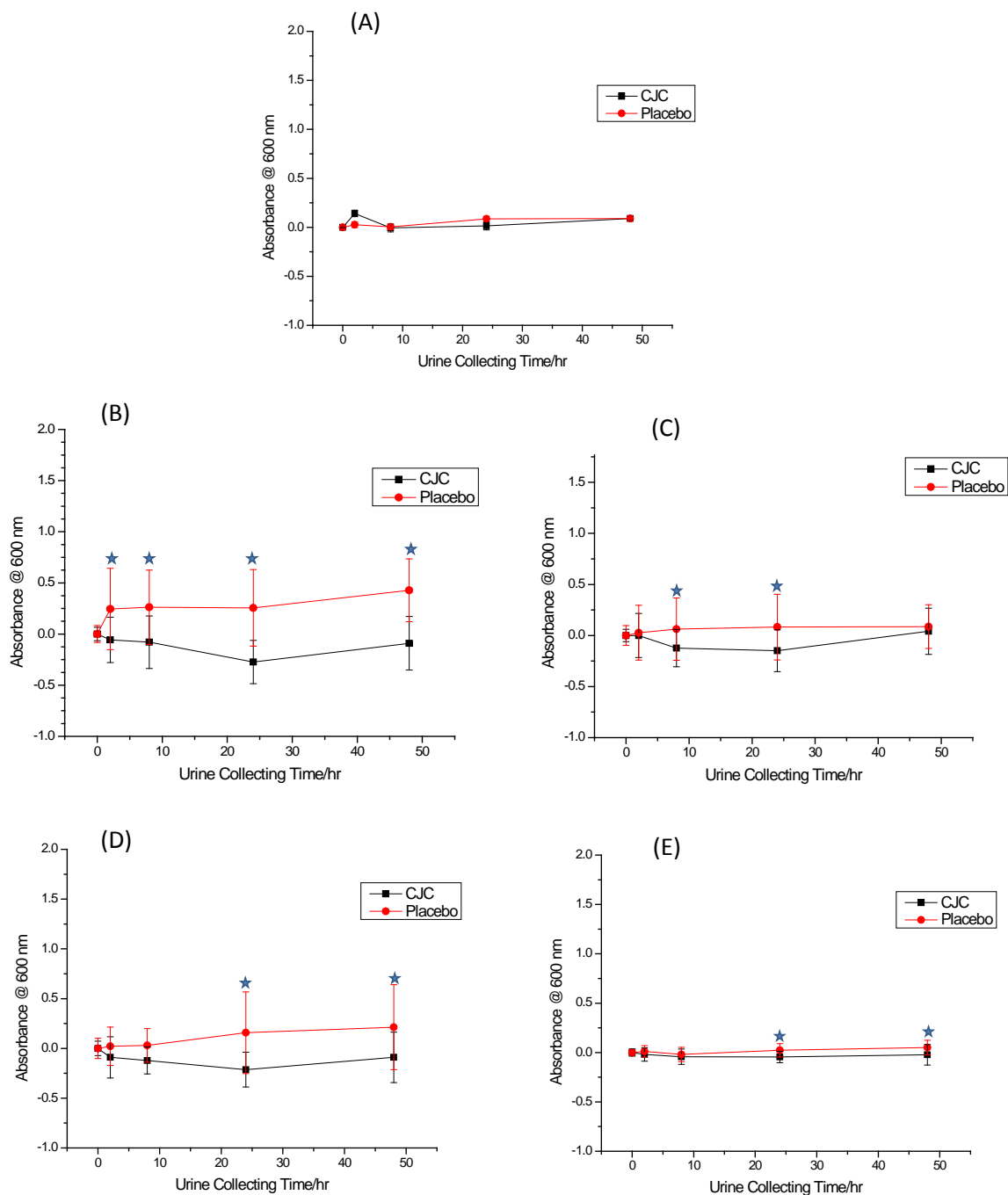


Figure 3.2 Amount of biofilm formed after incubation of 6 hour at 37 °C (A) *E. coli* HB101; (B) *S. aureus*; (C) *E. coli* CFT073; (D) *E. coli* B37; (E) *E. coli* BF1023. ★ indicates that biofilms formed in urine following ingestion of CJC or placebo were significantly different.

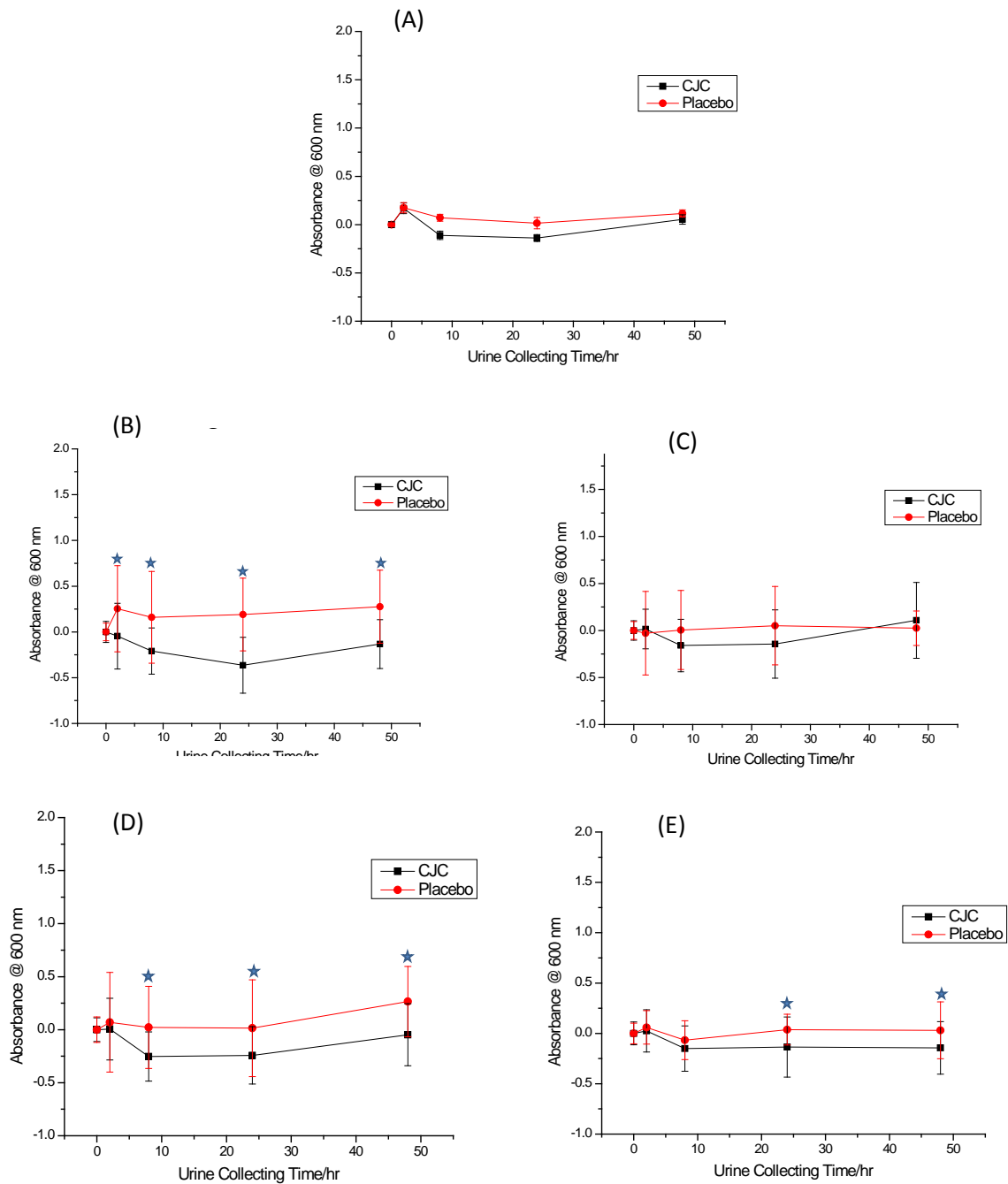


Figure 3.3 Amount of biofilm formed after incubation of 24 hour at 37 °C (A) *E. coli* HB101; (B) *S. aureus*; (C) *E. coli* CFT073; (D) *E. coli* B37; (E) *E. coli* BF1023. ★ indicates that biofilms formed in urine following ingestion of CJC or placebo were significantly different.

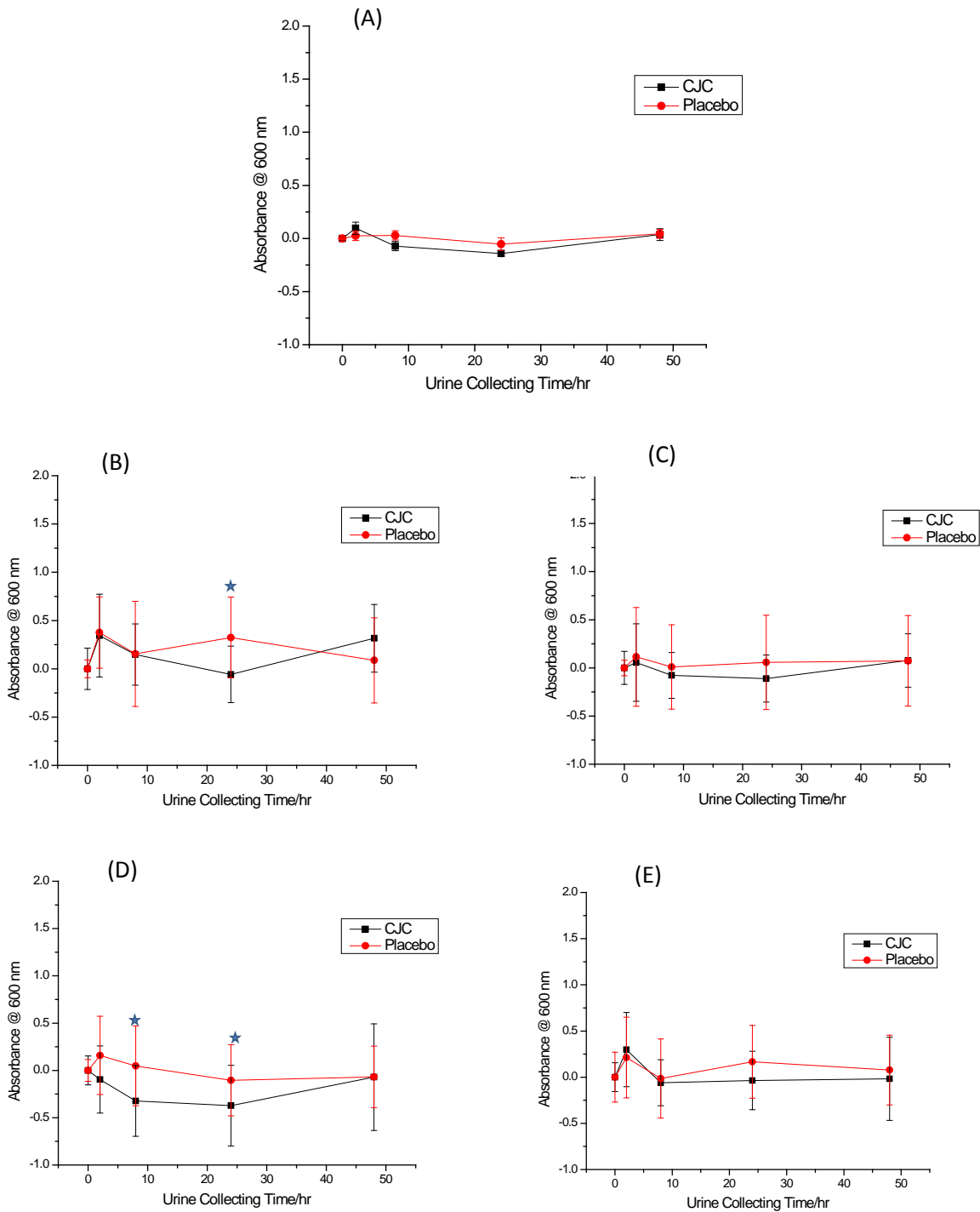


Figure 3.4 Amount of biofilm formed after incubation of 48 hour at 37 °C (A) *E. coli* HB101; (B) *S. aureus*; (C) *E. coli* CFT073; (D) *E. coli* B37; (E) *E. coli* BF1023. ★ indicates that biofilms formed in urine following ingestion of CJC or placebo were significantly different.

Table 3.1 Amount of biofilm formed after culturing in urine samples collected from volunteers drinking CJC or placebo for 6 hours. Biofilm amount at hour 0 serves as baseline.

Strain	Urine Sample Treatment	Mean Absorbance at 600 nm \pm SD				
		0hr	2hr	8hr	24hr	48hr
<i>E. coli</i> B37	Placebo	0.000	0.021 \pm 0.193	0.030 \pm 0.170	0.157 \pm 0.410	0.213 \pm 0.427
	CJC	0.000	-0.090 \pm 0.207	-0.121 \pm 0.136	-0.214 \pm 0.175*	-0.090 \pm 0.254*
<i>E. coli</i> CFT073	Placebo	0.000	0.028 \pm 0.268	0.063 \pm 0.305	0.082 \pm 0.321	0.087 \pm 0.214
	CJC	0.000	0.000 \pm 0.216	-0.123 \pm -0.182*	-0.148 \pm 0.205*	0.041 \pm 0.227
<i>E. coli</i> BF1023	Placebo	0.000	0.011 \pm 0.058	-0.019 \pm 0.073	0.023 \pm 0.067	0.051 \pm 0.073
	CJC	0.000	-0.020 \pm 0.067	-0.042 \pm 0.077	-0.045 \pm 0.058*	-0.023 \pm 0.105*
<i>S. aureus</i> (ATCC43866)	Placebo	0.000	0.245 \pm 0.398	0.263 \pm 0.363	0.256 \pm 0.375	0.428 \pm 0.307
	CJC	0.000	-0.057 \pm 0.222*	-0.080 \pm 0.256*	-0.274 \pm 0.212*	-0.090 \pm 0.262*
<i>E. coli</i> HB101	Placebo	0.000	0.026 \pm 0.028	0.005 \pm 0.027	0.087 \pm 0.026	0.089 \pm 0.023
	CJC	0.000	0.142 \pm 0.033	-0.006 \pm 0.040	0.014 \pm 0.040	0.091 \pm 0.021

* Statistically significant difference compared to the placebo group ($p < 0.05$).

After incubation for 6 hours at 37 °C (Figure 3.2), for all the strains except *E. coli* HB101, the amount of biofilm decreased within 24 hours after CJC consumption and slightly increased between 24 and 48 hours after CJC consumption (Table 3.1). The trend for the quantity of biofilm formed after 24 hours of incubation (Figure 3.3) is similar to those incubated for 6 hours for all strains except *E. coli* CFT073, where the biofilm amount for the sample treated with 48 hours post-CJC urine is a little higher than the one treated with 48 hours post-placebo urine. After 48 hours of incubation (Figure 3.4), the 24 hours post-CJC urine treatment reduced biofilm formation ability in all strains except *E. coli* HB101. However, only *S. aureus* and *E. coli* B37 showed a significant decrease of biofilm formation in urine following consumption of CJC compared to that in urine collected after drinking placebo at the same time point. The 48 hours post-CJC urine treatment formed the same amount of biofilm as 48 hours post-placebo urine treatment, except for *S. aureus*.

3.4.2 Individual Responses to CJC

The inhibitors to prevent biofilm formation against *E. coli* may be transitory and can be affected by a number of factors, dietary (on salt and water balance) and environmental. For a given strain of bacteria, an individual was considered to be responsive to CJC if the biofilm amount after culturing in post-CJC urine was significant lower than that in post-placebo urine ($P < 0.05$).

The number of volunteers who responded to CJC is summarized in Tables 3.2 - 3.5. The results demonstrated that the urine before drinking either CJC or placebo did not show any anti-adhesive effects for all strains, at all incubation time points.

Table 3.2 Number of volunteers that showed a significantly lower biofilm formation in urine following consumption of CJC (total number of volunteers is 10) after incubation for 0 hour at 37 °C.

Strain	# of volunteers showing difference between CJC and placebo				
	0hr	2hr	8hr	24hr	48hr
<i>E. coli</i> B37	0	0	1	1	3
<i>E. coli</i> CFT073	0	1	2	4	2
<i>E. coli</i> BF1023	0	3	4	4	3
<i>S.aureus</i> (ATCC43866)	0	2	1	6	6
<i>E. coli</i> HB101	0	1	2	1	2

After being incubated for 0 hours (Table 3.2), the number of volunteers who responded to CJC differed from strain to strain and there is no obvious trend among the treatment with urine collected before drinking CJC or placebo.

Table 3.3 Number of volunteers that showed a significantly lower biofilm formation in urine following consumption of CJC (total number of volunteers is 10) after incubation for 6 hours at 37 °C.

Strain	# of volunteers showing difference between CJC and placebo				
	0hr	2hr	8hr	24hr	48hr
<i>E. coli</i> B37	0	4	4	8	6
<i>E. coli</i> CFT073	0	3	5	5	5
<i>E. coli</i> BF1023	0	1	2	3	4
<i>S.aureus</i> (ATCC43866)	0	5	6	6	9
<i>E. coli</i> HB101	0	1	3	4	2

After incubation for 6 hours at 37 °C (Table 3.3), the results showed that within 24 hours after drinking CJC, the number of volunteers who showed a reduced biofilm formation increased, and this trend was similar in all the strains tested. The response at 48 hours after drinking CJC differed among the strains. *E. coli* BF1023 and *S. aureus* showed an increased number of responding volunteers at 48 hours compared to 24 hours, whereas for the other strains, the

number of volunteers who responded to CJC stayed unchanged or decreased, possibly because CJC has been washed out of the volunteer's body. The degree of biofilm reduction also varied with bacterial strain; *S. aureus* had the most significant biofilm decrease, whereas *E. coli* BF1023 showed very little change after treatment with post-CJC urine. These variations may result from the different susceptibility of each strain to CJC treatment, and variant surface properties such as adhesin type and density.

Table 3.4 Number of volunteers that showed a significantly lower biofilm formation in urine following consumption of CJC (total number of volunteers is 10) after incubation for 24 hours at 37 °C.

Strain	# of volunteers showing difference between CJC and placebo				
	0hr	2hr	8hr	24hr	48hr
<i>E. coli</i> B37	0	2	4	4	4
<i>E. coli</i> CFT073	0	3	4	4	2
<i>E. coli</i> BF1023	0	3	4	4	3
<i>S.aureus</i> (ATCC43866)	0	4	4	5	6
<i>E. coli</i> HB101	0	5	3	5	3

The trend after 24 hours incubation (Table 3.4) is the same compared to that with 6 hours incubation but had decreased number of volunteers who responded to CJC except *E. coli* BF1023.

Table 3.5 Number of volunteers that showed a significantly lower biofilm formation in urine following consumption of CJC (total number of volunteers is 10) after incubation for 48 hours at 37 °C.

Strain	# of volunteers showing difference between CJC and placebo				
	0hr	2hr	8hr	24hr	48hr
<i>E. coli</i> B37	0	3	5	4	5
<i>E. coli</i> CFT073	0	3	3	3	3
<i>E. coli</i> BF1023	0	1	2	3	3
<i>S.aureus</i> (ATCC43866)	0	1	2	6	0
<i>E. coli</i> HB101	0	1	2	3	1

After 48 hours incubation (Table 3.5), the number of responded volunteers further decreased compared to 24 hours incubation except *E. coli* B37. That is probably because the formed biofilm has already begun to decompose.

3.4.3 Viability Assay and Correlation with Biofilm Assay

To clarify whether cranberry juice metabolites behaved as anti-adhesive or anti-microbial agents, cell viability assay was measured.

The effects of post-CJC consumption urine on bacterial colony number are summarized in Table 3.6. Cell viability also differed from strain to strain, and no significant drop in the number of colonies was observed for all strains after ingestion of cranberry ($P < 0.5$). For example, for *E. coli* CFT073, volunteer 7, the plate count after 24 hours of incubation in post-CJC urine was 44.93 ± 32.63 , in comparison to 47.47 ± 35.50 when suspended in post-placebo urine for 24 hours. Similarly, *E. coli* HB101, a strain lacking fimbriae structure that served as a control, showed no difference in colony counts (post-CJC urine was 7.20 ± 3.76 , in comparison to 6.93 ± 3.10 in post-placebo urine, both for 24 hours). This indicates that the consumption of cranberry juice cocktail inhibits biofilm formation by possibly hindering bacterial adhesion activities rather than by killing the bacteria.

E. coli CFT073 had the most colonies after culturing in post-consumption urine; *S. aureus* had a high colony count as well. These results were consistent with the results of the biofilm formation assay, in that *E. coli* CFT073 and *S. aureus* formed relatively more biofilm in post-placebo urine among all the clinical strains.

Table 3.6 Colony count of bacteria strains after 24 hours incubation in post-CJC urine or post-Placebo urine.

Volunteer ID	Urine Sample Treatment	Strain				
		<i>E. coli</i> B37	<i>E. coli</i> BF1023	<i>E. coli</i> CFT073	<i>S. aureus</i>	<i>E. coli</i> HB101
1	CJC	8.73 ± 4.62	13.93 ± 7.65	44.93 ± 32.63	23.87 ± 10.88	7.20 ± 3.76
	Placebo	11.60 ± 4.24	14.53 ± 8.50	47.47 ± 35.50	25.07 ± 6.50	6.93 ± 3.10
2	CJC	18.27 ± 8.28	8.67 ± 5.68	32.13 ± 11.26	20.00 ± 11.18	7.07 ± 2.71
	Placebo	20.13 ± 8.85	9.25 ± 5.63	32.33 ± 8.82	23.33 ± 9.38	7.73 ± 2.69
3	CJC	3.67 ± 2.02	12.73 ± 8.47	18.47 ± 3.34	21.13 ± 7.12	4.87 ± 1.85
	Placebo	4.40 ± 2.78	13.20 ± 6.76	18.40 ± 4.50	19.80 ± 6.94	6.13 ± 2.80
4	CJC	23.00 ± 4.92	17.42 ± 8.87	21.25 ± 8.81	22.83 ± 9.52	5.08 ± 1.78
	Placebo	20.00 ± 3.02	17.92 ± 7.10	23.25 ± 10.97	22.00 ± 8.10	4.92 ± 1.68
5	CJC	8.93 ± 8.41	11.47 ± 2.88	26.87 ± 17.18	30.60 ± 6.19	6.40 ± 5.29
	Placebo	17.73 ± 29.00	11.33 ± 2.89	29.93 ± 26.55	29.20 ± 5.83	6.33 ± 3.77
6	CJC	18.33 ± 7.87	12.73 ± 5.01	36.00 ± 14.88	19.67 ± 7.07	5.40 ± 3.85
	Placebo	15.20 ± 6.73	13.27 ± 8.00	30.27 ± 10.82	23.93 ± 6.79	4.47 ± 2.85
7	CJC	12.07 ± 8.42	7.80 ± 5.61	18.80 ± 6.01	21.47 ± 10.52	2.60 ± 1.59
	Placebo	12.07 ± 4.70	11.67 ± 4.86	18.33 ± 4.39	19.60 ± 6.77	3.47 ± 2.36
8	CJC	7.47 ± 4.66	2.87 ± 1.19	76.40 ± 36.67	18.80 ± 6.98	5.73 ± 1.79
	Placebo	5.40 ± 4.91	4.60 ± 3.96	82.33 ± 36.73	19.47 ± 5.04	4.53 ± 2.26
9	CJC	14.33 ± 8.87	12.93 ± 8.92	nt	13.92 ± 7.63	4.93 ± 2.09
	Placebo	15.67 ± 8.23	9.87 ± 10.49	nt	15.50 ± 8.45	4.87 ± 2.36
10	CJC	12.67 ± 3.92	10.80 ± 4.48	126.93 ± 61.57	25.40 ± 6.52	5.20 ± 2.73
	Placebo	15.20 ± 4.52	12.07 ± 3.24	104.80 ± 23.21	29.67 ± 7.64	4.33 ± 2.77

All results are statistically non-significant ($P < 0.05$). nt=not tested

3.5 Discussion

3.5.1 Selection of Bacterial Strains

To determine the efficacy of the consumption of cranberry juice versus placebo with regard to the presence of *in vitro* bacterial anti-adherence activity, several clinical strains of *E. coli* were cultured in the urine of healthy volunteers and tested for their ability to form biofilm in this study. Since our focus is on the type of adhesion that can be affected by PACs, we concentrated primarily on P-fimbriated *E. coli* strains.

Uropathogenic *E. coli* strains typically demonstrate mannose-resistant adhesion in mannose-resistant hemagglutination (MRHA) experiments [64-66], but only cranberry can inhibit mannose-resistant adhesion [67, 68]. It is therefore important to investigate CJC's anti-adhesive activity against uropathogenic *E. coli* strains that demonstrate MRHA. Several MRHA strains (P-fimbriated *E. coli* B37, CFT073 and BF1023) [58] were selected in this study.

Table 3.7 Summary of properties and sources of five *E. coli* strains studied. All isolates also express type I fimbriae except *S. aureus* and HB101.

Strain	Fimbriae/Info	Source
B37	P-fimbriae from class II, ampicillin/sulfamethoxazole intermediate resistance	Dr. James Johnson, VA Medical Ctr, Minneapolis, MN; isolated from female with cystitis [69]
CFT073[WAM 2267]	Type P-fimbriae from class II	ATCC 700928; isolated from blood and urine of a woman with AP
BF1023	P-fimbriae, class I and class III	ATCC 700414; isolated from a female patient with cystitis [70]
<i>S. aureus</i>	Surface protein [71]	ATCC 43866
HB101	Non-fimbriated, lab strain (non-pathogenic control)	ATCC 33694

The strains chosen cover a variety of surface properties and types of fimbriae (Table 3.7). For comparison, a strain that does not have P-fimbriae structure but is known to cause cystitis (*S. aureus*) and a control that does not cause infections (non-fimbriated *E. coli* HB101) were included. To represent different types of adhesins, a strain that expresses P-fimbriae from class II (*E. coli* CFT073) and a strain that expresses P-fimbriae from class I and class III (*E. coli* BF1023) were selected. While there is no direct connection between antibiotic susceptibility and sensitivity to cranberry compounds as an anti-adhesive therapy, it may be helpful in getting these therapies adopted if their health effects against pathogens which cannot be treated with common antibiotics can be demonstrated. Therefore, a clinical *E. coli* strain with known antibiotic resistance (*E. coli* B37) was also included in this study.

3.5.2 Correlating Growth in Cranberry Metabolites and Development of Biofilms

According to the results of the biofilm formation assay, after the volunteers drank CJC, their urine reduced biofilm formation in bacteria strains that have P-fimbriae (*E. coli* B37, CFT073, and BF1023), and surface protein (*S. aureus* [71]), whereas the strain that does not have adhesins, *E. coli* HB101, was not affected (Table 3.1). These results indicated that drinking CJC prevents biofilm formation. The extent to which biofilm formation was inhibited could differ based on the adhesin density and type.

The duration of CJC's effects on biofilm formation after oral consumption was also investigated. A previous study using human cell agglutination assay showed anti-adhesion activity of urine lasted for 10 hours after CJC consumption [72]. In this study, the number of volunteers whose urine showed an inhibitory effect on biofilm formation

peaked between 8 and 24 hours after CJC consumption, and decreased in some strains after 48 hours. This phenomenon could be a result of cranberry components or metabolites' washing out from the body. This may explain why the amount of biofilm increased 48 hours after consumption of CJC.

3.5.3 Correlating Biofilm Formation and Pili Fimbriae Expression

In the natural environment, bacteria are often found as communities in the form of biofilms. The attachment of individual cells to an available solid surface in the aqueous phase is the first step which could lead to the formation of mature biofilms. But the bacterium has to overcome the physical forces that repel bacteria from binding. In this case, pili structure on bacterium aid it to anchor to the surfaces firmly and mediate stable attachment further.

E.coli CFT073 and B37 showed the most significant effects on biofilm formation, which is in a good agreement with the fact that majority of acute pyelonephritis cases (66%) are caused by P-fimbriated bacteria that contain Class II adhesins [73]. In addition, the bacterial cell viability assay demonstrated that for the amount of cranberry juice (16 oz.) used in this study, the cranberry juice metabolites in collected urine following consumption do not have antibiotic effects, thereby reducing selective pressures for antibiotic resistant bacteria. Instead, they worked as an inhibitor to block bacterial adhesive activity.

Although P-fimbriae with Class III adhesins are associated with 12% of cystitis cases in adult women [73], 37% of cystitis cases in children [74], and 13% of acute

pyelonephritis incidents [73], our results showed that cranberry juice does not work well with *E. coli* BF1023 which contains Class III adhesins besides Class I adhesins.

S. aureus is one of the major causes of numerous hospital- and community-acquired infections, including UTIs [71]. The widespread use of methicillin and other semisynthetic penicillins to treat it resulted in the emergence of methicillin resistant *S. aureus* [75]. Currently, greater than 60% of *S. aureus* isolates are resistant to methicillin [76]. *S. aureus* [75, 77] is therefore a growing public health problem in hospitals, nursing homes, and other institutions. This reinforced the critical need for new methods of control and treatment. Our results showed that there is essentially no biofilm formed in the *S. aureus* samples. This may suggest that oral consumption of CJC can be exploited as a treatment for Staphylococcal infections.

3.6 Conclusions

In this study, two experimental assays were optimized successfully to demonstrate bacterial anti-adhesion activity of cranberry metabolites in human urine following cranberry juice cocktail consumption. The inhibitory effects for preventing biofilm formation in the urine of healthy young women could persist for at least 24 hours after CJC ingestion. The anti-adhesive ability differed based on the type of bacteria, suggesting biofilm formation is closely associated with the kind of P-fimbriae. Furthermore, the viability test results indicated that the cranberry metabolites in post-CJC urine behaved as an inhibitor to lower adhesive abilities rather than a bactericide to kill the bacteria. In other words, cranberry products do not impair bacterial growth and will not sterilize the urinary tract, although they prevent bacterial adhesive ability, thus reducing the

development of UTI. Since bacterial adhesion is the primary step in initiation of UTI, consumption of cranberry may be useful to help prevent infections.

Chapter IV Inhibitory activity of cranberry juice metabolites on adherence of P-fimbriated *Escherichia coli* to bladder epithelial cells

4.1 Abstract

Research suggests that cranberry may interfere with bacterial adhesion to uroepithelial cells, thus preventing UTIs. To date, few research studies have been conducted to examine the potential interaction of *E. coli* and uroepithelial cells in the presence of post-cranberry consumption urine. The current study is a randomized, placebo-controlled, double-blind, crossover study to investigate the molecular-scale effect of cranberry juice metabolites on two P-fimbriated *E.coli* strains: B37 and CFT073 as assessed by atomic force microscopy (AFM). Three female subjects were asked to consume 8 oz cranberry juice cocktail or water. The washout period was 7 days. The urine samples were then collected at intervals of 2, 4, 6 hours post-ingestion of CJC or water. Additional urine before consumption of CJC was collected as a baseline control. The baseline average adhesion force between *E. coli* and uroepithelial cells was 13.09 ± 11.60 nN for CFT073 and 10.30 ± 5.50 nN for B37. For post-CJC urine treatment, the adhesion forces decreased to 2.94 ± 1.82 nN at 2 hours after consumption then increased slightly to 5.51 ± 2.78 nN at 6 hours after ingestion for CFT073, while it decreased to 4.77 ± 3.33 nN after consuming for 2 hours and seemed to be stable in the next 4 hours following consumption (5.52 ± 4.04 nN after drinking for 4 hours; 5.05 ± 4.42 nN after drinking for 6 hours) for B37. The adhesion forces in post-water consumption urine were

similar to that of the background for *E. coli* B37; meanwhile a downward trend for the adhesion forces in post-water consumption compared to the background was observed for *E. coli* CFT073. However, those adhesion forces in post-water consumption urine were still higher than those measured after CJC consumption at the same time intervals. The mean differences between the cranberry and placebo groups were statistically different according to two way ANOVA method followed by Holm-Sidak test. Our results suggest a significant inhibitory interaction between the daily consumption of 8 oz cranberry juice and bacteria adhesive activity.

4.2 Introduction

Urinary tract infection has been considered one of the most significant infections affecting humans today. It is usually caused by the presence of bacteria in the urinary tract system [19], especially *Escherichia coli* (*E. coli*) [3, 8]. Cranberry (*Vaccinium macrocarpon*) has been utilized to protect against UTIs for generations [6, 15, 16, 29]. The unique A-type proanthocyanidins (PACs) present uniquely in cranberry have been identified as the active agent to prevent P-fimbriated *E. coli* to attach to uroepithelial cells [17, 18]. However, the activity of cranberry metabolites in urine following ingestion of cranberry juice cocktail is still unclear and the active compounds remain unidentified. Due to the limited molecular knowledge of its inhibitory effect, cranberry products have not been integrated into established medical care regimes successfully.

As the first step in the development of infection, bacterial adhesion is of great research interest. In this study, a biofilm assay (Chapter III) was used to detect bacterial anti-adhesion activity of metabolites in urine following consumption of cranberry juice cocktail. Although interesting results were obtained, the biofilm assay does not describe adhesion at the molecular level. The lack of quantitative data on the adhesive interactions between bacterial cells and uroepithelial cells in the presence of urine which contains post-consumption cranberry metabolites limits its current use for UTI treatment. Understanding of how cranberry affects the interaction between bacteria and epithelial cells lining the urinary tract is important and helpful to provide the mechanisms by which cranberry metabolites inhibit the adhesion of *E. coli* to uroepithelial cells.

AFM is a sensitive tool and can be used to directly measure the nano-scale forces of attraction or repulsion between virtually any two surfaces. A technique to

quantitatively measure the adhesive interactions between a bacterium and a surface or between two biological surfaces is developed by our lab [78] using AFM. By directly quantifying such interactions between P-fimbriated *E. coli* and human uroepithelial cells exposed to post-CJC consumption urine, the molecular mechanisms by which cranberry juice cocktail (CJC) affects bacterial adhesion could be revealed and mechanistic understanding will allow for the development of the best therapies and measures to prevent infections.

In order to elucidate time-dependence of these mechanisms and variability for different volunteers, the adhesive interactions between P-fimbriated *E. coli* and uroepithelial cells will be directly and quantitatively characterized in this study. Two clinical Class II P-fimbriated *E. coli* strains (B37 and CFT073) that showed the most significant decreases in biofilm formation in Chapter III were chosen. The effect of the time to which a peak in anti-adhesive activity is reached will be quantified.

4.3 Materials and Methods

4.3.1 Subjects and Collection of Urine Samples

Female subjects were recruited by Dr. Amy Howell at Rutgers University (New Jersey). Although the present application is not a clinical study and does not involve human subjects, information is provided about the participants for information purposes. Volunteers were not allowed to eat or drink any cranberry products for at least three days prior to and on the day of urine collection. Volunteers were provided and asked to consume 8 oz. (240 mL) of commercial cranberry juice cocktail (CJC), or water. A washout period for at least seven days was used. On the day of the study, the urine before

consumption of CJC was collected as a baseline control. Additional urine samples were then collected at intervals of 2, 4, 6 hours post-ingestion of CJC or water. Samples were frozen immediately and sent to Dr. Terri Camesano's laboratory at Worcester Polytechnic Institute (WPI, MA) with a code number only for de-identified purposes. At WPI, samples were sterilized by filtering by polyethersulfone syringe filters (VWR International TM, West Chester, PA) with 0.8 μm and 0.2 μm membranes sequentially, and stored at -20 °C for future use.

4.3.2 Preparation of Bacteria Cell

Bacterial samples tested in this study were two clinical P-fimbriaed *E. coli* isolates (B37 and CFT073), The strains were cultured in Luria-Bertani broth (35g/L, Sigma-Aldrich, St. Louis, MO) at 37 °C with gentle shaking and harvested at late exponential phase (optical density = 0.9-1.0 at 600 nm), which allowed them to express P fimbriae. Harvested bacteria were washed to remove components of the growth media by centrifugation at 7000 RPM for 10 min.

4.3.3 Preparation of Biological Probes

A single *E. coli* bacterium was immobilized onto an AFM tip using a technique developed in our lab [5, 78]. Briefly, the probe was coated with poly-L-lysine (PLL) by immersing in 0.1% PLL for 30 min and air dried for 10 min. The probe was then immersed in a concentrated bacteria suspension for 10 min to allow bacteria to bind on the probe. After air drying for 10 min, the biologically functionalized tip was ready for future use to measure the adhesion forces between *E. coli* and a lawn of uroepithelial cells.

4.3.4 Uroepithelial Cell Culture

Human bladder uroepithelial cells were used in this study. SV-HUC-1 cell line was purchased from the American Type Culture Collection (ATCC) and maintained in liquid nitrogen vapor phase. The cells were grown in F-12K Medium (Kaighn's modification of Ham's F12 medium with L-glutamine, ATCC) supplemented with 10% fetal bovine serum (ATCC). Culture flasks were placed in a humidified atmosphere of 5% CO₂ in air at 37°C for seven days, and growth medium was replaced every two days. Trypsin-EDTA solution (0.25% Trypsin / 0.53 mM EDTA, ATCC) was added to harvest the uroepithelial cells as the low concentration of trypsin allowed us to detach the cells from the culture flasks without compromising their viability or surface properties [79]. The cells were kept in trypsin-EDTA for no more than 10 min in an incubator (5% CO₂, 37 °C), washed, centrifuged at 1200 RPM for 6 min, and resuspended in pre-warmed growth medium gently.

4.3.5 Preparation of Uroepithelial Cell Glass Slide for AFM Measurements

For bacterial adherence assay, early passage cells (passage < 50) were seeded to a sterilized glass slide (Corning Incorporated, Corning, NY) and grown to 90% confluence. Briefly, the resuspended cells were cultured in the 35 mm Tissue Culture Treated Dish (Sarstedt, Inc. Newton, NC) containing a glass slide with growth media. The culture dish was placed in an incubator (5% CO₂, 37 °C) to allow the cells to attach the surface of the glass slide. After two days incubation, the glass slide with a cell-layer lawn was obtained.

4.3.6 AFM Adherence Force Assays

Adhesion forces were measured with AFM (Asylum Research, Santa Barbara, CA), with all measurements performed in the presence of urine following consumption of

CJC or water (Figure 4.1). Silicon nitride AFM tips on a triangular cantilever (DNPS, Veeco Metrology) were used to acquire images in fluid in tapping mode. Before force measurements were made, the spring constant of the cantilever was measured using the thermal calibration method. The average spring constant was 513.3 ± 94.4 pN/nm. A human uroepithelial cell culture was maintained, and the adhesion force measurements between epithelial cells and bacteria were carried out in urine samples, and at least three individual cells were probed, where at least ten force cycles were recorded per bacterium per condition.

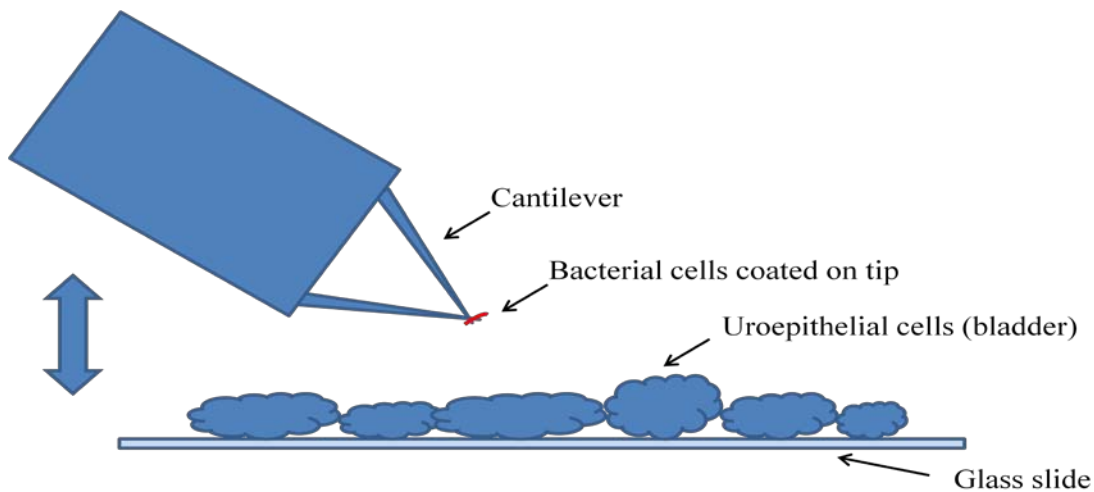


Figure 4.1 Schematic demonstrating AFM experiments to measure cell-cell interactions. An *E. coli* cell is binded to an AFM cantilever (silicon or silicon nitride have been used successfully in our lab). Interaction forces are measured between the biological functionalized probe and a lawn of epithelial cells, grown and attached on a glass slide.

4.3.7 Statistical Analysis

A two way ANOVA test was performed to compare the two groups that were treated with urine samples collected after CJC or water consumption. Background (bacteria treated with urine samples that were collected before drinking CJC) adhesion forces were also compared with adhesion forces measured on the bacteria treated with

urine samples that were collected after consumption of CJC or water. The statistical test used was Holm-Sidak method.

4.4 Results

4.4.1 Adhesion Forces between *E. coli* Bacteria and Uroepithelial Cells

For two bacterial strains, the P-fimbriated *E. coli* B37 and *E. coli* CFT073, adhesion forces to the bladder epithelial cells using bacterial probe were measured. The average adhesion forces between both of the P-fimbriated bacteria (*E. coli* B37 and CFT073) and uroepithelial cells were decreased in post-CJC consumption urine compared to the background (pre-CJC consumption urine) and the post-water consumption urine at the same time intervals as well.

In the absence of any treatment, the average adhesion force between *E. coli* CFT073 and uroepithelial cells was 13.09 ± 11.60 nN (Figure 4.3), while it was 10.30 ± 5.50 nN between *E. coli* B37 and uroepithelial cells (Figure 4.2). After CJC treatment, the adhesion forces between *E. coli* CFT 073 and uroepithelial cells decreased to 2.94 ± 1.82 nN at 2 hours after consumption, and then it increased slightly to 5.51 ± 2.78 nN at 6 hours after ingestion. The adhesion forces between *E. coli* B37 and uroepithelial cells decreased to 4.77 ± 3.33 nN after consuming for 2 hours and seemed to be stable in the next 4 hours following consumption (5.52 ± 4.04 nN after drinking for 4 hours; 5.05 ± 4.42 nN after drinking for 6 hours). The adhesion forces in post-water consumption urine were similar to that of the background for *E. coli* B37 (Figure 4.2); meanwhile a downward trend for the adhesion forces was observed for *E. coli* CFT073 (Figure 4.3),

especially at 4 hours. However, those adhesion forces in post-water consumption urine were still higher than those measured after CJC consumption at the same time intervals.

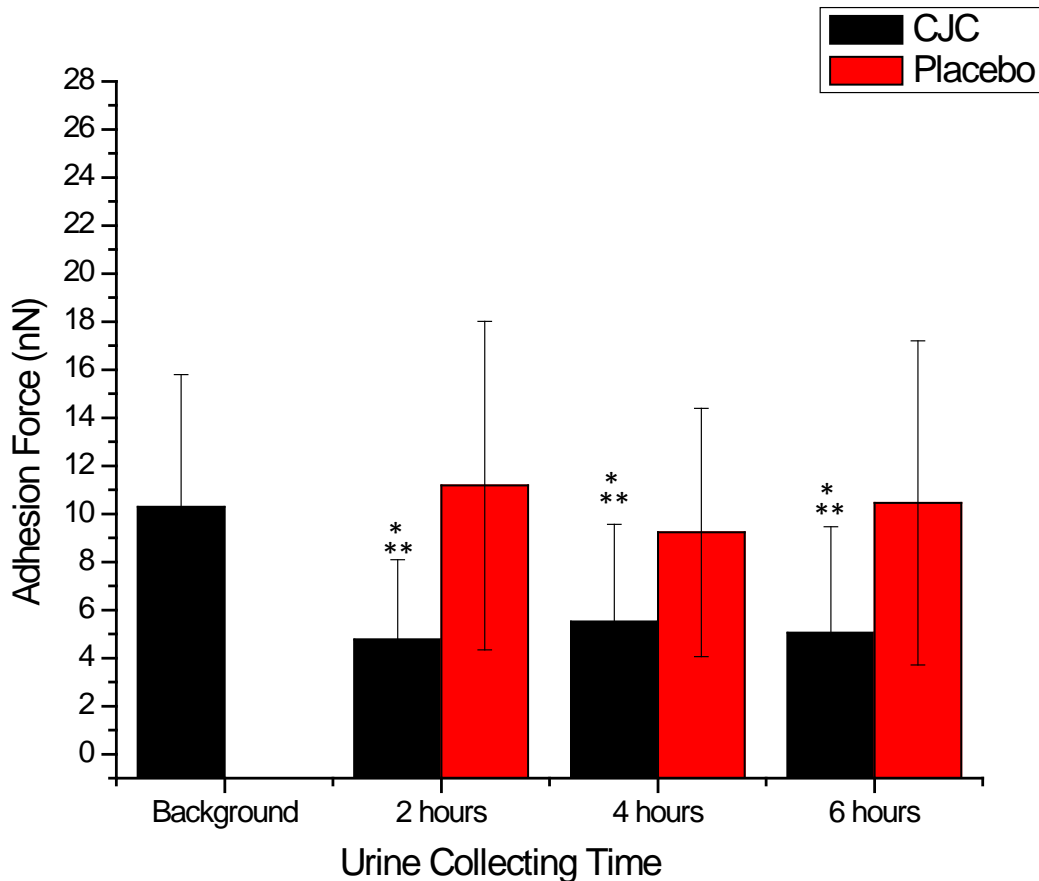


Figure 4.2 Average adhesion forces between *E. coli* B37 and uroepithelial cells as a function of urine collecting time (hour) after consumption of CJC or water. Data are mean \pm SD values. *Adhesion forces detected in urine following ingestion of CJC or placebo were significantly different. **The difference compared to background (pre-consumption urine) was significant.

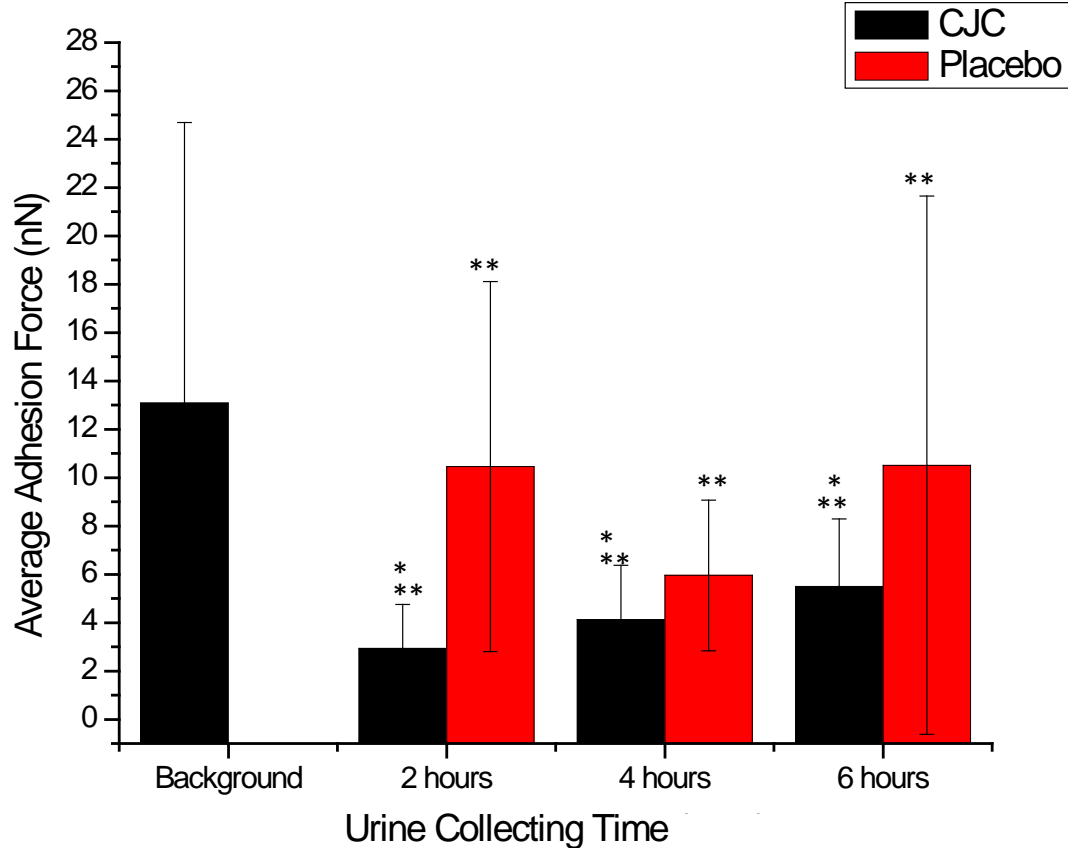


Figure 4.3 Average adhesion forces between *E. coli* CFT073 and uroepithelial cells as a function of urine collecting time (hour) after consumption of CJC or water. Data are mean \pm SD values. *Adhesion forces detected in urine following ingestion of CJC or placebo were significantly different. **The difference compared to background (pre-consumption urine) was significant.

When we compared each condition at same time point with one another, adhesion forces in all post-CJC urine treatment were significantly lower than that of post-water urine treatment for both the clinical strains. Only the adhesion forces between *E. coli* CFT073 and uroepithelial cells in post-water consumption urine were significantly different compared with background adhesion force levels.

4.4.2 Volunteer Variability

The inhibitory effects of cranberry juice metabolites on the two *E. coli* strains (B37 and CFT073) for individual volunteer are summarized in Figure 4.4. The anti-adhesive of ability on the tested bacteria differed from volunteer to volunteer. Adhesion reduction was found to be dependent on the time treatment of cranberry juice as well.

Table 4.1 Number of volunteers (total number of volunteers involved is 3) that showed a significantly lower adhesion forces between *E. coli* and uroepithelial cells in the presence of post-CJC consumption urine.

Strain	# of volunteers showing difference between CJC and placebo		
	2hr	4hr	6hr
<i>E. coli</i> B37	3	2	2
<i>E. coli</i> CFT073	3	2	1

All volunteers' urine (100%) had a significant anti-adhesive effect on *E. coli* strains at 2 hours following consumption, while at 4 hours, only 67% of volunteers responded to CJC. At 6 hours, *E. coli* B37 still works as it was on 4 hours, but *E. coli* CFT073 fell down to 33%.

4.5 Discussion

4.5.1 Bacteria Coated AFM Tips Preparation

To verify that the AFM tip was successfully coated with bacteria, adhesion forces between a bare silicon nitride probe and a clean glass slide were measured. The interactions detected between bare silicon nitride and glass was minimal compared to the force profile between the *E. coli* biological probe and the glass, which were generally in the nano-Newton (nN) range, indicating that bacteria were binded to AFM tip successfully using the method developed in our lab.

4.5.2 Role of Cranberries in P-fimbriated *E. coli* Adhesion to Epithelial Cells

Daily consumption of cranberry juice cocktail has been recommended to prevent urinary tract infections mainly caused by *E. coli*, and P-fimbriae is considered to play a significant role among virulence factors [80]. Therefore, this study focused on cranberry metabolites' ability to inhibit the binding of P-fimbriae on pathogenic *E. coli* to the galgal receptor on the epithelial cell, which is a very strong bond [18, 57].

It has been known that most kidney infections arise from bladder infections through an ascending route [81]. Therefore, the initial adhesion of the pathogen may occur to bladder epithelial cells, and then P-fimbriated uropathogens can migrate to the kidney, causing acute pyelonephritis [82]. That is the reason why bladder uroepithelial cell line rather than kidney uroepithelial cell line is chosen in this study.

Our AFM results showed that urine from subjects who drank CJC experienced a significant reduction in bacterial adherence to uroepithelial cells compared to when the same volunteer drank water. For *E. coli* B37 particularly, the antibiotic resistant strain, water consumption did not affect the adhesion forces compared to a significant decrease after CJC consumption, and adhesion was also insensitive to time after consumption (Figure 4.2). This is in a good agreement with one earlier study by Tao et al. from our laboratory [58]. These experiments demonstrated that the protocol and methodology developed in our lab can be used to detect molecular level differences in adhesion forces that are caused by consumption of CJC. The reduced anti-adhesive activity may be the result of some components in cranberry juice interacting with P-fimbriae directly, causing P-fimbriae to become compressed and less adhesive immediately. In addition, a

significant time-dependent decrease in bacterial *in vitro* adherence was encountered after cranberry consumption.

Although previous study showed that the presence of cranberry juice could change the surface characterization of bacteria significantly [57] and PACs in cranberry could block bacterial swarming motility completely [83], whether PACs are the only compounds responsible for preventing and treating UTIs remain unknown. It has been demonstrated the effects of PAC on bacterial adhesion to and invasion of kidney epithelial cells [84]. Furthermore, our study showed that cranberry metabolites could block bacterial adhesive ability and invasion of bladder epithelial cells. However, the role of PAC metabolites to this phenomena are still not clear, further studies are needed to be conducted to investigate whether the metabolites in urine are from uniquely PACs or non-PAC compounds. As invasion of uroepithelial cells is a key step in the development of a UTI, our results exhibited that active agents could survive from the digestive system and held the promising future to treat UTIs.

4.5.3 Time Dependence of CJC's Inhibitory Effects

In recent studies [85, 86], a significant bacterial anti-adhesion activity was observed in urine samples collected from volunteers that consumed cranberry powder compared to placebo. And it was dose-dependent [85, 86] and still working until 24 hours after consuming 72 mg of PAC [85]. Howell et al. [18] demonstrated that 0-6 hour following CJC consumption responded to the most important elimination period of PACs (which is recognized as the active agents in cranberry) in urines, that is why 0-6 hour (at intervals of every two hours) after CJC ingestion were chosen to collect the urine in this study.

This randomized, double-blind versus placebo study revealed a time dependent effect in *in vitro* model of *E. coli* adherence to bladder epithelial cells. We demonstrated a peak of urinary anti-adhesion activity 2 hours after consumption of 8 oz. commercial cranberry juice cocktail. One previous study in our lab also showed that anti-adhesive ability of post-consumption urine on *E. coli* strains peaked at 6 h after 16 oz. CJC ingestion and were still working after drinking 8 hours [58]. When it comes to 8 oz. in this study, most significant difference was observed at 2 hours following consumption, which is most probable because this effect has a dose-dependent relationship. In other words, the more CJC the volunteer received, the longer this anti-adhesive effect last.

For the experiments designed to test the effect of time after consumption, urine samples were collected at intervals of 0, 2, 4, and 6 hours after consumption of cranberry juice. The AFM results showed that cranberry juice has an immediate effect (after consuming CJC for 2 hours) on the P-fimbriated *E. coli* bacteria. When cultured with urine samples collected at different time intervals following CJC consumption, both of the clinical *E. coli* strains demonstrated decreased adhesion forces after initial CJC consumption but increased slightly with time (Figures 4.2-4.3). Although this is an *in vitro* model, our work suggests that continuous exposure to cranberry juice will be needed in order for *E. coli* to maintain an anti-adhesive behavior in the body.

4.6 Conclusions

UTIs are infections caused by invading of bacteria and the adhesive ability of these microorganisms seems to be an important pathogenic factor. Cranberry metabolites present in urine collected following consumption of CJC were examined for their potential to reduce the initial adhesion of uropathogenic bacteria to bladder epithelial

cells. For both of *E. coli* B37 and CFT073, two strains expressing P-fimbriae Class II, most significant inhibition of bacterial adhesion was observed for the condition where cranberry juice cocktail was taken after 2 hours and it was still working over 6 hours following consumption. The results of this study are promising for cranberry juice cocktail as a proposed dietary treatment which is independent of antibacterial mechanisms that may give rise to resistant strains.

Chapter V Research Summary

Although cranberry has been known for its health benefits to prevent urinary tract infections for quite a long time, the mechanisms are still not clear. The initial adhesion of pathogens to host tissue cells may be the first in a series of steps leading to the infection. In order to elucidate the anti-adhesive activity at molecular scale, this study investigated the inhibitory effects of cranberry metabolites in post-CJC consumption urine on pathogenic *E. coli*.

In Chapter III, we evaluated the persistence of cranberry metabolites in urine and its effects on biofilm formation and bacterial cell viability. The results demonstrated that biofilm formation was inhibited significantly for *S. aureus* and *E. coli* containing Class II P fimbriae, suggesting bacterial adhesive ability is closely associated with the expression of pili fimbriae. In addition, the viability test results confirmed that the cranberry metabolites in post-CJC urine behaved as an inhibitor to block the expression of pili fimbriae rather than a bactericide to kill the bacteria or restrain its growth.

In Chapter IV, we measured the adhesion forces directly between *E. coli* and uroepithelial cells in the presence of urine following consumption of CJC or water through atomic force microscopy. We determined that exposure to urine collected after drinking CJC reduced the adhesion forces significantly for both of our *E. coli* strains (P-fimbriated B37 and CFT073). This indicated that cranberry constituents could survive from the digestive system in our body and cranberry metabolites exhibited a significant bacterial anti-adhesive ability and this inhibition was clearly time-dependent.

Through these analyses, information on the dose of cranberry juice needed to provide an anti-adhesion benefit, the time of exposure acquired between *E. coli* and the cranberry metabolite in urine, and the variability in response due to different clinical strains of *E. coli* and the behavior of different volunteers was obtained. This work can help establish conditions that should be used in subsequent clinical trials.

Chapter VI Future Work

American cranberry has long been recognized for its preventive benefits to maintain urinary tract health; however, it is too acidic and astringent at full strength and may not be acceptable to many patients as a prophylactic therapy over a long period. Cranberry juice cocktail and cranberry tablets have been introduced in this context. However, few dose-response studies have been done to determine the optimal volume of juice or number of tablets needed to prevent infection. We will further detect the adhesion forces between uropathogenic *E. coli* and uroepithelial cells in presence of post-16 oz CJC urine through AFM and compare the results with adhesion forces obtained in the treatment of post-8 oz CJC urine. We might also extend the *E. coli* strains to cover more kind of adhesions.

Our results showed that urine collected after administration of cranberry juice can prevent biofilm formation by *E. coli* but the nature of the metabolites is unknown. To our knowledge, the identity of such metabolites has not been explored, nor their role in bacterial susceptibility. It therefore may be of interest to examine possible contributions by any of the compounds present in cranberry fruit as well as their metabolites found in the human body.

Although A-PACs are identified and widely recognized as the potential effective components in cranberry that reduce bacterial adhesion, it remains a question whether PACs are the only or major anti-adhesion components in cranberry, since some studies showed that cranberry juice had a higher anti-adhesion activity than A-PACs alone [60].

In addition, new research has suggested that bacteria that do not express P fimbriae, including other strains of *E. coli* as well as different species of bacteria, can also express anti-adhesive activity in the presence of cranberry juice [40, 60, 87]. These results suggest that compounds in cranberry, in addition to PACs, also have anti-adhesion activity. Further, PACs have not been detected in urine. Therefore, further experiments needed to determine the relative anti-biofilm efficacy of the CJC, cranberry PACs, and non-PAC components of cranberry juice.

The kinetic information of binding between cranberry compounds and pathogenic bacteria and/or the uroepithelial cell is still lacking, even though direct measurements of adhesion forces between uropathogenic *E. coli* and uroepithelial cells are made. To understand the dynamics and efficacy of cranberry compounds as an anti-adhesive therapy, the kinetic information is particularly of importance. Therefore, we will quantify the binding affinity and monitoring the kinetics of bacterial binding with epithelial cells, using Quartz crystal microbalance with dissipation monitoring (QCM-D).

References

1. Foxman, B., et al., *Urinary tract infection: Self reported incidence and associated costs*. Annals of Epidemiology, 2000. **10**(8): p. 509-515.
2. Foxman, B., *Epidemiology of urinary tract infections: Incidence, morbidity, and economic costs*. American Journal of Medicine, 2002. **113**: p. 5S-13S.
3. Russo, T.A. and J.R. Johnson, *Medical and economic impact of extraintestinal infections due to Escherichia coli: focus on an increasingly important endemic problem*. Microbes and Infection, 2003. **5**(5): p. 449-456.
4. Gupta, K., U. Scholes, and W.E. Stamm, *Increasing prevalence of antimicrobial resistance among uropathogens causing acute uncomplicated cystitis in women*. Jama-Journal of the American Medical Association, 1999. **281**(8): p. 736-738.
5. Camesano, T.A., Y.T. Liu, and P.A. Pinzon-Arango, *Cranberry prevents the adhesion of bacteria: overview of relevant health benefits*. Agro Food Industry Hi-Tech, 2007. **18**(1): p. 24-27.
6. Di Martino, P., et al., *Reduction of Escherichia coli adherence to uroepithelial bladder cells after consumption of cranberry juice: a double-blind randomized placebo-controlled cross-over trial*. World Journal of Urology, 2006. **24**(1): p. 21-27.
7. Kuzminski, L.N., *Cranberry juice and urinary tract infections: Is there a beneficial relationship?* Nutrition Reviews, 1996. **54**(11): p. S87-S90.
8. Perez-Lopez, F.R., J. Haya, and P. Chedraui, *Vaccinium macrocarpon: An interesting option for women with recurrent urinary tract infections and other health benefits*. Journal of Obstetrics and Gynaecology Research, 2009. **35**(4): p. 630-639.
9. Lynch, D.M., *Cranberry for prevention of urinary tract infections*. American Family Physician, 2004. **70**(11): p. 2175-2177.
10. Blatherwick, N.L., ML, *Studies of Urinary Acidity II: The Increased Acidity Produced by Eating Prunes and Cranberries*. J. Biol. Chem., 1923. **57**: p. 815-818.
11. Kinney, A.B. and M. Blount, *Effect of cranberry juice on urinary pH*. Nurs Res, 1979. **28**(5): p. 287-90.
12. Jackson, B. and L.E. Hicks, *Effect of cranberry juice on urinary pH in older adults*. Home Healthc Nurse, 1997. **15**(3): p. 198-202.

13. Nickey, K., *Effect of prescribed regimens of cranberry juice and ascorbic acid*. Arch Physical Med Rehab, 1975. **56**: p. 556.
14. Avorn, J., et al., *Reduction of Bacteriuria and Pyuria after Ingestion of Cranberry Juice*. Jama-Journal of the American Medical Association, 1994. **271**(10): p. 751-754.
15. Sobota, A.E., *Inhibition of Bacterial Adherence by Cranberry Juice - Potential Use for the Treatment of Urinary-Tract Infections*. Journal of Urology, 1984. **131**(5): p. 1013-1016.
16. Zafriri, D., et al., *Inhibitory activity of cranberry juice on adherence of type 1 and type P fimbriated Escherichia coli to eucaryotic cells*. Antimicrob Agents Chemother, 1989. **33**(1): p. 92-8.
17. Foo, L.Y., et al., *The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated Escherichia coli in vitro*. Phytochemistry, 2000. **54**(2): p. 173-181.
18. Howell, A.B., et al., *A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity*. Phytochemistry, 2005. **66**(18): p. 2281-2291.
19. Johnson, C.C., *Definitions, classification, and clinical presentation of urinary tract infections*. Med Clin North Am, 1991. **75**(2): p. 241-52.
20. Moore, E.E., et al., *Urinary incontinence and urinary tract infection - Temporal relationships in postmenopausal women*. Obstetrics and Gynecology, 2008. **111**(2): p. 317-323.
21. Leahy, M., J. Speroni, and M. Starr, *Latest developments in cranberry health research*. Pharmaceutical Biology, 2002. **40**: p. 50-54.
22. Barbosa-Cesnik, C., et al., *Cranberry Juice Fails to Prevent Recurrent Urinary Tract Infection: Results From a Randomized Placebo-Controlled Trial*. Clinical Infectious Diseases, 2011. **52**(1): p. 23-30.
23. Mysorekar, I.U. and S.J. Hultgren, *Mechanisms of uropathogenic Escherichia coli persistence and eradication from the urinary tract*. Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(38): p. 14170-14175.
24. Bahrani-Mougeot, F.K., et al., *Type 1 fimbriae and extracellular polysaccharides are preeminent uropathogenic Escherichia coli virulence determinants in the murine urinary tract*. Molecular Microbiology, 2002. **45**(4): p. 1079-1093.
25. Wullt, B., *The role of P fimbriae for Escherichia Coli establishment and mucosal inflammation in the human urinary tract (vol 19, pg 522, 2002)*. International Journal of Antimicrobial Agents, 2003. **21**(6): p. 604-621.

26. Emody, L., M. Kerenyi, and G. Nagy, *Virulence factors of uropathogenic Escherichia coli*. International Journal of Antimicrobial Agents, 2003. **22**: p. S29-S33.
27. Ruiz, J., et al., *Differences in virulence factors among clinical isolates of Escherichia coli causing cystitis and pyelonephritis in women and prostatitis in men*. Journal of Clinical Microbiology, 2002. **40**(12): p. 4445-4449.
28. Gaffney, R.A., et al., *Effect of Vaginal Fluid on Adherence of Type-1 Piliated Escherichia-Coli to Epithelial-Cells*. Journal of Infectious Diseases, 1995. **172**(6): p. 1528-1535.
29. Jepson, R.G. and J.C. Craig, *Cranberries for preventing urinary tract infections*. Cochrane Database of Systematic Reviews, 2008(1): p. -.
30. Raz, R., B. Chazan, and M. Dan, *Cranberry juice and urinary tract infection*. Clinical Infectious Diseases, 2004. **38**(10): p. 1413-1419.
31. Costerton, J.W., *Overview of Microbial Biofilms*. Journal of Industrial Microbiology, 1995. **15**(3): p. 137-140.
32. Naves, P., et al., *Correlation between virulence factors and in vitro biofilm formation by Escherichia coli strains*. Microbial Pathogenesis, 2008. **45**(2): p. 86-91.
33. Davies, D.G., A.M. Chakrabarty, and G.G. Geesey, *Exopolysaccharide Production in Biofilms - Substratum Activation of Alginate Gene-Expression by Pseudomonas-Aeruginosa*. Applied and Environmental Microbiology, 1993. **59**(4): p. 1181-1186.
34. Soto, S.M., et al., *Implication of biofilm formation in the persistence of urinary tract infection caused by uropathogenic Escherichia coli*. Clinical Microbiology and Infection, 2006. **12**(10): p. 1034-1036.
35. Raad, I.I., et al., *The role of chelators in preventing biofilm formation and catheter-related bloodstream infections*. Current Opinion in Infectious Diseases, 2008. **21**(4): p. 385-392.
36. Marsh, P.D., *Dental plaque as a microbial biofilm*. Caries Research, 2004. **38**(3): p. 204-211.
37. Zelic, O., S. Cakic, and N. Lukovic, *The Effect of Two Different Oral Antiseptics on Dental Plaque Formation (De Novo Biofilm) and on Gingival Inflammation*. Srpski Arhiv Za Celokupno Lekarstvo, 2009. **137**(1-2): p. 6-9.
38. Randler, C., et al., *A three-phase in-vitro system for studying Pseudomonas aeruginosa adhesion and biofilm formation upon hydrogel contact lenses*. BMC Microbiology, 2010. **10**: p. -.

39. Darouiche, R.O., *Device-associated infections: A macroproblem that starts with microadherence*. *Clinical Infectious Diseases*, 2001. **33**(9): p. 1567-1572.
40. Johnson-White, B., et al., *Prevention of nonspecific bacterial cell adhesion in immunoassays by use of cranberry juice*. *Analytical Chemistry*, 2006. **78**(3): p. 853-857.
41. Franceschi, S., et al., *Role of different types of vegetables and fruit in the prevention of cancer of the colon, rectum, and breast*. *Epidemiology*, 1998. **9**(3): p. 338-341.
42. Vainio, H. and E. Weiderpass, *Fruit and vegetables in cancer prevention*. *Nutrition and Cancer-an International Journal*, 2006. **54**(1): p. 111-142.
43. Temple, N.J. and K.K. Gladwin, *Fruit, vegetables, and the prevention of cancer: Research challenges*. *Nutrition*, 2003. **19**(5): p. 467-470.
44. Siciliano, A., *Cranberry*. *HerbalGram*, 1996. **38**: p. 51-54.
45. Blumenthal, M.F., GKL., *Total Sales of Herbal Supplements in United States Show Steady Growth*. *HerbalGram*, 2006. **71**: p. 64-66.
46. Duthie, S.J., et al., *The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers*. *European Journal of Nutrition*, 2006. **45**(2): p. 113-122.
47. Vinson, J.A., et al., *Single dose and supplementation studies with cranberry juice relevant to its role as an antioxidant and heart disease*. *Faseb Journal*, 2003. **17**(5): p. A1097-A1098.
48. Neto, C.C., *Cranberry and blueberry: Evidence for protective effects against cancer and vascular diseases*. *Molecular Nutrition & Food Research*, 2007. **51**(6): p. 652-664.
49. Lilja, J.J., J.T. Backman, and P.J. Neuvonen, *Effects of daily ingestion of cranberry juice on the pharmacokinetics of warfarin, tizanidine, and midazolam - Probes of CYP2C9, CYP1A2, and CYP3A4*. *Clinical Pharmacology & Therapeutics*, 2007. **81**(6): p. 833-839.
50. Li Z, S.N., Carpenter CL, Thames G, Minutti C, Bowerman S., *Cranberry does not affect prothrombin time in male subjects on warfarin*. *J Am Diet Assoc.*, 2006. **106**(12): p. 2057-61.
51. Ahuja, S., B. Kaack, and J. Roberts, *Loss of fimbrial adhesion with the addition of *Vaccinium macrocarpon* to the growth medium of *P-fimbriated Escherichia coli**. *J Urol*, 1998. **159**(2): p. 559-62.

52. Ofek, I., J. Goldhar, and N. Sharon, *Anti-Escherichia coli adhesin activity of cranberry and blueberry juices*. *Toward Anti-Adhesion Therapy for Microbial Diseases*, 1996. **408**: p. 179-183,288.
53. Camesano, T.A., Y.T. Liu, and M. Datta, *Measuring bacterial adhesion at environmental interfaces with single-cell and single-molecule techniques*. *Advances in Water Resources*, 2007. **30**(6-7): p. 1470-1491.
54. Kontiokari, T., et al., *Randomised trial of cranberry-lingonberry juice and Lactobacillus GG drink for the prevention of urinary tract infections in women*. *British Medical Journal*, 2001. **322**(7302): p. 1571-1573.
55. Stothers, L., *A randomized trial to evaluate effectiveness and cost effectiveness of naturopathic cranberry products as prophylaxis against urinary tract infection in women*. *Can J Urol*, 2002. **9**(3): p. 1558-62.
56. Liu, Y., et al., *Direct adhesion force measurements between E. coli and human uroepithelial cells in cranberry juice cocktail*. *Molecular Nutrition & Food Research*, 2010. **54**(12): p. 1744-1752.
57. Liu, Y.T., et al., *Role of cranberry juice on molecular-scale surface characteristics and adhesion behavior of Escherichia coli*. *Biotechnology and Bioengineering*, 2006. **93**(2): p. 297-305.
58. Tao, Y., et al., *Oral Consumption of Cranberry Juice Cocktail Inhibits Molecular-Scale Adhesion of Clinical Uropathogenic Escherichia coli*. *Journal of Medicinal Food*, 2011.
59. Liu, Y., et al., *Cranberry changes the physicochemical surface properties of E. coli and adhesion with uroepithelial cells*. *Colloids Surf., B*, 2008. **65**(1): p. 35-42.
60. Pinzon-Arango, P.A., Y. Liu, and T.A. Camesano, *Role of Cranberry on Bacterial Adhesion Forces and Implications for Escherichia coli-Uroepithelial Cell Attachment*. *J. Med. Food*, 2009. **12**(2): p. 259-270.
61. O'Toole, G.A., et al., *Genetic approaches to study of biofilms*. *Methods Enzymol*, 1999. **310**: p. 91-109.
62. Howell, A.B. and B. Foxman, *Cranberry juice and adhesion of antibiotic-resistant uropathogens*. *Jama-Journal of the American Medical Association*, 2002. **287**(23): p. 3082-3083.
63. Martino, P.D., et al., *Effects of cranberry juice on uropathogenic Escherichia coli in vitro biofilm formation*. *J. Chemother*, 2005. **17**(5): p. 563-565.
64. Duguid, J.P., S. Clegg, and M.I. Wilson, *The fimbrial and non-fimbrial haemagglutinins of Escherichia coli*. *J Med Microbiol*, 1979. **12**(2): p. 213-27.

65. Evans, D.J., Jr., et al., *Hemolysin and K antigens in relation to serotype and hemagglutination type of Escherichia coli isolated from extraintestinal infections*. J Clin Microbiol, 1981. **13**(1): p. 171-8.
66. Green, C.P. and V.L. Thomas, *Hemagglutination of human type O erythrocytes, hemolysin production, and serogrouping of Escherichia coli isolates from patients with acute pyelonephritis, cystitis, and asymptomatic bacteriuria*. Infect Immun 1981. **31**(1): p. 309-15.
67. Beachey, E.H., *Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces*. J. Infect. Dis. , 1981. **143**(3): p. 325-45.
68. Howell, A.B., et al., *Inhibition of the adherence of P-fimbriated Escherichia coli to uroepithelial-cell surfaces by proanthocyanidin extracts from cranberries*. N Engl J Med 1998. **339**(15): p. 1085-6.
69. Colgan, R., et al., *Risk factors for trimethoprim-sulfamethoxazole resistance in patients with acute uncomplicated cystitis*. Antimicrob Agents Chemother, 2008. **52**(3): p. 846-51.
70. Johnson, J.R., J.J. Brown, and P. Ahmed, *Diversity of hemagglutination phenotypes among P-fimbriated wild-type strains of Escherichia coli in relation to papG allele repertoire*. Clinical and Diagnostic Laboratory Immunology, 1998. **5**: p. 160-170.
71. Gill, S.R., et al., *Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain*. Journal of Bacteriology, 2005. **187**(7): p. 2426-2438.
72. Howell, A.B. and B. Foxman, *Cranberry juice and adhesion of antibiotic-resistant uropathogens*. Jama, 2002. **287**(23): p. 3082-3.
73. Karkkainen, U.M., et al., *Rapid and specific detection of three different G adhesin classes of P-fimbriae in uropathogenic Escherichia coli by polymerase chain reaction*. Journal of Microbiological Methods, 1998. **34**(1): p. 23-29.
74. Johanson, I.M., et al., *Pap, Papg and Prsg DNA-Sequences in Escherichia-Coli from the Fecal Flora and the Urinary-Tract*. Microbial Pathogenesis, 1993. **15**(2): p. 121-129.
75. Stevens, D.L., *Community-acquired Staphylococcus aureus infections: Increasing virulence and emerging methicillin resistance in the new millennium*. Current Opinion in Infectious Diseases, 2003. **16**(3): p. 189-91.

76. Cardo, D., et al., *National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004*. American Journal of Infection Control, 2004. **32**(8): p. 470-485.
77. Etienne, J., et al., *Community-acquired methicillin-resistant Staphylococcus aureus carrying Panton-Valentine leukocidin genes: Worldwide emergence*. Emerging Infectious Diseases, 2003. **9**(8): p. 978-984.
78. Camesano, T., A.; Liu, Yatao; Gallardo-Moreno, Amparo *Methods for measuring cell-cell or cell-matrix adhesive forces and compounds for disrupting adhesive forces in biological systems*. 2007: US Patent.
79. Svensson, M., et al., *Glycolipid depletion in antimicrobial therapy*. Molecular Microbiology, 2003. **47**(2): p. 453-461.
80. Kontiokari, T., M. Nuutinen, and M. Uhari, *Dietary factors affecting susceptibility to urinary tract infection*. Pediatric Nephrology, 2004. **19**(4): p. 378-383.
81. Lane, M.C., et al., *Expression of flagella is coincident with uropathogenic Escherichia coli ascension to the upper urinary tract*. Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(42): p. 16669-16674.
82. Lane, M.C. and H.L.T. Mobley, *Role of P-fimbrial-mediated adherence in pyelonephritis and persistence of uropathogenic Escherichia coli (UPEC) in the mammalian kidney*. Kidney International, 2007. **72**(1): p. 19-25.
83. Tufenkji, N. and C. O'may, *The Swarming Motility of Pseudomonas aeruginosa Is Blocked by Cranberry Proanthocyanidins and Other Tannin-Containing Materials*. Applied and Environmental Microbiology, 2011. **77**(9): p. 3061-3067.
84. Tufenkji, N., et al., *Cranberry derived proanthocyanidins can prevent pathogen invasion of kidney epithelial cells*. Food Research International, 2010. **43**(3): p. 922-924.
85. Lavigne, J.P., et al., *Dosage effect on uropathogenic Escherichia coli anti-adhesion activity in urine following consumption of cranberry powder standardized for proanthocyanidin content: a multicentric randomized double blind study*. BMC Infectious Diseases, 2010. **10**.
86. Chou, M.Y., et al., *Cranberry products inhibit adherence of P-fimbriated Escherichia coli to primary cultured bladder and vaginal epithelial cells*. Journal of Urology, 2007. **177**(6): p. 2357-2360.
87. Eydelnant, I.A. and N. Tufenkji, *Cranberry derived proanthocyanidins reduce bacterial adhesion to selected biomaterials*. Langmuir, 2008. **24**(18): p. 10273-10281.

Appendices

Appendix A: Support File for Chapter III

Part I: An example of relative amount of biofilm formed after bacteria strain was incubated in urine following consumption of CJC or placebo for an individual (volunteer 1). Biofilm amount at hour 0 serves as baseline.

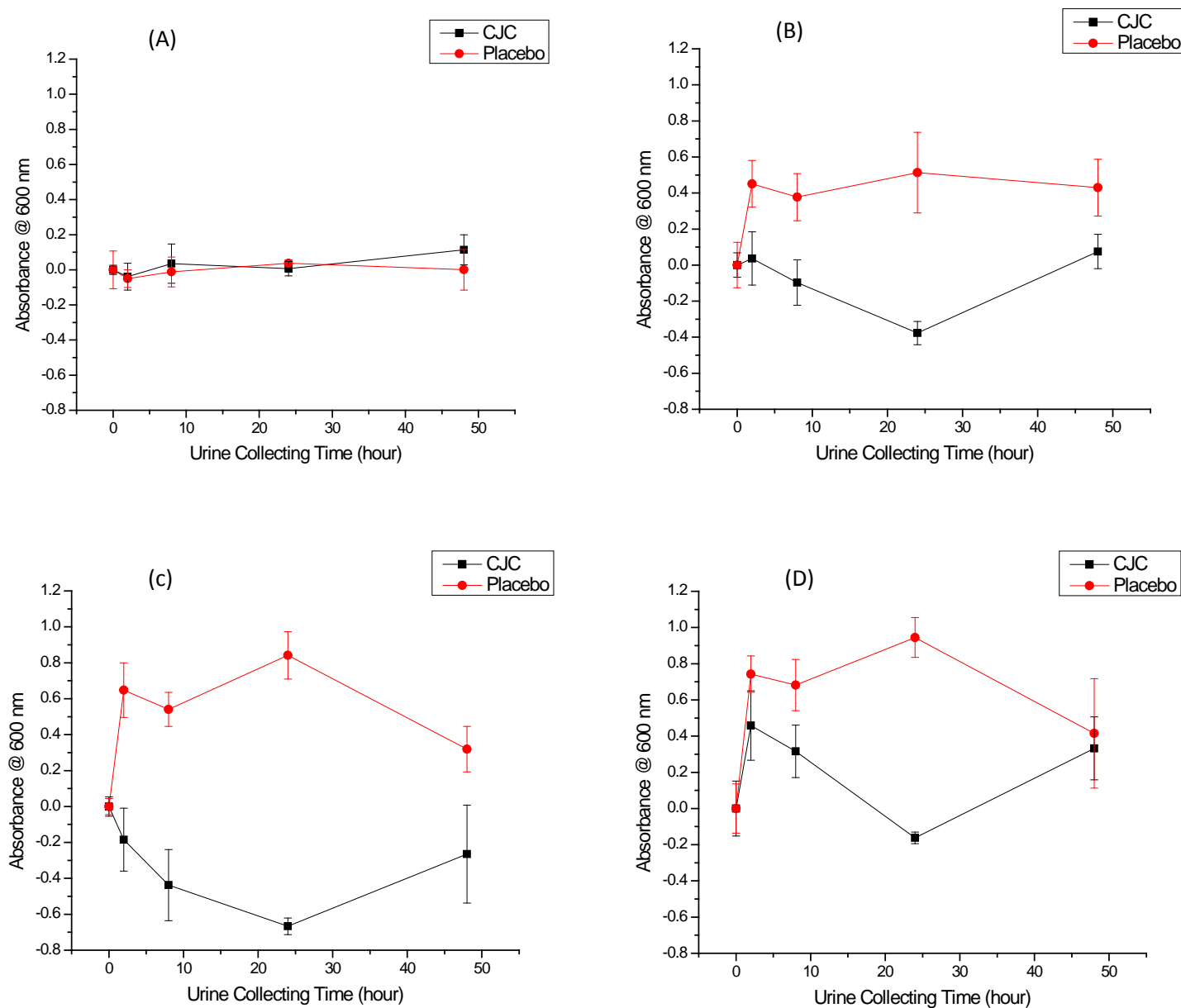


Figure S.1 Amount of biofilm formed for *S. aureus* in urine of volunteer 1 collected following consumption of CJC or placebo after incubation for (A) 0 hour; (B) 6 hours; (C) 24 hours; (D) 48 hours

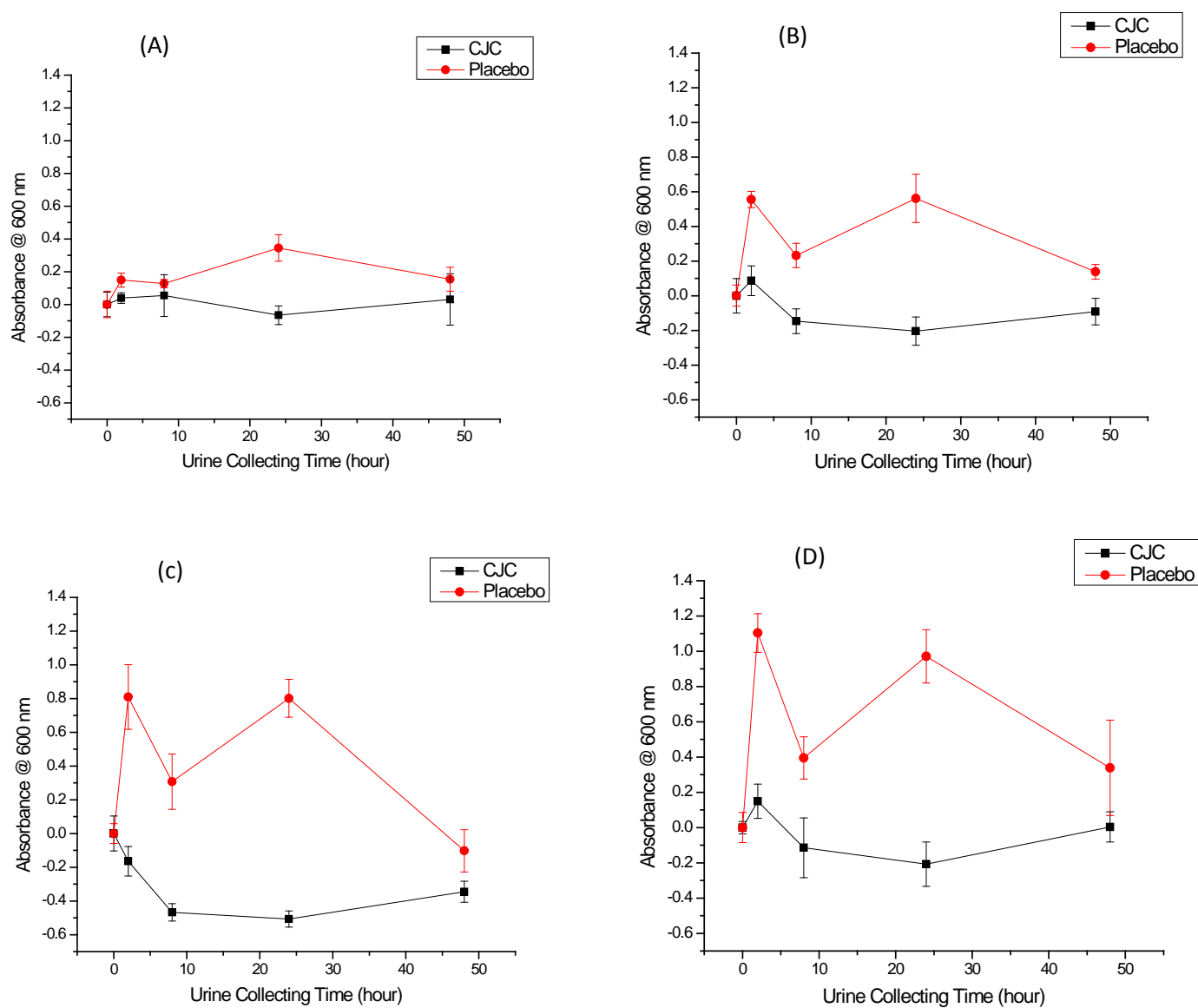


Figure S.2 Amount of biofilm formed for *E. coli* CFT073 in urine of volunteer 1 collected following consumption of CJC or placebo after incubation for (A) 0 hour; (B) 6 hours; (C) 24 hours; (D) 48 hours.

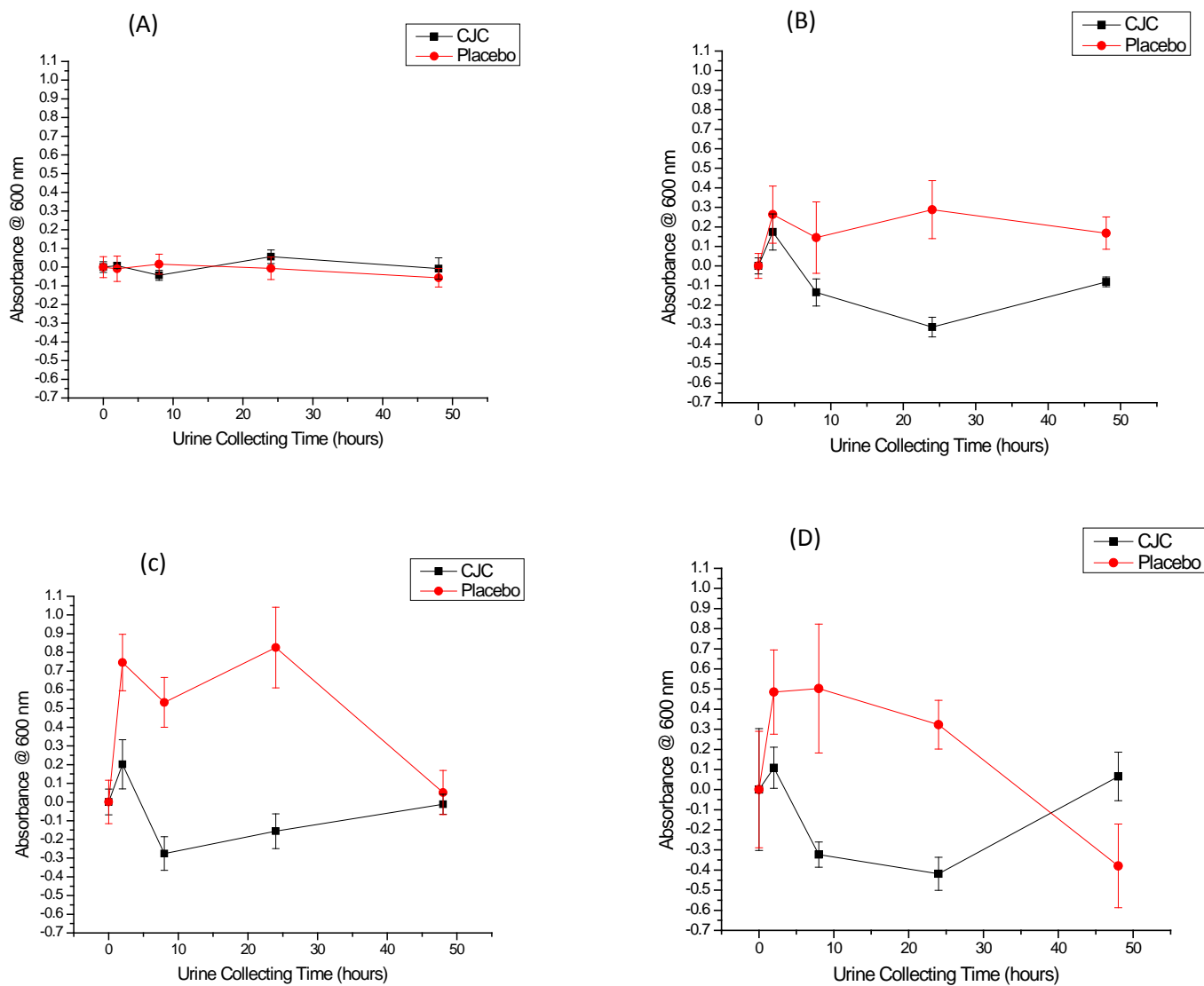


Figure S.3 Amount of biofilm formed for *E. coli* B37 in urine of volunteer 1 collected following consumption of CJC or placebo after incubation for (A) 0 hour; (B) 6 hours; (C) 24 hours; (D) 48 hours.

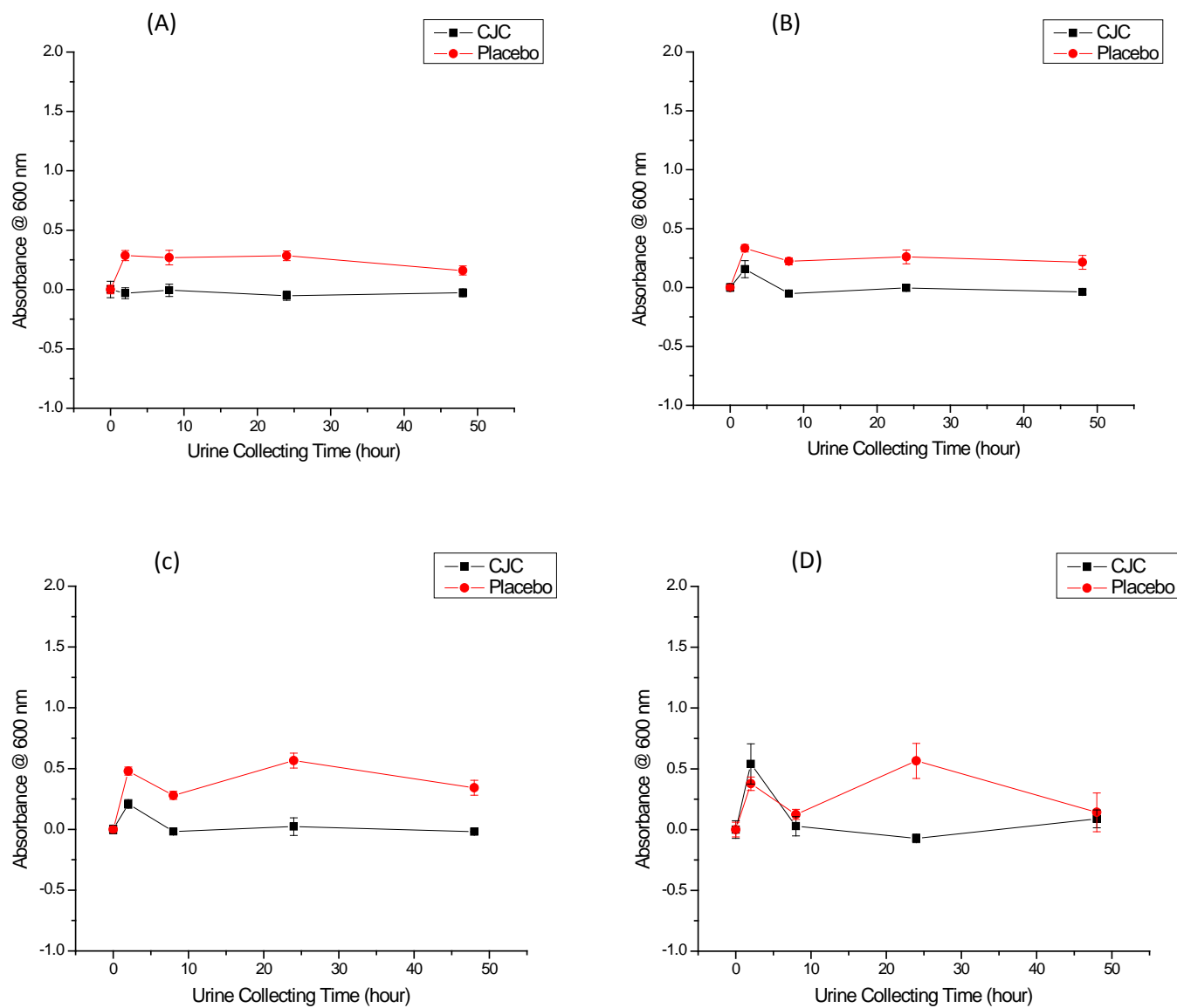


Figure S.4 Amount of biofilm formed for *E. coli* HB101 in urine of volunteer 1 collected following consumption of CJC or placebo after incubation for (A) 0 hour; (B) 6 hours; (C) 24 hours; (D) 48 hours.

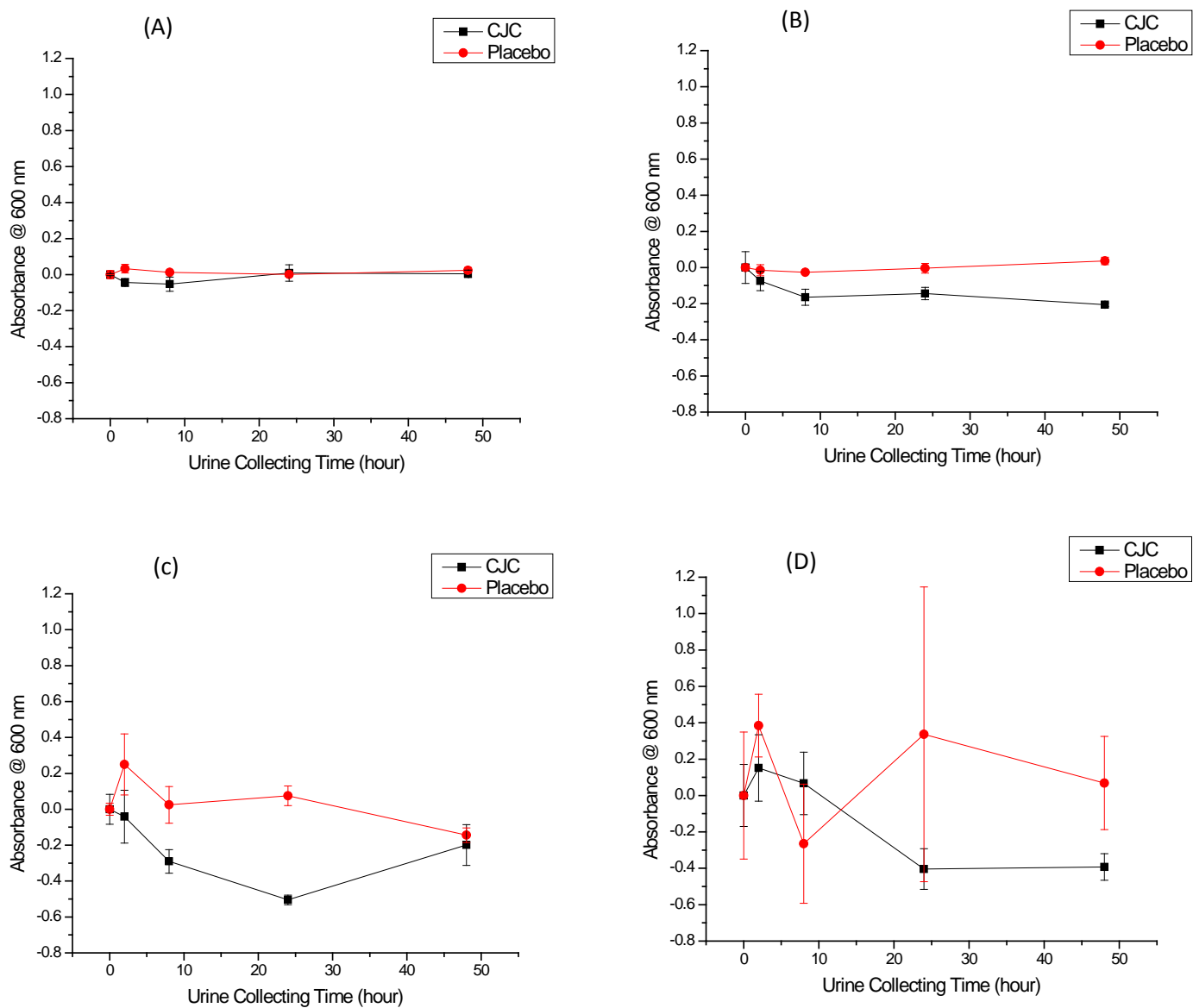


Figure S.5 Amount of biofilm formed for *E. coli* BF1023 in urine of volunteer 1 collected following consumption of CJC or placebo after incubation for (A) 0 hour; (B) 6 hours; (C) 24 hours; (D) 48 hours.

Part II: Individual responded to CJC in biofilm formation assay recorded in detail. 1 (in black) indicates biofilm formation in post-CJC urine was significantly lower than that in post-Placebo urine. 1 (in grey) indicates biofilm formation in post-CJC urine was significantly higher than that in post-Placebo urine. 0 indicates biofilm formation after culturing in urine collected following consumption of CJC or placebo are non-significant ($P < 0.5$).

Table S.1 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *S. aureus* incubated for 0 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	1	0	0	0	1	0	1	0	0
8	0	0	0	0	0	1	0	0	1	1	0	0
24	0	1	1	1	0	0	1	1	0	1	1	1
48	0	1	1	1	0	0	1	1	0	1	1	1

Table S.2 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *S. aureus* incubated for 6 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	0	0	0	1	1	1	0	0	1	1
8	1	1	1	0	0	1	1	1	1	0	1	1
24	1	1	0	0	0	1	1	1	1	0	1	1
48	1	1	1	1	0	1	1	1	1	1	1	1

Table S.3 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *S. aureus* incubated for 24 hours.

Urine collecting time (hours)	Volunteer ID										
	1	2	3	4	5	6	7	8	9	10	ALL
0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	0	0	0	1	0	1	0	0	1
8	1	0	0	1	0	1	1	1	0	0	1
24	1	0	1	0	0	1	0	1	0	1	1
48	1	1	0	0	0	1	1	1	0	1	1

Table S.4 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *S. aureus* incubated for 48 hours.

Urine collecting time (hours)	Volunteer ID										
	1	2	3	4	5	6	7	8	9	10	ALL
0	0	0	0	0	0	0	0	0	0	0	0
2	0	1	0	1	0	0	0	0	0	1	0
8	0	0	1	1	0	0	1	0	1	1	0
24	1	0	0	1	1	1	0	1	1	1	1
48	0	0	1	1	1	0	0	0	0	1	0

Table S.5 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* CFT073 incubated for 0 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	1	0	0	0	0	0	0	0	0
8	0	0	0	0	1	0	0	1	1	1	0	0
24	1	0	0	1	1	0	0	0	0	1	1	1
48	0	0	0	1	0	0	0	0	0	1	1	1

Table S.6 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* CFT073 incubated for 6 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	0	1	0	0	0	0	1	1	0	0
8	1	1	0	1	1	1	0	1	0	1	1	1
24	1	1	0	0	1	0	1	1	1	0	1	1
48	1	1	0	1	1	1	1	0	1	0	0	0

Table S.7 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* CFT073 incubated for 24 hours.

Urine collecting time (hours)	Volunteer ID										
	1	2	3	4	5	6	7	8	9	10	ALL
0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	0	1	0	0	0	1	1	1	0
8	1	1	0	1	1	0	0	1	1	1	0
24	1	1	0	1	1	0	0	1	1	0	0
48	0	1	0	1	1	0	0	1	1	0	0

Table S.8 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* CFT073 incubated for 48 hours.

Urine collecting time (hours)	Volunteer ID										
	1	2	3	4	5	6	7	8	9	10	ALL
0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	1	0	0	0	1	0	1	0	0
8	1	1	0	0	0	0	0	1	1	0	0
24	1	1	0	1	0	0	0	1	1	1	0
48	1	1	0	0	1	0	0	1	1	0	0

Table S.9 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* B37 incubated for 0 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	1	1	1	0	0
8	0	0	1	1	0	0	0	1	0	1	0	0
24	0	0	0	1	0	0	0	1	1	0	0	0
48	0	1	0	0	0	1	0	1	1	1	0	0

Table S.10 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* B37 incubated 6 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	1	1	1	0	1	0	0	0	0	0	0
8	1	0	1	1	1	0	0	0	0	0	0	0
24	1	1	1	1	1	1	0	0	1	1	1	1
48	1	1	1	1	0	1	0	0	0	1	1	1

Table S.11 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* B37 incubated for 24 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	0	0	0	0	0	0	0	0	1	0	0
8	1	0	0	0	0	1	0	1	0	1	1	1
24	1	0	0	0	0	1	0	1	0	1	1	1
48	0	1	1	1	0	0	0	0	0	1	1	1

Table S.12 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* B37 incubated for 48 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	1	1	0	0	0	0	0	0	1	0	0
8	1	1	1	0	0	1	0	0	0	1	1	1
24	1	1	1	0	0	0	0	0	0	1	1	1
48	0	1	1	0	1	0	0	0	1	1	0	0

Table S.13 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* HB101 incubated for 0 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	0	0	0	1	0	0	0	0	0	0
8	1	0	0	1	1	0	1	0	0	1	0	0
24	1	0	0	0	0	1	1	0	0	0	0	0
48	0	1	0	0	0	0	1	0	1	0	0	0

Table S.14 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* HB101 incubated for 6 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	1	1	1	0	0	0	0	1	0	0
8	1	0	1	1	1	0	0	1	0	0	0	0
24	1	0	1	1	0	0	0	1	1	0	0	0
48	1	0	0	1	0	0	0	0	1	0	0	0

Table S.15 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* HB101 incubated for 24 hours.

Urine collecting time (hours)	Volunteer ID										ALL	
	1	2	3	4	5	6	7	8	9	10		
0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	0	1	1	0	0	1	1	1	1	0	0
8	1	1	1	0	0	0	0	0	0	1	0	0
24	1	1	1	0	0	0	0	1	1	0	0	0
48	1	1	0	0	0	0	1	0	1	0	0	0

Table S.16 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* HB101 incubated for 48 hours.

Urine collecting time (hours)	Volunteer ID										ALL	
	1	2	3	4	5	6	7	8	9	10		
0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	1	1	0	0	0	0	0	0	1	0	0
8	0	0	1	0	0	0	0	1	0	0	0	0
24	1	0	1	0	0	0	1	1	0	0	0	0
48	0	1	0	0	0	0	1	0	0	0	0	0

Table S.17 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* BF1023 incubated for 0 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	0	0	1	1	1	0	1	0	0	0	0
8	1	1	0	1	1	0	0	1	0	1	0	0
24	0	1	0	0	1	0	1	1	1	0	0	0
48	0	1	0	0	1	0	1	1	0	0	0	0

Table S.18 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* BF1023 incubated for 6 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	1	0	0	0
8	1	0	0	0	0	0	0	0	1	0	0	0
24	1	0	0	0	0	0	0	1	1	0	1	1
48	1	0	1	0	0	0	0	1	1	0	1	1

Table S.19 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* BF1023 incubated for 24 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	1	1	0	0	0	0	0	0	0	0
8	1	1	0	1	0	0	1	0	1	0	0	0
24	1	1	1	1	1	0	0	0	1	0	1	1
48	0	1	1	1	0	1	0	0	1	0	1	1

Table S.20 Individual volunteer responded to CJC in biofilm formation assay recorded in detail for *E.coli* BF1023 incubated for 48 hours.

Urine collecting time (hours)	Volunteer ID											
	1	2	3	4	5	6	7	8	9	10	ALL	
0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	1	1	0	0	0	0	0	0	0	0
8	0	1	1	1	0	0	0	0	0	0	0	0
24	0	1	1	1	0	0	1	0	1	0	0	0
48	0	1	1	1	0	0	0	0	1	0	0	0

Part III: An example of measured absorbance at 600 nm took by a microtiter plate reader.

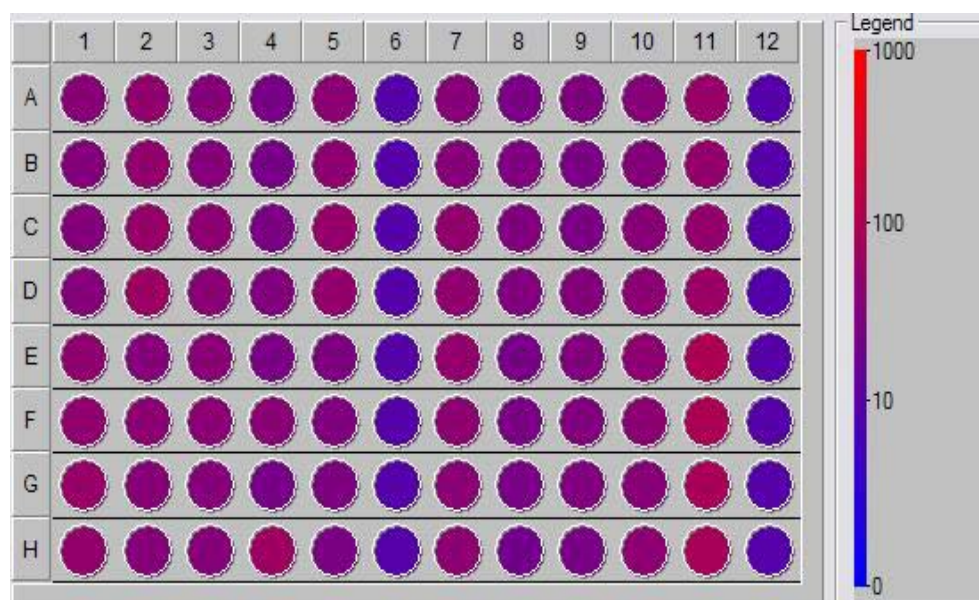


Figure S.6 An example of measured absorbance at 600 nm took by a microtiter plate reader. 1-5 indicate biofilm formation of *E. coli* CFT073 after culturing in urine collected following consumption of CJC for 0, 2, 8, 24 and 48 hours, respectively. 7-11 represent biofilm formation of *E. coli* CFT073 after incubating in culturing collected following ingestion of placebo for 0, 2, 8, 24 and 48 hours, respectively. 6 and 12 are blank.

Part IV: An example of colony count in bacterial cell viability assay.

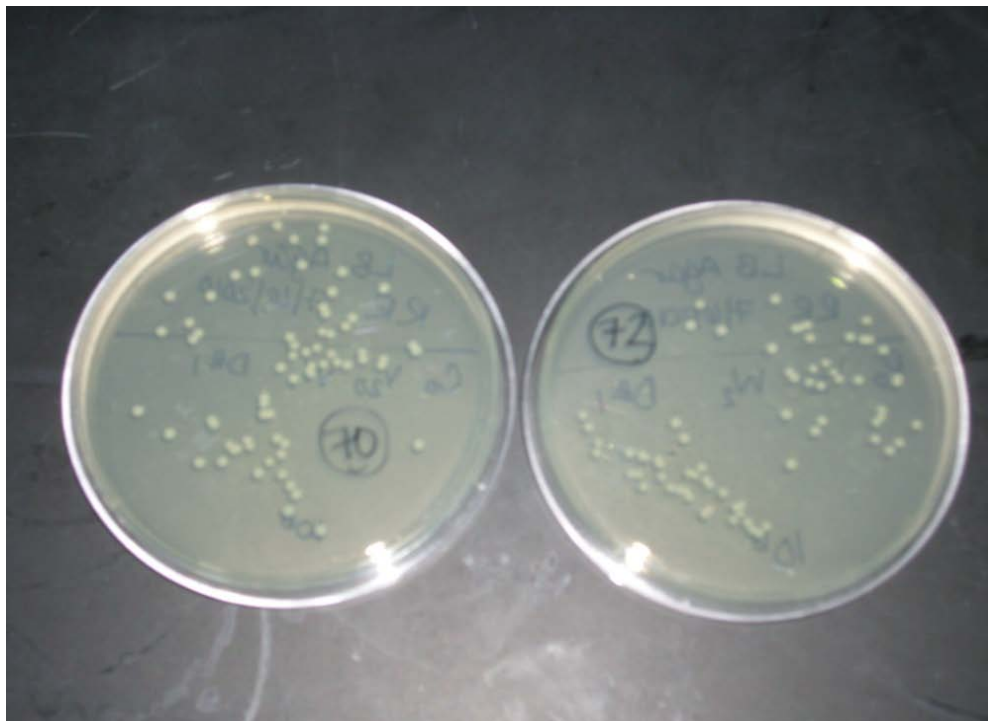


Figure S.7 A photograph of a representative example of colony count of *E. coli* CFT073 after incubated in urine collected following consumption of CJC (left) or placebo (right) for 24 hours.

Appendix B: Support File for Chapter IV

Part I: Average adhesion forces between pathogenic *E. coli* and uroepithelial cells detected by atomic force microscope for each volunteer.

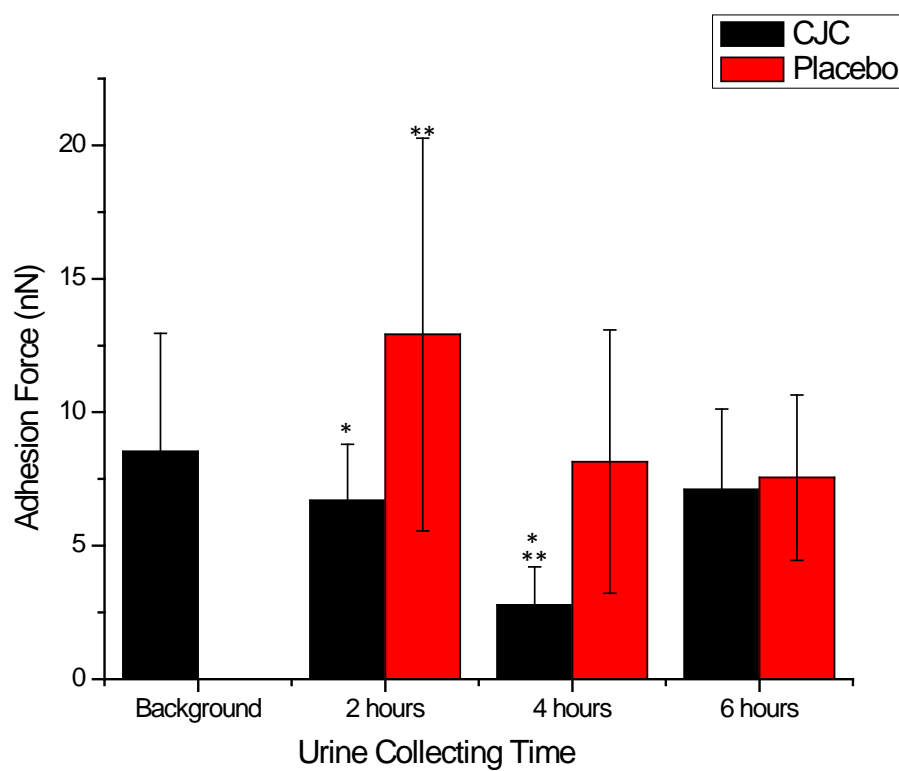


Figure S.8 Adhesion forces between *E. coli* B37 and uroepithelial cells as a function of urine collecting time (hour) after consumption of CJC or water for volunteer 1. *Adhesion forces detected in urine following ingestion of CJC or placebo were significantly different. **The difference compared to background (pre-consumption urine) was significant.

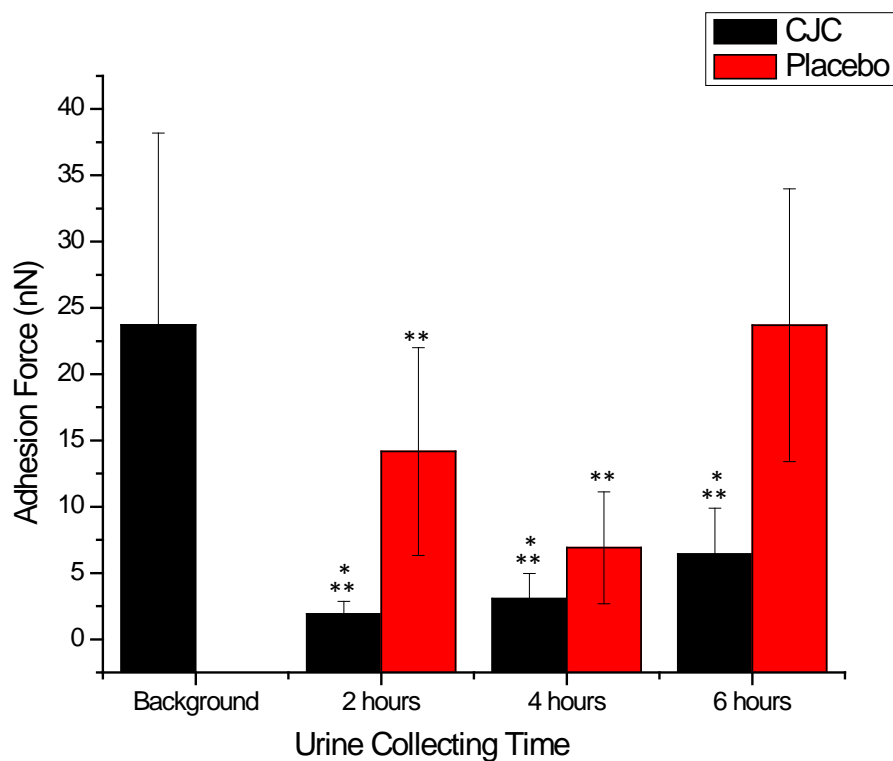


Figure S.9 Adhesion forces between *E. coli* CFT073 and uroepithelial cells as a function of urine collecting time (hour) after consumption of CJC or water for volunteer 1. *Adhesion forces detected in urine following ingestion of CJC or placebo were significantly different. **The difference compared to background (pre-consumption urine) was significant.

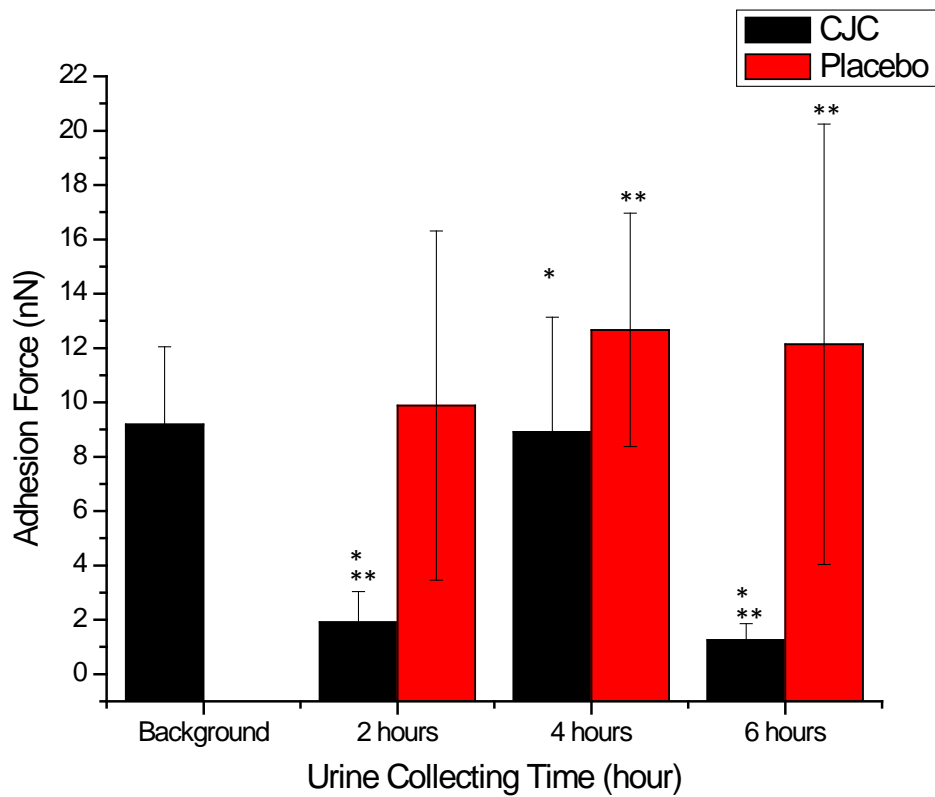


Figure S.10 Adhesion forces between *E. coli* B37 and uroepithelial cells as a function of urine collecting time (hour) after consumption of CJC or water for volunteer 2. *Adhesion forces detected in urine following ingestion of CJC or placebo were significantly different. **The difference compared to background (pre-consumption urine) was significant.

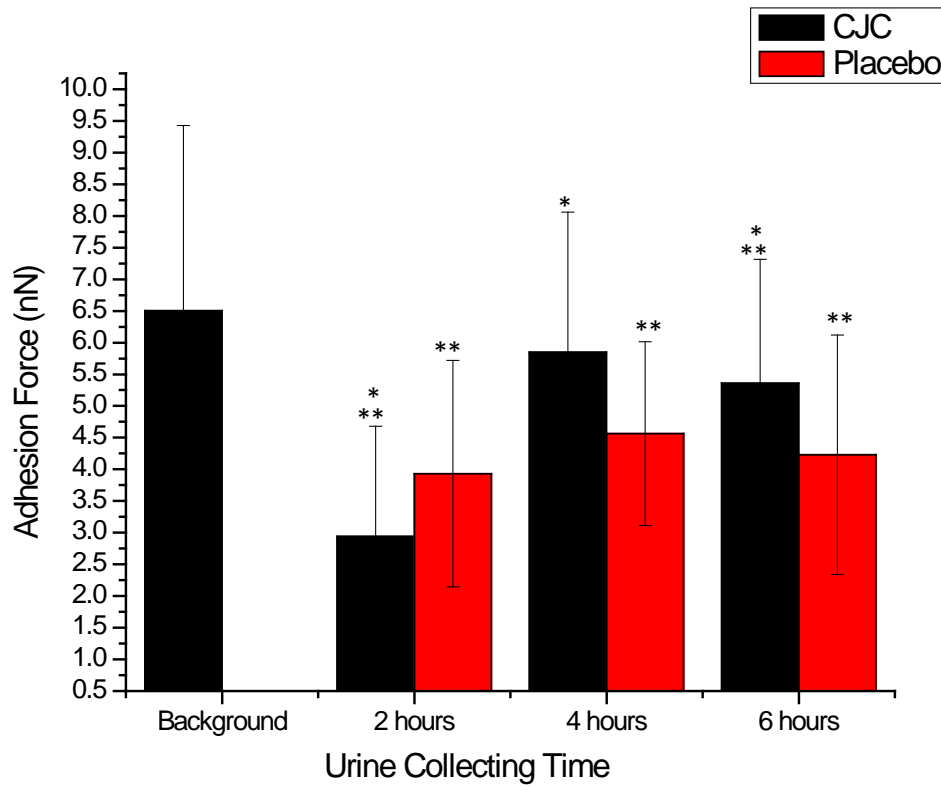


Figure S.11 Adhesion forces between *E. coli* CFT073 and uroepithelial cells as a function of urine collecting time (hour) after consumption of CJC or water for volunteer 2. *Adhesion forces detected in urine following ingestion of CJC or placebo were significantly different. **The difference compared to background (pre-consumption urine) was significant.

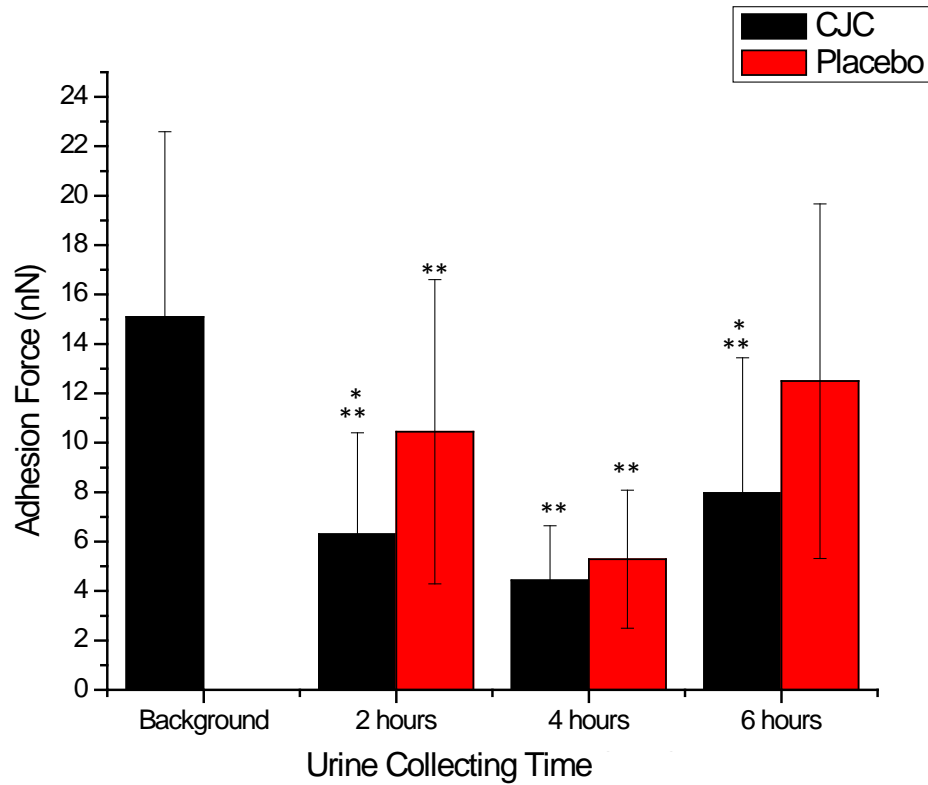


Figure S.12 Adhesion forces between *E. coli* B37 and uroepithelial cells as a function of urine collecting time (hour) after consumption of CJC or water for volunteer 3. *Adhesion forces detected in urine following ingestion of CJC or placebo were significantly different. **The difference compared to background (pre-consumption urine) was significant.

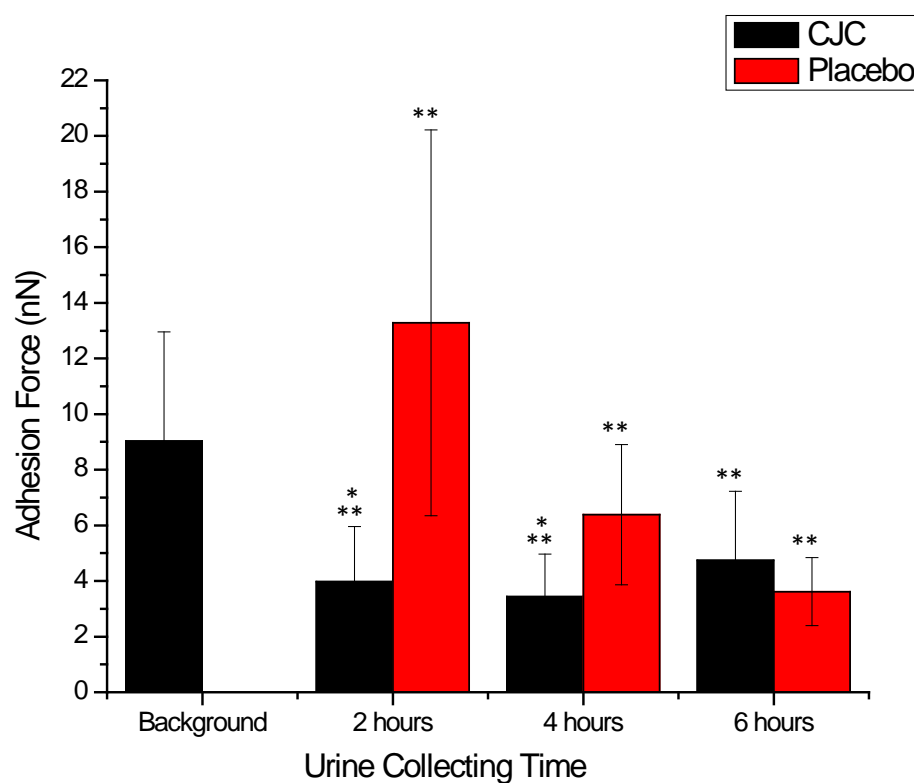


Figure S.13 Adhesion forces between *E. coli* CFT073 and uroepithelial cells as a function of urine collecting time (hour) after consumption of CJC or water for volunteer 3. *Adhesion forces detected in urine following ingestion of CJC or placebo were significantly different. **The difference compared to background (pre-consumption urine) was significant.