

# Using Atomic Force Microscopy to Measure Anti-Adhesion Effects on Uropathogenic Bacteria, Observed in Urine after Cranberry Juice Consumption

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## ABSTRACT

A volunteer was given cranberry juice cocktail (CJC) or water to drink, and urine was collected at 2 and 8 hours after consumption, in order to quantitatively determine whether adhesion forces were changed for the volunteer after CJC consumption. Atomic force microscopy (AFM) was used to measure adhesion forces between bacteria and a silicon nitride tip. Forces between *Escherichia coli* or *Staphylococcus aureus* and the AFM tip were lower in the urine after the volunteer consumed CJC, compared to drinking water. A steric model was applied to the AFM data, in order to quantify how the urine changed the properties of the bacterial surfaces. There was a small decrease in the equilibrium length of surface molecules on the bacteria when in the post-CJC urine, compared to the post-water urine. However, these changes were not statistically significant. We hypothesize that post-CJC urine imparts subtle changes on the molecules of the bacterial surfaces, and that these changes lead to the reduction in adhesion with the AFM probe.

**Keywords:** *Vaccinium macrocarpon*; Urinary Tract Infections; *E. coli*; *S. aureus*; AFM

## 1. Introduction

Considered to be one of the most common bacterial infections, urinary tract infections will affect almost half of all women in their lifetime, and nearly 1 in 3 women will have a UTI requiring antibiotics before age 24 [1]. Alternative therapies for UTIs have drawn increasing interest due to concern of antibiotic resistant bacteria. The American cranberry (*Vaccinium macrocarpon*) is a potential therapeutic for UTIs, and several clinical studies have shown that consumption of cranberry can help prevent UTIs or recurrences of UTIs among elderly people [2,3], children [4], and sexually active women with previous UTIs [5], although it has been difficult to demonstrate longer term benefits of cranberry as a means to prevent UTI recurrence over long times [6]. Cranberry products are consumed by many healthy women as a preventive measure, but further research is needed to elucidate how oral consumption of cranberry affects the activity of uropathogenic bacteria, and to better understand the type of cases where cranberry can be effective for UTI prevention. The timing and dose needed for this therapeutic effect are also being investigated [7].

*E. coli* spp. are by far the most predominant species that cause UTIs, accounting for 70% - 95% of all UTIs. In addition, P-fimbriated *E. coli* strains are the primary cause for 90% of acute pyelonephritis cases [8]. Remaining UTIs are caused by various organisms, including staphylococci, proteus organisms, *Klebsiella* spp., and *Enterococcus faecalis*. The incidence of UTIs related to *S. aureus* was found to be 3.5%, based on an analysis of 321 patients over a 3-year period [9]. Pathogenic bacteria have the ability to adhere to inert surfaces, such as a catheter, which leads to device-associated infections. Catheter-related UTIs tend to be caused by Gram-negative bacilli [10], including pseudomonads [11], fungi, and Gram-positive cocci, which includes staphylococci [10,12,13].

The first step in the initiation of a UTI, or any bacterial infection, is that bacteria must attach to a surface, such as a catheter placed inside the body or the epithelial cells that line the urinary tract. The attachment of bacteria to abiotic materials is a complicated process that is affected by various physicochemical properties of both bacterial cells and substratum surface. Understanding the role of cranberry juice components on interactions within this process is critical in developing novel strategies for the

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efficient control of bacterial attachment and biofilm formation.

Exposure to cranberry juice has been shown to decrease bacterial attachment, adhesion, and biofilm formation. For example, cranberry juice inhibited the attachment of *E. coli* to human uroepithelial cells, red blood cells, and resin beads coated with receptors that bind to P-fimbriae, the molecules on uropathogenic *E. coli* cell surfaces that facilitate adhesion to uroepithelial cells [14-16]. In an attempt to gain more quantitative information on how cranberry juice affects bacterial adhesion, adhesion force measurements have been made using atomic force microscopy (AFM). AFM experiments demonstrated that P-fimbriated bacteria alter the conformation of their fimbriae in the presence of cranberry juice cocktail (CJC) [17], and adhesion forces between *E. coli* and an AFM silicon nitride tip were reduced when bacteria were grown in the presence of light CJC or in proanthocyanidins extracted from the same juice [14]. In addition to studies with juice, the urine of volunteers who have consumed CJC has been investigated to determine if anti-adhesive or anti-biofilm compounds remain in urine. We previously reported that AFM adhesion force measurements between uropathogenic *E. coli* were decreased in the presence of urine from a volunteer who had consumed CJC [18]. In addition, DiMartino *et al.* showed that cranberry juice consumption decreased biofilm development of uropathogenic *E. coli* in the urine compared with placebo [19,20].

In recent years, AFM has emerged as a valuable and powerful tool for understanding the nanoscale properties of living cells. A major advantage of AFM is that it allows measurements of the forces associated with single biomolecules in aqueous media. This is ideal for biological applications, where AFM can be used to investigate the individual bacterial cells in buffer solution, without the need of fixing, staining, or labeling. AFM has been extremely useful for providing high-resolution topographic images of cell surfaces and biomolecules [21-23]. In addition, quantitative information on bacterial interaction forces and adhesion can be provided through analysis of AFM data [24]. Furthermore, in order to quantify AFM data and use it to interpret behavior of bacterial surfaces, a steric model can be applied to force vs. distance profiles [25,26]. This model allows us to characterize the physicochemical properties of polymers on a bacterial surface.

In this work, AFM was used to investigate the interactions between bacteria (*S. aureus* or *E. coli*) and a model surface (silicon nitride tip) in the presence of urine samples containing cranberry juice components. We focused on representative bacterial pathogens in this study, namely, two types of clinical *E. coli* that are associated

with UTIs (strains BF1023 and CFT073, both of which have P-fimbriae), and *S. aureus* ATCC43866. While *E. coli* is the most common uropathogen, *S. aureus* is a pathogen frequently involved in device-centered infections, has the ability to adhere to catheters and other medical devices and form biofilm [13], and is a growing concern due to multi-drug antibiotic resistance. The steric model was applied so that changes in the bacterial surfaces due to exposure to the urine of a volunteer who had consumed CJC could be quantified. To our knowledge this is the first study to characterize the effect of CJC byproducts in urine on the adhesion properties of *Staphylococcus aureus* (*S. aureus*) using AFM.

## 2. Materials and Methods

### 2.1. Urine Samples

De-identified urine samples were sent to Worcester Polytechnic Institute (WPI) from Rutgers University. An arbitrary, crossover design was applied, with a single volunteer. The volunteer was a healthy Caucasian male, aged 42. He drank 16 oz. (240 mL) of commercial cranberry juice cocktail (CJC) or water. After consuming the single dose, urine was collected at two time intervals (2 and 8 hours). Samples were immediately frozen and shipped to WPI, where they were stored at  $-20^{\circ}\text{C}$ . Urine samples were later thawed and filtered through  $0.8\ \mu\text{m}$  (Pall Corp., East Hills, NY) and  $0.2\ \mu\text{m}$  (VWR International<sup>TM</sup>, West Chester, PA) polyethersulfone syringe filters, sequentially.

### 2.2. Bacteria Cultures

Two *E. coli* clinical strains that cause acute pyelonephritis (AP) or cystitis, and one *S. aureus* strain (ATCC 43866) were selected as model bacteria. All bacteria were purchased from ATCC (Manassas, VA). The two *E. coli* strains were; *E. coli* CFT073 and *E. coli* BF1023, both are P-fimbriated strains from different classes. CFT073 expresses P-fimbriae from class II and was isolated from the blood and urine of a woman with AP, and BF1023 expresses P-fimbria from classes I and III and was isolated from a female patient with cystitis.

*E. coli* strains were cultured at  $37^{\circ}\text{C}$  in colonizing factor antigen (CFA) media. CFA media is composed of 1% (w/v) casamino acids (Bacto<sup>TM</sup>, Sparks, MD 21152), 0.078% (w/v) yeast extract (Bacto<sup>TM</sup>, Sparks, MD 21152), 0.4 mM  $\text{MgSO}_4$  (Sigma-Aldrich), 0.04 mM  $\text{MnCl}_2$  (Sigma-Aldrich) in ultrapure water, and the pH was adjusted to 7.4 using sodium hydroxide (Sigma-Aldrich). For culture plates, 2% agar (Bacto<sup>TM</sup>, Sparks, MD 21152) was added to the media. *S. aureus* cultures were grown overnight in Luria-Bertani (LB) medium at  $37^{\circ}\text{C}$ . Bacte-

ria were harvested at late exponential phase, when the absorbance of the culture was 0.9 - 1.1 at a wavelength of 600 nm, measured with a spectrophotometer (Thermo Spectronic, Rochester, NY).

### 2.3. Bacterial Cell Preparation for AFM

Bacteria were immobilized to acid-cleaned glass slides using a cross-linking reaction between amine and carboxyl groups [18]. The cleaned glass slides were immersed in 10% 3-aminopropyltrimethoxysilane in methanol for 20 min. Glass slides were rinsed with methanol and ultrapure water. Bacterial cultures were washed three times with ultrapure water by centrifuging at 7000 rpm for 10 min, removing the supernatant, and resuspending the bacterial pellets in ultrapure water. A mixture of 300  $\mu$ l of 100 mM EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and 10 ml of the bacterial solution was prepared and incubated for 10 minutes at 37°C with rotation at 18 rpm. After 10 min, a 600  $\mu$ l aliquot of 40 mM NHS (N-hydroxysulfosuccinimide) solution was added to the bacterial solution and incubated at 37°C for 10 min with rotation at 18 rpm. The final solution was then added to the aminosilane-treated glass slides and agitated at 70 rpm for 4 h to allow bacteria to bind to the glass slides. Previous work has shown that the viability and morphology of bacterial cells is not affected by the binding process [27].

### 2.4. AFM Force Measurements

AFM interaction forces were measured using an MFP-3D-BIO AFM (Asylum Research, Santa Barbara, CA). Silicon nitride cantilevers were used, with an average spring constant of  $0.12 \pm 0.02$  N/m (DNPS, Veeco Metrology) calibrated and estimated from the power spectral density of the thermal noise fluctuations in air. The normal photodiode sensitivity was determined in liquid by measuring the slope of the constant compliance region of the force curve on a hard surface. Before experiments, the cantilevers were exposed to ultraviolet light for 1 h to remove any potential organic contamination. All force measurements were performed with the bacterial-coated slides and cantilevers immersed in the urine sample. During this time, we found that the bacterial cells remain viable but are not actively growing. For each condition studied, at least five individual bacterial cells were probed, and ten force measurements were recorded in the center of each bacterium.

### 2.5. Steric Model

The repulsive interactions between the bacterium and AFM tip were fit using a model for steric interactions.

The steric model describes the forces between interacting surfaces that have thermally mobile surface groups [28]. A common type of thermally-diffuse surface is a surface covered with chain molecules that dangle out into the solution, such as polymers. When another surface is approaching these dangling chains in a well-defined volume, a repulsive force known as “steric” force arises. The steric model originally developed by Alexander [29] and de Gennes [28] (AdG) to describe the forces between two flat surfaces with high coverage of grafted or adsorbed polymers. In addition, the AdG model has been successfully applied to the characterization of bacterial polymers [24,26] and to quantify interactions between a polymer-covered surface, such as hyaluronic acid [30], with model surfaces. Adaption of the AdG model to account for the spherical AFM probe interacting with a flat surface was performed [25], where the steric model takes the following form

$$F(D) = \frac{8kTR\pi L_o}{35s^3} \left[ 7 \left( \frac{L_o}{d+\delta} \right)^{5/4} + 5 \left( \frac{d+\delta}{L_o} \right)^{7/4} - 12 \right],$$

where  $F$  is the steric repulsive force measured by AFM, as a function of the separation distance ( $D$ ),  $k$  is the Boltzmann constant,  $T$  is the absolute temperature,  $R$  is the tip radius,  $s$  is the average distance between grafting sites, and  $L_o$  is the equilibrium length of the polymer layer. The separation distance,  $D$ , was used as the sum of the measured distance ( $d$ ) and the offset distance ( $\delta$ ), according to the procedure of Chang *et al.* [30]. Although modeling has sometimes been done without this parameter, the inclusion of  $\delta$  offset enables improved fitting because it provides an estimate for the layer thickness at the maximal applied force. By fitting the steric model to the approach portions of the AFM force cycles, the equilibrium polymer length, spacing, and offset distance were calculated. For each bacterium and condition studied, average values of the equilibrium polymer length and spacing were reported in plots, but individual values (with no averaging) of these parameters were statistically compared. At each time point, the two way ANOVA test was used to compare the data sets from the two treatments (water or CJC).

### 2.6. Adhesion Force Analysis

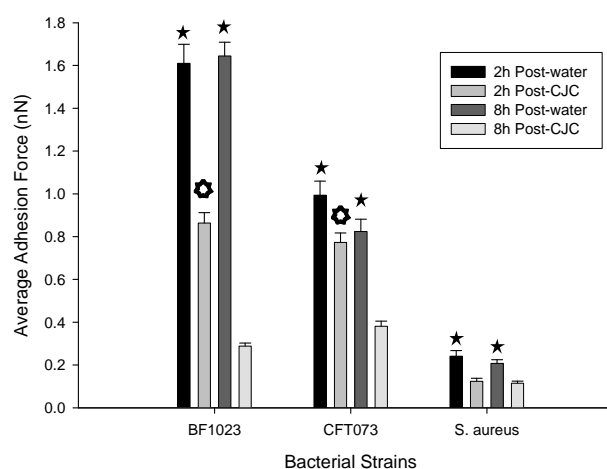
Each AFM force cycle has two parts, the approach of the AFM tip to the bacterium, and the retraction of the tip away from the bacterial sample. During the approach portion of the force cycle, repulsive steric forces are often observed, as described. However, the retraction portion of the cycle is useful to analyze because this is where adhesion forces between the AFM tip and molecules protruding from the bacterial surface can be ob-

served. For each experimental condition studied, we collected the data from all adhesion events and calculated an average adhesion force. Both individual (representative) retraction force profiles are presented, along with averaged adhesion forces for each experimental condition. Statistical analyses using SigmaStat<sup>®</sup> 2.03 (Systat Software, San Jose, CA) were also performed. The two way ANOVA test was used to compare the two groups that were treated with urine samples collected after (2 hours and 8 hours) of water or CJC consumption.

### 3. Results and Discussion

#### 3.1. Analysis of Adhesion Forces

The retraction portions of the AFM force cycles were examined to gain information regarding the adhesion forces between biomolecules on the bacterial surface and AFM tip. Individual adhesion forces from 50 retraction curves were combined and used to calculate an average adhesion force between the bacterial cell surface and AFM tip. **Figure 1** shows the average adhesion forces for each bacterial strain in the urine collected after CJC consumption (post-CJC urine) and after water consumption (post-water urine), at two time intervals (2 and 8 h) following beverage consumption. In addition, adhesion forces for different treatments and at different time points were compared statistically to obtain more reliable results (**Figure 1**). Tested data groups were considered significantly different if the resulting P-value was less than 0.05. For all bacterial strains, the adhesive forces between bacterial cells and AFM tip in the post-CJC urine were significantly less than the adhesive forces between bacterial cells and AFM in the post-water urine (**Figure 1**). *E. coli* strains demonstrated a significant decrease in the adhesive forces with time after CJC consumption and showed no significant changes in the adhesive forces with time after water consumption. Strain BF1023 showed a greater reduction in the adhesive forces with time after CJC consumption than CFT073. For BF1023, the average adhesive forces decreased from  $(0.86 \pm 0.05 \text{ nN})$  at 2 h to  $(0.29 \pm 0.01 \text{ nN})$  at 8 h following CJC consumption, while CFT073 adhesive forces decreased from  $(0.77 \pm 0.04 \text{ nN})$  at 2 h to  $(0.38 \pm 0.02 \text{ nN})$  at 8 h following CJC consumption. On the other hand, adhesion forces between *S. aureus* and the AFM tip at 2 h  $(0.124 \pm 0.01 \text{ nN})$  and 8 h  $(0.115 \pm 0.01 \text{ nN})$  after CJC consumption were not statistically different from one another. Similarly, the adhesive forces between *S. aureus* and the AFM tip at 2 h  $(0.24 \pm 0.03 \text{ nN})$  and 8 h  $(0.21 \pm 0.02 \text{ nN})$  after water consumption were not statistically different from one another. Comparing all strains under various conditions, BF1023 had the highest adhesion forces with the AFM tip and *S. aureus* had the

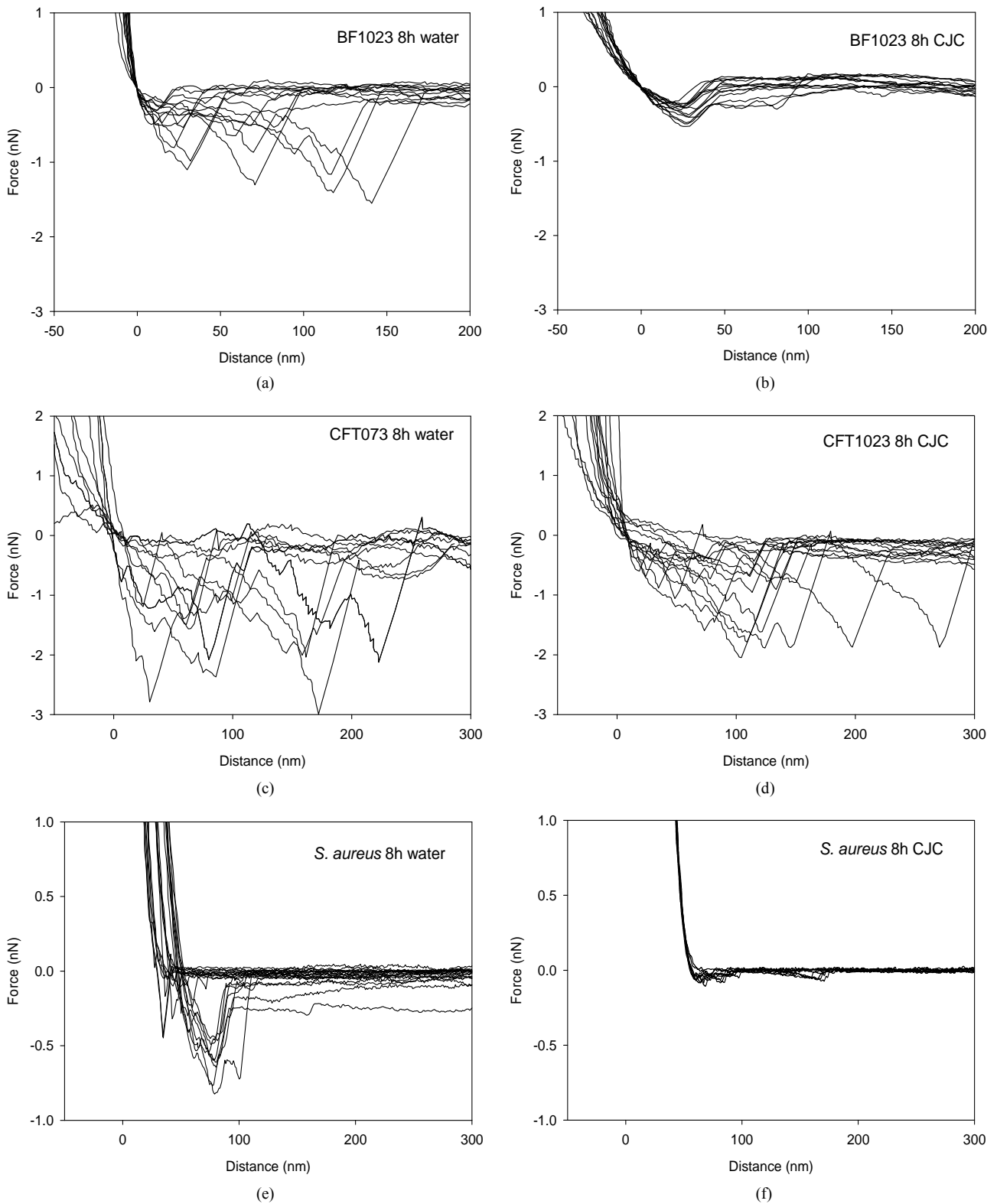


**Figure 1.** Average adhesion forces for *E. coli* BF1023, *E. coli* CFT073, and *S. aureus* after 2 h and 8 h of beverage consumption (water or CJC). Error bar indicates the standard error of the mean. 5-point black star indicates a statistically significant difference between two treatments at one time point. 7-point black star indicates a statistically significant difference between two times at one treatment condition.

weakest adhesion forces with the AFM tip. For example, at 2 h after water consumption, the adhesion forces between the AFM tip and BF1023, CFT073, and *S. aureus* were  $(1.61 \pm 0.09)$ ,  $(0.99 \pm 0.07)$ , and  $(0.24 \pm 0.03)$ , respectively. The silicon nitride AFM tip has a weakly negative electrostatic charge in solution. It is not meant to represent a catheter or an epithelial cell exactly, but can be used as a model probe to screen for non-specific physicochemical interactions between the bacterium and the probe material. It can also be used to help rank the strength of different samples or treatments.

In all the strains tested, there was a significant decrease in the adhesion forces between bacteria and AFM tip in the post-CJC urine as compared to the post-water urine both at 2 and 8 h after beverage consumption. These results confirm the existence of anti-adhesive molecules in the urine after oral consumption of CJC. This trend is also easily visualized by examining representative adhesion force profiles for the 8 h conditions (**Figure 2**). All 3 bacterial strains tested show an obvious decrease in the magnitude of the adhesion forces, as well as the distance at which those force extend, in the presence of post-CJC urine at 8 h, compared to post-water urine at 8 h. A similar trend in the data was observed for the 2 h post condition (data not shown), in which adhesion forces were lower and extended over shorter distances in post-CJC urine than in post-water urine.

However, the effect of time post-consumption on the adhesion forces differed among the strains. Within two hours of drinking CJC, *S. aureus* had the highest reduction in the adhesion forces between the post-CJC urine



**Figure 2. Representative adhesion force data corresponding to 8 hours after consumption of water or CJC for all three bacterial strains. In all cases, the right panels (b), (d), and (f) show much lower adhesion forces than the left panels (a), (c), and (e), which correspond to CJC and water, respectively. Similar types of data trends were observed 2 h post-beverage consumption (data not shown). For each condition, 50 force curves were collected. For clarity, only representative plots are displayed.**

and post-water urine, followed by *E. coli* BF1023 and finally by *E. coli* CFT 073 (**Figure 1**). However, after 8 hs of beverage consumption, the scenario was different, *E. coli* BF1023 had the highest reduction in the adhesion forces between the post-CJC urine and post-water urine followed by *E. coli* CFT073 and then by *S. aureus*. By considering changes in the post-CJC urine data as a function of time, our results demonstrated that the adhesion forces for the two *E. coli* strains after drinking CJC continued to decrease over the eight hour measurement period while the adhesion forces of *S. aureus* appeared to reach a plateau 2 hs post-CJC. Furthermore, the adhesive forces between AFM tip and *S. aureus* were lower than the adhesive forces between *E. coli* strains and the AFM tip.

The differences in how *E. coli* and *S. aureus*, and even among the two *E. coli* strains, reacted to post-CJC urine will also depend in part on the nature of the bacterial surfaces. *E. coli* CFT073 and BF1023 each exhibit mannose resistant hemagglutination. CFT073 expresses P-fimbriae, while the adhesins on BF1023 have not been characterized. *S. aureus* has surface protein adhesins that are smaller than *E. coli* fimbriae [31]. There are no published reports in which AFM has been used to characterize how *S. aureus* is affected by cranberry juice or its byproducts. However, a recent study found that cranberry extracts could inhibit growth and biofilm formation for *S. aureus* and other Staphylococcus spp., as well as inhibiting biofilm formation (but not growth), for *E. coli* [32]. The difference in activity of cranberry extracts against Gram-negative and Gram-positive bacteria was suggested by LaPlante *et al.* to be due to the lack of outer-membrane lipopolysaccharides for the Gram-positive bacteria, which could then make it easier for cranberry or other antimicrobial compounds to penetrate the cell membrane.

### 3.2. Bacteria Surface Characterization and Steric Model Application

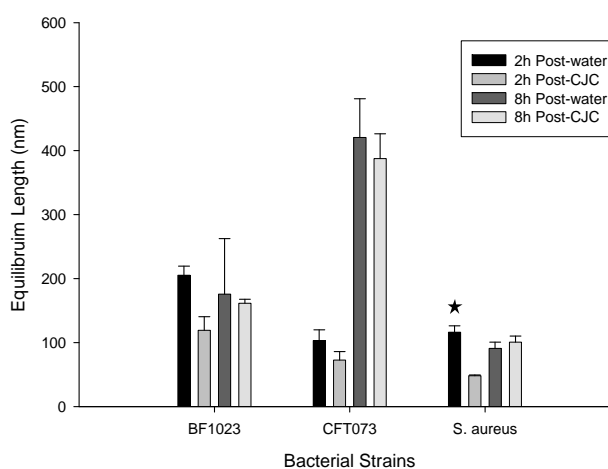
Data from all 50 force curves were used to determine the parameters of the steric model, the equilibrium length, the spacing between polymer units, and the offset. For strain *S. aureus*, the correlation coefficient values obtained by fitting the steric model to 50 approach curves were higher than 0.98 ( $R^2 > 0.98$ ), while for strains CFT073 and BF1023, the correlation coefficient values were ( $R^2 > 0.95$ ) and ( $R^2 > 0.90$ ), respectively.

For each bacterial strain, the two way ANOVA test was applied to the steric model parameters for two time intervals (2 and 8 h) and at two treatments (post-water and post-CJC). A given set of two tested groups was considered significantly different if the resulting P-value

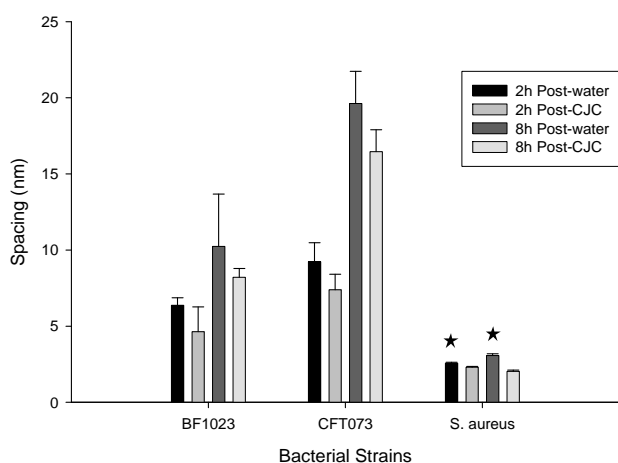
was less than 0.05. **Figure 3** shows the average equilibrium length for each given strain as a function of time and treatment after beverage consumption. For *S. aureus*, our statistical analysis demonstrated that there was a significant difference between the equilibrium lengths in the urine collected 2 h after water consumption and the urine collected 2 h after CJC consumption. The equilibrium polymer length in the urine 2 h post-CJC consumption was 60% less than its equivalent value in urine 2 h post-water consumption. However, the decrease in the equilibrium length did not continue with time, since the post-water urine and the post-CJC urine groups showed no significant difference in their average equilibrium lengths after 8 h of beverage consumption.

Neither *E. coli* strains, BF1023 nor CFT073, showed a significant change in the average equilibrium length between the post-water urine group and the post-CJC urine group during the 2 h or the 8 h following beverage consumption. In general, *S. aureus* average equilibrium polymer lengths were shorter than the average equilibrium polymer lengths of *E. coli* strains BF1023 and CFT073. The average equilibrium polymer lengths of strain *S. aureus* were in the range of 48 - 116 nm and the average equilibrium polymer lengths of strains BF1023 and CFT073 were in the range of 119 - 205 nm and 73 - 420 nm, respectively.

Another parameter obtained from fitting the steric model to the AFM approach curves is the spacing between polymers sites, which can represent how densely the bacterial surface is covered with interacting molecules (**Figure 4**). In all cases, *S. aureus* had smaller average spacing (2.0 - 3.1 nm) compared to the average spac-



**Figure 3.** Average equilibrium lengths of *E. coli* BF1023, *E. coli* CFT073, and *S. aureus* at two time intervals (2 and 8 h) after beverage consumption (water or CJC). Error bar indicates the standard error of the mean. 5-point black star indicates a statistically significant difference between two treatments at one time point.



**Figure 4.** Average spacing values of *E. coli* BF1023, *E. coli* CFT073, and *S. aureus* at two time intervals (2 and 8 h) after beverage consumption (water or CJC). Error bar indicate the standard error of the mean. 5-point black star indicates a statistically significant difference between two treatments at one time point.

ing for BF1023 (4.6 - 10.2 nm) and CFT073 (7.4 - 19.6 nm) (Figure 4). Furthermore, when tested at 2 and 8 h following beverage consumption, *S. aureus* showed a significant decrease in the spacing between grafting sites in the post-CJC urine group compared to its equivalent value in the post-water urine group (Figure 4). This is an indication that the decrease in the spacing between *S. aureus* polymer brushes as a result of drinking CJC lasted up to 8 hours. However, statistical analysis applied on the average spacing values for both *E. coli* strains revealed that there was no significant difference between the spacing in post-CJC urine and post-water urine for both strains after 2 and 8 h following beverage consumption.

#### 4. Conclusion

Exposure to the urine of a volunteer who consumed CJC imparted changes in bacterial adhesion and in the physicochemical properties of bacterial surface molecules, which were not observed in post-water urine. The changes were also dependent on properties of the bacteria. For two *E. coli* strains, adhesion forces were decreased in post-CJC urine, as quantified via AFM. However, we did observe statistically significant changes in the conformation of the polymers on the *E. coli* surface in post-CJC urine, compared to post-water urine. For *S. aureus*, we observed both a decrease in adhesion force in post-CJC urine, and statistically significant changes in the conformation of bacterial surface polymers in post-CJC urine. Since the nature of the surface molecules on *E. coli* and *S. aureus* are very different, these results suggest that there are multiple mechanisms by which post-CJC

compounds in urine can interact with Gram-negative and Gram-positive bacteria.

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