

**EFFECTS OF CRANBERRY JUICE COCTAIL ON SURFACE ADHESION AND
BIOFILM FORMATION OF UROPATHOGENIC BACTERIA**

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Abstract

American cranberry (*Vaccinium macrocarpon*) has been long known for its benefits in maintaining urinary tract health. Clinical trials have shown that drinking cranberry juice can prevent urinary tract infections (UTIs) in various subpopulations that are prone to UTIs, especially women, but the mechanisms by which cranberry acts against uropathogenic bacteria are still unclear. Studies showed that when exposed to cranberry juice or A-PACs, a group of tannins that are unique to cranberry, the adhesion activity and biofilm formation of uropathogenic bacteria were reduced. However, the metabolism of cranberry juice has not been elucidated, therefore further study is needed to find out whether the anti-bacterial components in cranberry could survive the digestive system and reach the urinary tract, and how the components or metabolites remaining in urine act against uropathogenic bacteria. We used atomic force microscopy (AFM) to study the surface adhesion force of uropathogenic *E. coli* incubated with urine samples that were collected from volunteers after drinking 16 oz. of cranberry juice cocktail (CJC) or water. The urine samples were collected at 0, 2, 4, 6, and 8 hours after CJC or water consumption. When incubated with post-water urine, the adhesion forces of pathogenic bacteria that have fimbriae (*E. coli* B37, B73, B78, BF1023, CFT 073, and J96) did not change; whereas the adhesion forces of these strains decreased over the 8 hour period after CJC consumption. The control strain that does not have fimbriae, *E. coli* HB101, showed low adhesion force when incubated with post-water and post-CJC urine. In a human red blood cell agglutination (HRBC) assay, the attachment of pathogenic *E. coli* to red blood cells was significantly lower after exposed to post-CJC urine, compared to those exposed to post-water urine. These results indicate the anti-bacteria components or metabolites of CJC stay active in urine, and these compounds prevent adhesion of *E. coli* by reducing fimbriae-mediated

adhesion. We also examined the effects of drinking CJC on biofilm formation of uropathogenic bacteria. Female volunteers were given 16 oz. of CJC or placebo, and their urine was collected at 0, 2, 8, 24, and 48 hours after consumption. Bacteria (*E. coli* B37, CFT073, BF1023, HB101, and *S. aureus* ATCC43866) were cultured in a mixture of urine and growth media in 96 well microtiter. The biofilm formed was quantified by staining the biofilm dissolved in a solvent with crystal violet and measuring the absorbance at 600 nm. The results showed that biofilm formation was reduced within 24 hours after CJC consumption, and it started to increase after 48 hours, possibly due to the washout of CJC in the system. These studies suggest that CJC can be an effective preventive measure for UTIs as it inhibits adhesion and biofilm formation of uropathogenic bacteria.

Authorship

The contents of this thesis are a representation of the work done by the main author. Contributions to this project were made by Paola A. Pinzón-Arango, a Ph.D. candidate in Biomolecular Engineering at Worcester Polytechnic Institute. She did part of the adhesion force measurements and some experiments within the biofilm formation assay. Yuxian Zhang, a Master's degree candidate in Chemical Engineering at Worcester Polytechnic Institute, contributed to the biofilm formation assay and data analysis for the experiments. Dr. Amy B. Howell from Rutgers University, NJ provided us urine samples for the AFM study. Regina Roberto from Health Services, Worcester Polytechnic Institute, helped us with recruiting volunteers and sample collection for the clinical trial.

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Chapter 1: Background

1.1 Urinary Tract Infections (UTIs)

1.1.1 Overview

Following respiratory tract infections, urinary tract infections (UTIs) are the second most frequent infections in human body [2]. Defined by a certain threshold number of bacteria in the urine (normally 10^4 to 10^5 CFU per ml [2]), UTIs consist of urethritis, cystitis, urethral syndrome, and pyelonephritis. Most UTIs develop in an ascending pattern (Figure 1.1), in which uropathogenic organisms first colonize in periurethral tissues and pass through the urethra, reach the bladder and in some cases continue progressing upward to infect the ureters and eventually the kidneys [3].

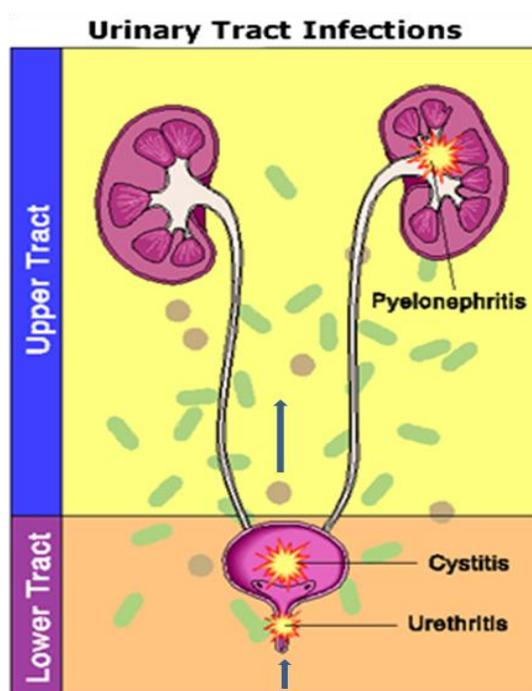


Figure 2.1 The ascending pattern of UTIs

UTIs occurring in an ascending pattern are mostly caused by Gram-negative bacteria such as *Escherichia coli* and *Staphylococcus saprophyticus*, whereas the much less frequent descending UTIs are mostly caused by Gram-positive bacteria such as group B *Streptococcus* [4]. Clinically, UTIs are commonly categorized as uncomplicated and complicated UTIs. Complicated UTIs refer to the infections associated with conditions that raise the risk of the treatment failure, such as anatomic or functional abnormalities of the urinary tract, the presence of foreign bodies (in many cases catheters), multi-antibiotic resistant pathogens, and immunosuppression. Most of the acute UTI cases in pre-menopausal women that do not have these conditions are uncomplicated [5]. Men are significantly less susceptible to UTIs compared to women due to the difference in the structure of male urinary tract, and complicated UTI cases are very rare in young men. Subpopulations with a higher risk to UTIs include infants [6], women [7], the elderly having medical conditions that induce complicated UTIs [8], patients with spinal cord injury and/or catheters [9], patients of acquired immunodeficiency disease syndrome [10], and patients of diabetes [11].

1.1.2 Statistics

As the most susceptible sub-population to uncomplicated UTIs, 1 in 3 women will have at least one UTI by the age of 24 which requires antibiotic treatment, and 40% to 50% of women will have at least one UTI in their lifetime [1]. The risk of UTIs in women increases with age (Figure 1.2), and women above 70 years old are most prone to UTIs [12]. An estimated 11.3 million women in the U.S. are diagnosed with UTIs and treated with antibiotics each year, and the yearly cost of UTIs is estimated to be \$1.6 billion [13]. As a result of medical condition, and instrumentation, most commonly catheterization, there is an increased risk of UTIs during

hospitalization. According to a report in 2007, UTIs account for 36% of nosocomial infections, being the most common nosocomial infections followed by surgical site infections [14].

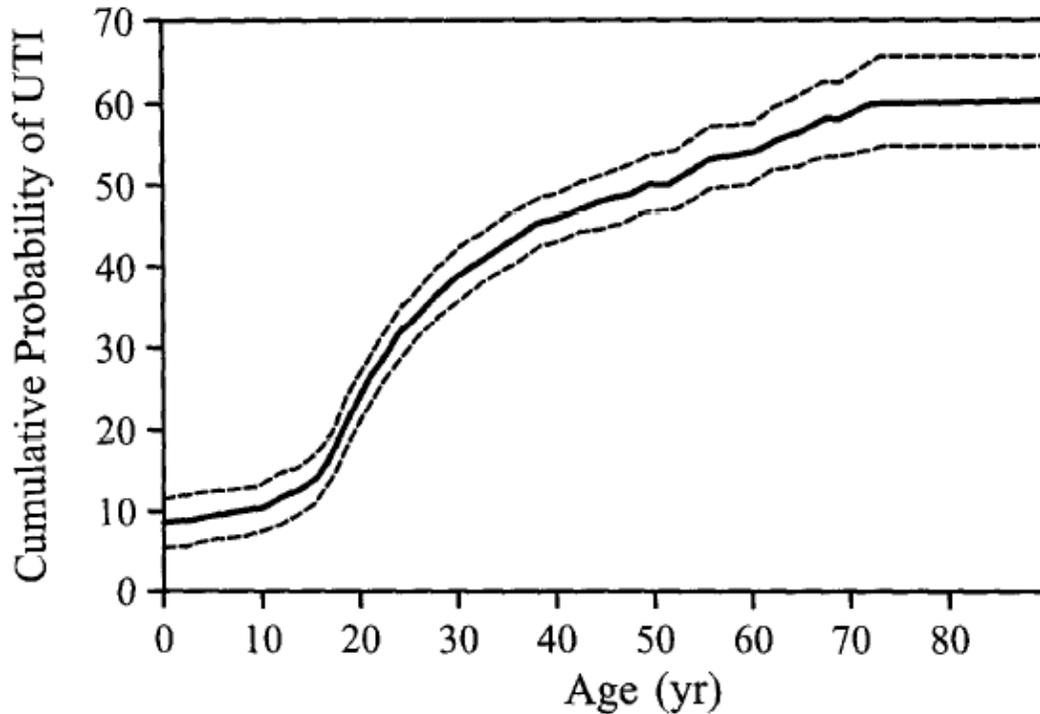


Figure 1.2 Cumulative probability of physician diagnosed UTIs by age among U.S. women. [1].

1.1.3 Treatments and problems

In hospital care, a multiple antibiotic treatment protocol is usually used to treat UTIs [15]. In the U.S., the Trimethoprim-Sulphamethoxazole (TMP-SMX) is considered the standard antibiotic treatment for UTIs; the treatment guidelines vary in countries, depending on many factors such as antibiotic prescription history, population susceptibility [15].

As a result of extensive antibiotic use to treat UTIs, urinary pathogens are inevitably becoming resistant to beta lactams and even TMP-SMX. Fluoroquinolones have been used as a replacement of TMP-SMX, but resistance to this group of antibiotics is increasing too. Some

physicians have returned to old antibiotics such as Nitrofurantoin and Fosfomycin because resistance to these drugs has stayed low, although these antibiotics either do not reach an ideal serum concentration within a short time or have a limited uropathogen spectrum [16]. Antibiotic resistance is an increasingly serious problem in treatment of UTIs particularly because women are prone to recurrent UTIs once they have their first UTI episode [13], and repeatedly treatment using antibiotics leads to development of resistance.

1.2 Uropathogenic Bacteria and Virulence Factors

An international survey on the prevalence and antimicrobial resistance of UTI pathogens revealed that over 80% of UTIs are caused by *Escherichia coli*, followed by the second common urinary pathogen *Staphylococcus saprophyticus*, accounting for 10-15% UTIs. Other pathogens include *Klebsiella*, *Enterobacter*, *Enterococci* and *Proteus* species [17]. Many uropathogenic *E. coli* are resistant to frequently prescribed antibiotics including ampicillin, Trimethoprim, Sulphamethoxazole, TMP-SMX, and nalidixic acid, whereas the antibiotic resistance is less severe in other uropathogens [18].

As the most prevalent uropathogen, *E. coli* has been intensively studied for its virulence factors. Similar to many pathogenic microorganisms, uropathogenic *E. coli* initiate infection by adhering to host tissues, in most cases the urinary tract lining [19]. By attaching to the epithelial cells lining the urinary tract, *E. coli* cells avoid being swept away by urine flow, therefore are able to colonize the urinary mucosa and cause infections. Although many pathogens are found to adhere to human erythrocytes, the adherence is usually inhibited in the presence of mannose, whereas the adherence of *E. coli* to uroepithelial cells is typically resistant to mannose, often referred to as mannose resistant hemagglutination (MRHA) [20]. The association of MRHA with *E. coli* adhesion to epithelial cells is a result of the involvement of fimbriae in both cases [21, 22].

E. coli fimbriae could be roughly categorized as type 1, type P, and X fimbriae. Type 1 fimbriae exist in many *E. coli* strains, and they demonstrate mannose-sensitive hemagglutination. Hemagglutination of type P fimbriae and a group of other less common fimbriae named X fimbriae is mannose resistant, and strains with these fimbriae are often associated with pyelonephritis [19]. P fimbriae are named after the P blood type antigens they bind to, which are a family of oligosaccharides containing a terminal or internal Gal α (1 \rightarrow 4)Gal β moiety. The Gal-Gal moiety is the receptor for P fimbriae. P-fimbriated *E. coli* are mostly associated with acute pyelonephritis, less with cystitis and rare with asymptomatic bacteriuria [23, 24]. The X fimbriae bind to urinary tract lining at various locations and cause infections in both upper and lower urinary tract. This family of fimbriae consists of several subgroups that have structures distinct from P fimbriae including some unidentified fimbriae, and the binding mechanisms of X fimbriae are not thoroughly understood.

1.3 Cranberry and UTIs

1.3.1 Overview

American cranberry (*Vaccinium macrocarpon*) is a member of the North American fruit family. The fruit of cranberry is widely consumed in a variety of food products such as juices, fruits, dried fruits, sauces and tablets. It has been long known that cranberry juice is beneficial in maintaining urinary tract health, reports that relate cranberry to urinary tract health trace back to the early 1900s [25]. Ever since 1966 when the first clinical trial was conducted to study cranberry's effects on UTIs [26], about a dozen of clinical studies have been performed to evaluate cranberry's effect on various subpopulations [27-36].

Reduction of bacteriuria and pyuria by cranberry juice in postmenopausal women was studied in a double blind, placebo controlled clinical trial in 1994 [27]. 153 subjects were given 300mL of either cranberry juice or placebo beverage daily for six months. The odds of bacteriuria and pyuria in cranberry group were 42% of that of the control group, demonstrating the efficacy of cranberry in reducing the two types of infections. In another randomized trial for 150 women with previous UTIs [30], cranberry-lingonberry juice and *Lactobacillus* (a probiotic) drink's effects in preventing UTIs were examined. During the first six months of the study, the cranberry-lingonberry group showed a much lower recurrence rate (16%) compared to the *Lactobacillus* group (39%) and the control group (36%) (Figure 1.3). The results indicated cranberry is effective in preventing recurrent UTIs. Other clinical trials also suggested American cranberry's inhibitive effects on UTIs among the elderly [32] and sexually active women [34].

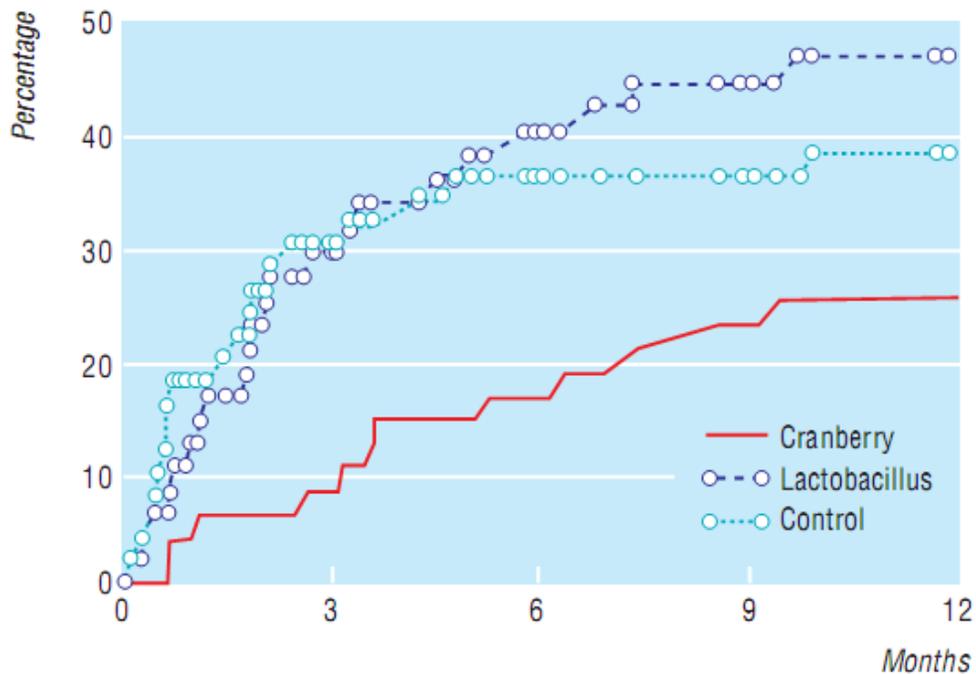


Figure 1.3 Cumulative rate of first recurrence of UTI during 12 month follow up in women receiving cranberry-lingonberry mixture for six months, Lactobacillus drink for 12 months, or no intervention [30]

1.3.2 Cranberry preventing UTIs: mechanisms

There have been several hypotheses of how cranberry interacts with uropathogens to reduce infections. It was suggested that the acidity of urine, due to hippuric and benzoic acid contained in cranberry, could inhibit bacterial growth and thus prevent infections [37]. However, clinical trials showed that drinking cranberry juice could not [27] or could only transiently acidify the urine [38]. It was also proposed that the increase of anti-inflammatory compound level (typically salicylic acid) associated with cranberry juice consumption was a possible mechanism of cranberry's anti-infection effects [39]. But this theory could not explain why consumption of many other natural products does not prevent UTIs, since salicylic acid is a common compound contained in many plants.

The current understanding on cranberry's beneficial effects in the urinary tract are focused on the fact that cranberry prevents bacterial adhesion [40-43]. In recent years, a group of tannins called proanthocyanidins (PACs) extracted from cranberry have drawn much attention among researchers. PACs were first identified in 1998 as the anti-adhesion components in cranberry [40]. The structure of these PACs were elucidated in 2000 [44], where it was found that cranberry PACs have the A-type linkage in the molecular structure, which is unique to cranberry and has not been found in procyanidins or PACs from other fruits. The A-type linkage refers to the double bonds between the epicatehin/catechin units in PACs molecules (Figure 1.4), as opposed to B-type linkages which are single bonds. There could be one or more A-type linkage in a PAC molecule. While B-PACs, which are contained in many natural products including green tea, grape, and chocolate, do not demonstrate anti-adhesion effects [45], A-PACs showed the ability to inhibit adhesion of uropathogenic bacteria *in vitro* [46].

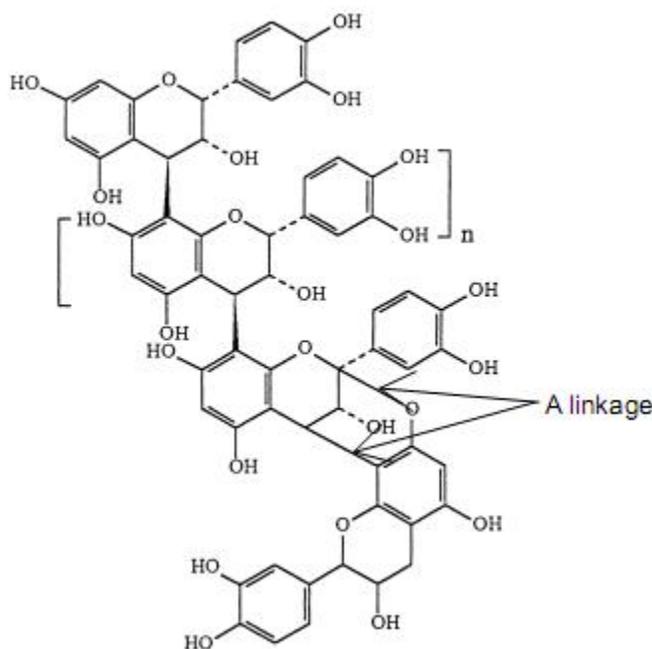


Figure 1.4 Molecular structure of A-PACs [44].

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 [47]. The mechanisms of how cranberry prevents uropathogenic bacteria from binding to host tissue are also still not clear. It has been shown that the molecular conformation [48] and agglutination ability [49] of *E. coli* P-fimbriae changed after incubation with cranberry juice; cranberry was also found to change the zeta potential of *E. coli* [50]. In addition, it has been shown that incubating with cranberry juice could significantly reduce the amount of biofilm formed by *E. coli* [47]. Although these studies demonstrated cranberry can change the surface properties and biofilm formation ability of *E. coli*, many of them were done with cranberry juice or A-PACs; the metabolism of cranberry

components is not thoroughly understood, and the anti-adhesion effects of cranberry metabolites remaining in urine need further investigation.

This work will address cranberry's effects on the surface properties and biofilm formation ability of uropathogenic bacteria after oral consumption.

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Chapter 2: Oral Consumption of Cranberry Juice Cocktail Inhibits Molecular-Scale Adhesion of Clinical Uropathogenic *Escherichia coli*

2.1 Abstract

Cranberry juice cocktail (CJC) has been shown to inhibit the formation of biofilm by uropathogenic *E. coli*. In order to investigate whether the anti-adhesive components could reach the urinary tract after oral consumption of CJC, a volunteer was given 16 oz. of either water or CJC. Urine samples were collected at 0, 2, 4, 6, and 8 hours after consumption of a single dose. The ability of compounds in the urine to influence bacterial adhesion was tested for six clinical uropathogenic *E. coli* strains, including four P-fimbriated strains (B37, CFT073, BF1023, J96) and two strains not expressing P-fimbriae but exhibiting mannose-resistant hemagglutination (MRHA) (B73 and B78). A non-fimbriated strain, HB101, was used as a control. Atomic force microscopy (AFM) was used to measure the adhesion force between a silicon nitride probe and bacteria cells treated with urine samples. Within two 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 eight hour measurement period. The adhesion forces of bacteria after exposure to urine collected following water consumption did not change. HB101 showed low adhesion forces following both water and CJC consumption and these did not change over time. The AFM adhesion force measurements were consistent with

the results of a hemagglutination assay, confirming that oral consumption of CJC could acts against adhesion of uropathogenic *E. coli* cells.

Key Words: Cranberry, anti-adhesion, urinary tract tinfections, *Escherichia. coli*

2.2 Introduction

Urinary tract infections (UTIs) are the most common community-required infections affecting the human body [1], with yearly costs estimated to be \$1.6 billion in the U.S [1, 2]. The main UTI pathogen is *E. coli*, which causes 90% of community-acquired UTIs and 30% of nosocomial UTIs [3, 4]. Infections are initiated when pathogenic bacteria attach to uroepithelial cells via the binding of bacterial adhesins to the receptors on the host cell membrane [5]. Typical adhesins of uropathogenic *E. coli* include type 1 fimbriae, P fimbriae, and X adhesins [5]. Type 1 fimbriae-mediated adherence can be blocked by D-mannose, α -methylmannoside, and many other mannose analogs, and therefore this mechanism is termed mannose-sensitive adhesion [6]. The binding of P fimbriae and X adhesins to uroepithelial cells cannot be blocked by mannose or its analogs, and thus this mechanism is termed mannose-resistant adhesion [7, 8].

UTIs are conventionally treated with antibiotics but there are concerns due to the development of antibiotic resistance and the problem of recurrent UTIs that affect some patients. Therefore, alternative therapies are drawing increasing interest among researchers. The American cranberry (*Vaccinium macrocarpon*) was shown to prevent UTIs in some studies [9-11], but the underlying mechanisms have not been fully explored. A family of phytochemicals in cranberry, A-type proanthocyanidins (A-PACs), were identified as the anti-adhesive components [12]. The non-dialyzable material (NDM) in cranberries containing mainly A-PACs and some

unidentified molecules, has been used in some studies and had anti-adhesive activity [13]. Incubation of P-fimbriated *E. coli* with 60 µg/mL A-PACs significantly decreased mannose resistant human red blood cell (HRBC) hemagglutination, and similar effects were observed when A-PAC solution was substituted with urine that was collected from volunteers who drank cranberry juice cocktail [14]. In our previous study using atomic force microscopy (AFM) to measure adhesion forces between P-fimbriated *E. coli* and a silicon nitride probe, the average adhesion forces decreased after 12 cultures in the presence of light CJC (from 1.60 ± 0.71 nN to 0.56 ± 0.3 nN) or PACs (from 1.60 ± 0.71 nN to 0.42 ± 0.2 nN), and the frequency distribution of adhesion forces shifted to lower values as the concentration of cranberry juice in the culture media increased [15, 16].

Previous studies have not addressed whether cranberry juice compounds have molecular-scale anti-adhesive activity after passing through the digestive system. Therefore, we investigated CJC's effects on adhesion forces of clinical *E. coli* strains, including antibiotic resistant strains that were isolated from patients with cystitis or acute pyelonephritis. Using direct adhesion force measurements with AFM, the effects of cranberry components or metabolites remaining in urine on the adhesion of *E. coli* were investigated. The adhesion forces measured with AFM were correlated to the results of a macroscopic assay, namely the agglutination of red blood cells exposed to specific *E. coli* agglutinins. To our knowledge, this is the first molecular-scale study to confirm the existence of anti-adhesive molecules in urine after oral consumption of CJC.

2.3 Materials and Methods

2.3.1 Urine samples

Urine samples had been collected for other IRB-approved research studies at Rutgers University. The samples sent to Worcester Polytechnic Institute (WPI) were de-identified, and no personal information was provided. A random, 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 intervals of 0, 2, 4, 6, and 8 hours. Samples were immediately frozen and shipped to WPI, where they were stored at -20 °C. Urine samples were later thawed and filtered through 0.8 µm (Pall Corp., East Hills, NY) and 0.2 µm (VWR InternationalTM, West Chester, PA) polyethersulfone syringe filters, sequentially.

2.3.2 Bacteria cultures

Six *E. coli* clinical strains that cause acute pyelonephritis or cystitis were selected, and a lab strain with no fimbriae was used as a control. The clinical strains possess a variety of surface properties, adhesin types, and antibiotic sensitivities (Table 2.1). To study different types of adhesins we chose two strains from each of the most common subgroups: i) strains that demonstrate mannose hemagglutination (MRHA) but no P-fimbriae (B73, B78); ii) strains that express P-fimbriae from class II (B37, CFT073); and iii) strains that express P-fimbriae from class III (BF1023, J96).

Table 2.1 Summary of properties and sources of 7 *E.coli* strains studied. All isolates also express type 1 fimbriae except HB101.

Strain	Fimbriae/Info	Source
B73	No P-fimbriae but exhibits MRHA (may be Dr adhesins); trimethoprin-sulfamethoxazole resistant	Dr. James Johnson, VA Medical Ctr, Minneapolis, MN; isolated from female with cystitis [39]
B78	No P-fimbriae but exhibits MRHA (may be Dr adhesins); ampicillin/sulfamethoxazole intermediate resistance	Dr. James Johnson, VA Medical Ctr, Minneapolis, MN; isolated from female with cystitis [39]
B37	P-fimbriae from class II, ampicillin/sulfamethoxazole intermediate resistance	Dr. James Johnson, VA Medical Ctr, Minneapolis, MN; isolated from female with cystitis [39]
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 [40]
J96	P-fimbriae, class I and class III	ATCC 700336; isolated from a patient with pyelonephritis; [41, 42]
HB101	Non-fimbriated, lab strain (non-pathogenic control)	ATCC 33694

Bacteria were cultured at 37 °C in colonizing factor antigen (CFA) media. CFA media is composed of 1% (w/v) casamino acids (Bacto™, Sparks, MD 21152), 0.078% (w/v) yeast extract (Bacto™, Sparks, MD 21152), 0.4mM MgSO₄ (Sigma-Aldrich), 0.04mM MnCl₂ (Sigma-Aldrich) in ultrapure water, and the pH was adjusted to 7.4 using sodium hydroxide (Sigma-Aldrich). For culture plates, 2% agar (Bacto™, Sparks, MD 21152) was added to the media. Bacteria were grown overnight and 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). Bacteria were washed three times with ultrapure water by centrifuging the solution at 2000 g and removing the supernatant.

2.3.3 AFM adhesion force measurements

Bacteria were attached to glass slides before AFM experiments [17, 18]. Glass slides were treated with 3:1 (vol/vol) HCl/HNO₃ solution (Fisher Chemical, Fair Lawn, NJ) for 45 minutes and rinsed with ultrapure water. Slides were soaked with 7:3 (vol/vol) H₂SO₄/H₂O₂ solution (Fisher Chemical) and rinsed with ultrapure water. The acid cleaned glass slides were stored at 4 °C in ultrapure water until use. Glass slides were functionalized with an amine group to facilitate bacterial attachment. Glass slides were treated with ethanol (Sigma-Aldrich), methanol (Sigma-Aldrich), and then immersed in solution of 35% 3-aminopropyltrimethoxysilane (Sigma-Aldrich) in methanol for 15 minutes followed by rinsing with ultrapure water. A 300 μL solution of 100 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Pierce, Rockford, IL) was added to the washed *E. coli* cells and incubated at 37 °C for 10 minutes with rotation at 18 rpm, followed by addition of a 600 μL solution of 40 mM N-hydroxysulfosuccinimide (NHS) (Pierce, Rockford, IL) solution and incubation at 37 °C for 10 minutes with rotation at 18 rpm. Bacteria solution incubated with EDC and NHS was added to the 3-aminopropyltrimethoxysilane -treated glass slides and agitated at 70 rpm for 4 hours to allow bacteria to bind onto the slides. Viability and morphology of bacteria cells were maintained during the binding process [19].

AFM adhesion force measurements were performed using a Dimension 3100 instrument with Nanoscope IIIa controller (Veeco Metrology, Santa Barbara, CA). Silicon nitride cantilevers with spring constants of 0.12 ± 0.02 N/m (DNPS, Veeco Metrology) were used to acquire images and force data. Before experiments, the cantilevers were cleaned by immersing in ethanol and exposing to ultraviolet light. All the measurements were done with the slides and cantilevers immersed in the same urine sample that the slides were treated with, in order to

mimic the physiological environment. From each slide, five bacteria were probed, and ten force measurements were made in the center of each bacterium. Force data were exported in ASCII format and analyzed as described previously [20] to acquire adhesion force-separation distance data. During the portion of the force profile where the AFM tip retracts from the bacterial surface, adhesion peaks are often observed. On each retraction curve the peaks represent the moment when the AFM probe pulls off from the cell surface, and the values of the forces at the peaks are defined as adhesion forces between the probe and the cell surface.

2.3.5 Human Red Blood Cell (HRBC) agglutination assay

Whole blood (type O positive) acquired from a volunteer was stored with EDTA at 4 °C. Before the agglutination experiment, the red blood cells were separated from whole blood by centrifugation at 200 g for 10 minutes, in order to remove the platelets and plasma. The resulting red blood cells were suspended in phosphate buffered saline (PBS) and washed three times. After each wash the supernatant was removed and the remaining cells were resuspended in PBS. The concentration of red blood cells in the suspension was measured using a hemacytometer (Hausser Scientific, Horsham, PA). The suspension was diluted with urine to a concentration of 10^7 red blood cells/ mL. The urine samples used in the HRBC agglutination assay were those collected at intervals of 0-2 hours or 6-8 hours after water or CJC consumption, as described above.

E. coli cells harvested at late exponential stage were diluted in urine to 10^9 cells/ mL. Red blood cells and bacteria were incubated separately in urine at 37 °C for three hours. Then the two were co-incubated for 90 minutes to allow agglutination. The number of bacteria that attached to

red blood cells was counted under a light microscope (Nikon Eclipse E400). For each urine sample, the number of attached *E. coli* bacteria was counted on 20 red blood cells.

2.4 Results

2.4.1 Adhesion force measurements and analysis

When the clinical *E. coli* strains were incubated with the background urine sample (urine collected just before water or CJC consumption), this resulted typically in adhesion forces above 1nN, which varied somewhat depending on the strain (Table 2.2). *E. coli* BF1023 had the highest background adhesion force of 1.68 ± 1.01 nN, while B73, B37, B78, CFT073 and J96 had background adhesion forces between 1.00 and 1.3 nN. HB101, the lab strain that has no fimbriae, showed the lowest background adhesion force of 0.40 ± 0.26 nN.

When cultured with urine samples collected at different time intervals following CJC consumption, all the clinical *E. coli* strains demonstrated decreasing adhesion forces with time after initial CJC consumption (Figure 2.1). The adhesion forces of clinical bacteria cultured with

Table 2.2 Mean adhesion force between a silicon nitride AFM probe and bacteria cells and standard deviation (SD) measured after treatment with urine samples collected before (background) and after consumption of 16 oz water or cranberry juice (CJC)

strain	Urine Sample Treatment	Mean Adhesion Force \pm SD (nN)				
		background	0-2hr	2-4hr	4-6hr	6-8hr
B73	Water		1.42 \pm 0.63	1.15 \pm 0.61	1.13 \pm 0.50	1.26 \pm 0.77
	CJC	1.30 \pm 0.77	0.52 \pm 0.31*'	0.42 \pm 0.25*'	0.30 \pm 0.17*'	0.29 \pm 0.25*'
B78	Water		1.37 \pm 0.72	1.33 \pm 0.88	1.44 \pm 0.91	1.33 \pm 0.82
	CJC	1.30 \pm 0.86	0.98 \pm 0.73*'	0.73 \pm 0.45*'	0.66 \pm 0.35*'	0.49 \pm 0.24*'
B37	Water		0.84 \pm 0.49*	0.75 \pm 0.40*	1.04 \pm 0.62	0.96 \pm 0.42
	CJC	1.07 \pm 0.41	0.69 \pm 0.30*	0.61 \pm 0.32*	0.47 \pm 0.21*'	0.47 \pm 0.22*'
CFT073	Water		0.99 \pm 0.70	1.02 \pm 0.60	0.89 \pm 0.50	0.82 \pm 0.47
	CJC	1.12 \pm 0.75	0.77 \pm 0.50*'	0.47 \pm 0.36*'	0.44 \pm 0.27*'	0.38 \pm 0.18*'
BF1023	Water		1.61 \pm 1.1	1.82 \pm 1.20	1.75 \pm 1.21	1.64 \pm 0.94
	CJC	1.68 \pm 1.01	0.86 \pm 0.44*'	0.72 \pm 0.34*'	0.45 \pm 0.25*'	0.29 \pm 0.12*'
J96	Water		1.29 \pm 0.59	1.29 \pm 0.62	1.35 \pm 0.75	1.30 \pm 0.42
	CJC	1.25 \pm 0.78	0.88 \pm 0.52*'	0.48 \pm 0.26*'	0.49 \pm 0.19*'	0.38 \pm 0.33*'
HB101	Water		0.42 \pm 0.31	0.45 \pm 0.32	0.43 \pm 0.36	0.46 \pm 0.37
	CJC	0.40 \pm 0.26	0.41 \pm 0.22	0.35 \pm 0.27	0.43 \pm 0.46	0.41 \pm 0.22

*Statistically significant difference compared to background ($p < 0.05$).

' Statistically significant difference compared to treatment with urine sample collected at the same time point after water consumption ($p > 0.05$).

urine collected 0-2 hours after drinking CJC decreased to below 1 nN for all the clinical strains. Adhesion forces continued to decrease for 8 hours, when the adhesion forces became as low as 0.2 - 0.5 nN. The control strain, HB101, did not show significant changes in the adhesion forces during the 8 hours following CJC consumption.

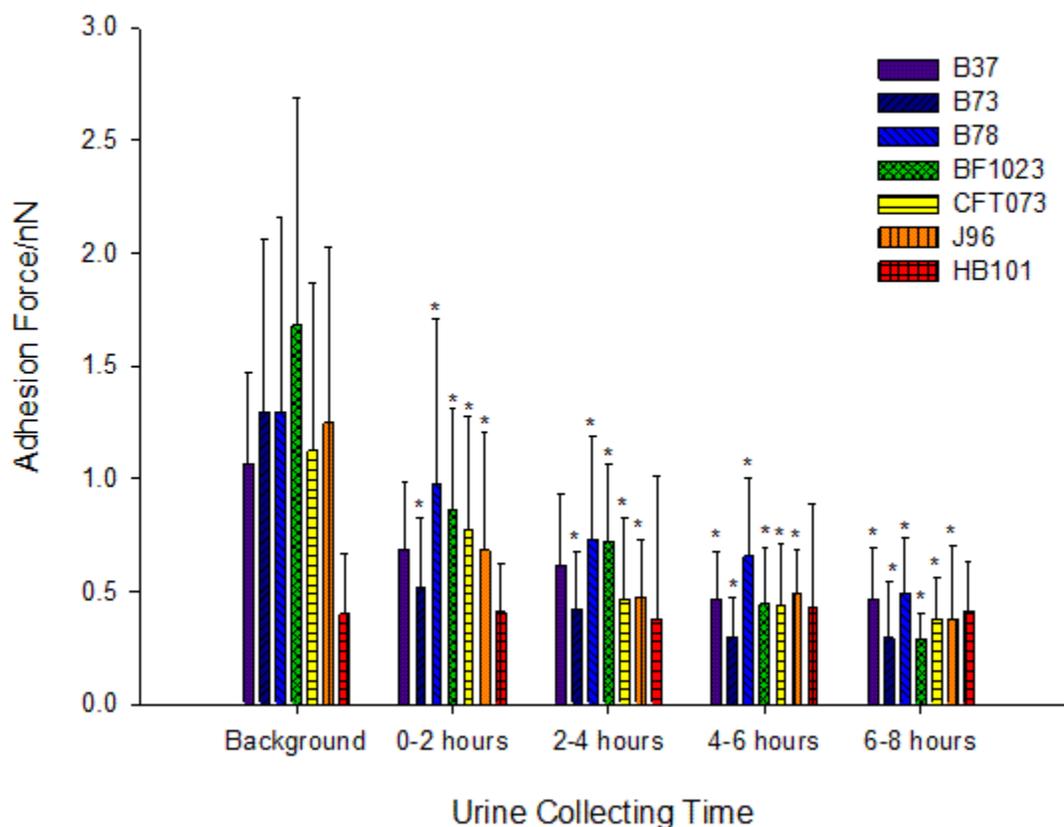


Figure 2.1 Adhesion forces of E.coli cells measured by AFM after incubating with urine.

Water consumption did not affect the adhesion forces of the clinical and lab strains, with the exception of B37 (Table 2.2). Following water consumption, B37 showed an adhesion force of 0.84 ± 0.49 nN at 0 – 2 hours and 0.75 ± 0.40 nN at 2 – 4 hours, which were significantly

different from the background; but those adhesion forces were still higher than adhesion forces measured after CJC consumption at the same time intervals. For B73, B78, CFT073, BF1023 and J96, the adhesion forces stayed above 1nN or slightly below 1nN with no significant differences from the background. The adhesion forces of HB101 remained between 0.3 - 0.5 nN with no significant differences from the background.

The ANOVA tests showed that for all the clinical strains, adhesion forces after CJC consumption were significantly lower than the background adhesion force levels (Table 2.2). The adhesion forces measured after CJC consumption also were lower compared to those measured at the same time intervals after water consumption. B37 showed higher adhesion forces with treatment of urine collected after water consumption, but within 4 hours after consumption, the adhesion forces after water consumption were only slightly higher than those after CJC consumption, and the difference between the two was not statistically different. After 4 hours the adhesion forces after water consumption increased and became significantly higher than the CJC group. For the majority of clinical strains, namely B73, B78, CFT073, BF1023 and J96, the difference of adhesion forces between CJC and water was statistically significant at each time interval, with adhesion forces after water consumption higher than CJC consumption.

2.4.2 Human Red blood cell (HRBC) agglutination assay

Co-culturing clinical strains with urine samples collected 6-8 hours after CJC consumption (CJC (6-8)) resulted in high bacteria cell retention on red blood cells (RBC) (Figure 2.2). The mean number of attached *E. coli* cells per RBC was above six with two strains having especially high retention (11.35 bacteria/ RBC for CFT073 and 11.85 bacteria/ RBC for BF1023).

The retention after CJC (6-8) culturing was significantly lower, being typically below two bacteria/ RBC.

The control strain HB101 demonstrated low retention to RBCs after culturing in either water (6-8) (0.15 bacteria/ RBC) or CJC (6-8) (0.35 bacteria/ RBC), and there was no statistically significant difference between water and CJC.

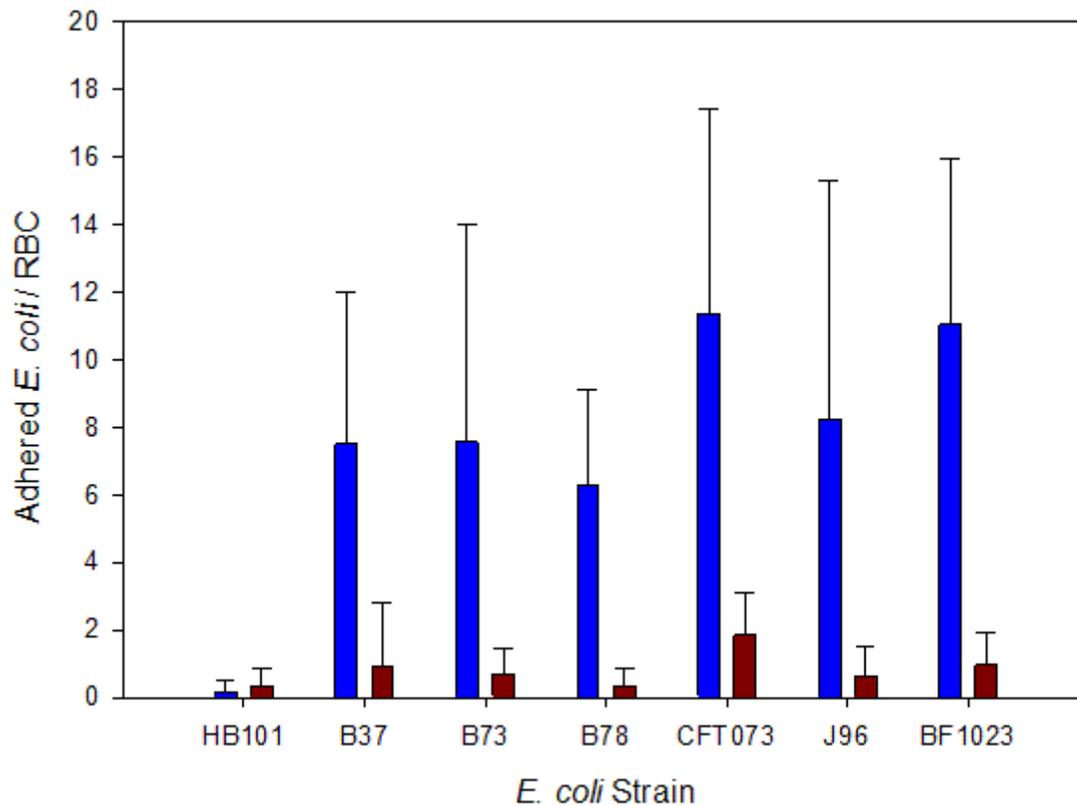


Figure 2.2 Attachment of *E. coli* cells to red blood cells (RBC).

2.5 Discussion

2.5.1 Adhesion forces of *E. coli*

Antibiotic resistance has been a major problem in the treatment of bacterial infections. CJC is a promising preventive therapy for UTIs since it works as an anti-adhesive instead of a

bactericide, and thus does not induce the development of antibiotic resistance. In this study, three antibiotic resistant strains isolated from female patients with cystitis were selected to investigate CJC's effects on these bacteria (Table 1). All of the antibiotic resistant strains showed little change in adhesion forces after water consumption compared to a significant decrease after CJC consumption, indicating that cranberry juice is effective in preventing non-specific adhesion of these antibiotic resistant *E. coli* strains.

For many years, it has been recognized that uropathogenic *E. coli* strains typically demonstrate mannose-resistant adhesion in mannose-resistant hemagglutination (MRHA) experiments [21-23]. While many phytochemicals in other fruits can inhibit mannose-sensitive adhesion, cranberry is one of the only fruits found to inhibit mannose-resistant adhesion [24, 25]. Therefore, it is important to investigate the adhesive properties of uropathogenic *E. coli* strains that demonstrate MRHA, especially how oral consumption of cranberry juice affects the adhesion of these strains in a simulated physiological environment (urine collected from a volunteer after CJC consumption). Therefore, strains used in this study are from two MRHA positive subcategories. B37, CFT073, BF1023, and J96 have P-fimbriae. Strains B73 and B78 do not have P-fimbriae, but exhibit MRHA.

P-fimbriae are considered the dominant virulence factors in upper UTIs [26]. They were named after the P blood group antigens (a family of oligosaccharides with the Gal α (1 \rightarrow 4)Gal β moiety) that they bind to. Although Gal α (1 \rightarrow 4)Gal β containing receptors are not abundant in the membrane of shed human epithelial cells [27], they are dominant in human renal cell membranes [28]. P-fimbriae adhere to epithelial cells in multiple tissues in upper urinary tract [29], explaining the high probability of P-fimbriae-mediated upper UTIs, such as acute pyelonephritis.

P-fimbriae also adhere to the epithelial and muscular layers of the bladder [30], indicating their involvement in lower UTIs. In addition, human polymorphonuclear leukocytes (hPMNLs) in blood only have trace amounts of glycolipids containing Gal α (1 \rightarrow 4)Gal β [31, 32], rendering a poor binding of hPMNLs with pathogenic P-fimbriated bacteria and thus the killing of these bacteria. We showed a significant decrease in adhesion of P-fimbriated strains (B37, CFT073, BF1023, and J96) after incubation with urine samples that were collected after CJC consumption, compared to the non-fimbriated strain HB101 which demonstrated low adhesion forces throughout the 8 hours after CJC consumption. These results indicated that the anti-adhesive components or metabolites of CJC remaining in urine have an inhibitive effect on uropathogenic *E. coli* adhesion.

The adhesins of the other two MRHA positive strains, B73 and B78, have not been clearly identified yet, they may be Dr adhesins which belong to the X adhesin family. The X adhesin family consists of Dr adhesins, S fimbriae, F1C fimbriae, M adhesins, G fimbriae, and other unidentified X adhesins [5]. The Dr adhesins differ from fimbriae because they are not distinct filaments. They can exist as a fine mesh, a coil-like structure, or a filamentous capsular coating [33]. The receptors for Dr adhesins are the Dr blood group antigens located on the decay-accelerating factor, a group of cell membrane proteins regulating the complement cascade [34]. The binding site of Dr adhesins in the urinary tract include the renal interstitium, Bowmans capsule, tubular basement membrane, ureteral transitional epithelial cells, and exfoliated cells in urine [34].

B73 and B78 were isolated from cystitis patients, and the Dr adhesin family constitutes 78% of X adhesin strains among cystitis patients isolates [33]. Therefore, it is likely that B73 and

B78 have Dr adhesins. Our results showed that similar to P-fimbriated strains, B73 and B78 showed decreased adhesion forces after being treated with urine from the volunteer who consumed CJC. Although the molecular binding mechanisms of Dr adhesin and P-fimbriae are different in the urinary tract, CJC can inhibit the adhesion of both, which could be a result of decreased non-specific adhesion induced by CJC metabolites remaining in urine. Using contact angle measurements, a previous study [35] showed that cranberry juice could induce a decrease in non-specific adhesion between P-fimbriated *E. coli* and uroepithelial cells. Since non-specific adhesive interactions, including van der Waals forces, electrostatic forces, and hydrophobic interactions, do not involve the specific adhesin-receptor binding, it is likely that CJC metabolites influence the strains without P-fimbriae in the similar way they affect P-fimbriated strains by diminishing non-specific interactions [36, 37]. It is also possible that CJC metabolites can change the amount and conformation of *E. coli* surface macromolecules and thus the adhesion activity [15, 38]. We showed previously that cranberry juice reduced the equilibrium length of *E. coli* P-fimbriae from ~148 nm to ~48 nm [15], suggesting the compressing of surface macromolecules on *E. coli* could be a mechanism of decreased adhesion, which could explain the non-specific adhesion decrease after CJC consumption in both P-fimbriated and non P-fimbriated *E. coli* strains.

2.5.2 RBC agglutination assay

While the adhesion forces measured with a silicon nitride AFM probe represent the non-specific adhesion of *E. coli* cell surfaces including van der Waals forces, electrostatic and hydrophobic interactions, biological interactions also include specific types of adhesion, such as receptor-ligand bonds. AFM adhesion force measurements represent non-specific adhesion

between the silicon nitride AFM probe and the bacterial surfaces, whereas the RBC agglutination assay represents overall interaction between bacteria cells and human cells, which contains both non-specific and specific adhesion.

Compared to the control strain HB101, all the clinical strains demonstrated high retention to RBCs after co-culturing with the urine sample collected 6-8 hours after water consumption (water (6-8)) (Figure 2), which can be explained by the fact that HB101 does not have any adhesins. When co-cultured with the urine sample collected 6-8 hours after CJC consumption (CJC (6-8)), the retention of HB101 did not change compared to water (6-8). These results suggest that CJC metabolites remaining in urine can inhibit overall adhesion of uropathogenic *E. coli*.

In conclusion, by incubating uropathogenic *E. coli* with urine collected after water or CJC consumption, and measuring the resulting change of adhesion force between *E. coli* cell surface and an AFM probe, we were able to demonstrate that the anti-adhesive components in CJC could reach the urinary tract, and these components were active in preventing non-specific adhesion. We also confirmed that CJC components remaining in urine played a role in inhibiting specific adhesion of *E. coli* by means of HRBC agglutination assay.

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Chapter 3: A Pilot Clinical Study of Cranberry Juice Cocktail for Preventing Biofilm Formation of Uropathogenic Bacteria

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 11 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. 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 indicate 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.

3.2 Introduction

Considered to be one of the most common bacterial infections, urinary tract infections (UTIs) account for nearly 7 million physician visits per year with estimated costs of \$1.6 billion in the U.S [1]. Women are much more prone to UTIs compared to men, and by the age of 24, a third of women are estimated to have had at least one physician-diagnosed UTI [2].

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 among elderly people [3], elderly women [4], and sexually active women with previous UTIs [5]. Cranberry products are consumed by many healthy women as a preventive measure, research is needed to elucidate how oral consumption of cranberry affects the activity of uropathogenic bacteria.

Cranberry juice has been shown to reduce the surface adhesion ability of *E. coli* measured by atomic force microscopy [6], it was also shown to inhibit the attachment of *E. coli* to human uroepithelial cells, red blood cells, and resin beads coated with receptors that bind to P-fimbriae, the filaments on *E. coli* cell surface that facilitate adhesion. [6-8]. But whether drinking cranberry juice helps prevent UTIs by inhibiting biofilm formation in the urinary tract is still unclear. As the first step of bacterial infection, biofilm formation is of significant interest to researchers [9]. 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 healthy women.

3.3 Participants and Methods

3.3.1 Study subjects

After advertising through email and fliers, female students from Worcester Polytechnic Institute (MA) between the ages of 18 and 27 were screened for enrollment in this trial. Volunteers were tested for UTIs using a dipstick test, and those with UTIs were excluded from the study and were referred for treatment at the University Health Services. Twenty volunteers qualified, and 16 oz of CJC and 16 oz of placebo (Ocean Spray) were provided to each them. Each volunteer drank CJC, followed by placebo at least one week after CJC consumption to avoid interference between the two treatments. Volunteers were asked not to consume any other berries or juices during this time, in order to exclude the intervention of metabolites possibly similar to cranberry metabolites. The study was double blind (volunteers and researchers). Urine samples were collected at 0, 2, 4, and 8 hours after CJC or placebo consumption, with the 0 hour sample serving as a baseline. Four volunteers dropped out because they admitted to not drinking the correct amount of beverage or did not drink the entire beverage at a single time, and five volunteers were excluded since they provided an insufficient amount of urine or did not supply the correct time points.

3.3.2 Biofilm formation assay

Urine samples were centrifuged at 2000g to remove epithelial cells and filtered using 0.8 μm and 0.25 μm polyethersulfone syringe filters (VWR InternationalTM, West Chester, PA) sequentially to remove bacteria or other particles. Bacterial samples tested were three clinical *E. coli* isolates (CFT073, B37, and BF1023), a non-pathogenic lab strain of *E. coli* with no fimbriae (HB101), and a *Staphylococcus aureus* strain (ATCC 43866). Bacteria were cultured in LB

media (35g/L, Sigma-Aldrich, St. Louis, MO), harvested at late exponential stage, which corresponded to an absorbance of 0.9-1.1 measured at a wavelength of 600nm using a spectrophotometer (Thermo Spectronic, Rochester, NY), and then mixed with urine samples at a ratio of 1: 1. The mixed suspension was transferred into a 96-well PVC microtiter plate and incubated at 37 °C. After six hours of incubation, 20 µL of crystal violet was added to stain bacteria cells for 10 minutes, and the microtiter plate was washed three times with ultrapure water to remove planktonic bacteria. The biofilm was extracted using 400 µL extracting agent (20% acetone in ethanol v/v). The absorbance of the extract was measured at the wavelength of 600 nm, as the indicator of the amount of biofilm [9]. Each urine sample was tested in three repeats.

3.3.4 Data analysis

For each volunteer, the post-CJC and post-placebo urine samples' effects on biofilm formation were compared using the Student's t-test. The difference of biofilm amount across all 11 volunteers between post-CJC and post-placebo urine were also tested using the Student's t-test. The significance level of the test was 0.05, and the time point zero samples were used as a baseline.

3.4 Results

3.4.1 Individual responses to CJC

For a given strain of bacteria, an individual was considered to be responsive to CJC if the difference in biofilm formation between post-CJC urine and post-placebo urine was significant ($P < 0.05$). 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 (Table 3.1). 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 numbers of these volunteers stayed unchanged or decreased (Table 3.1). 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 3.1. Number of volunteers that showed a significant difference between CJC and placebo (total number of volunteers is 11) . Significant level 0.05 in Student’s test.

Strain	# of volunteers showing difference between CJC and placebo				
	0hr	2hr	8hr	24hr	48hr
<i>E. coli</i> B37	0	4	4	8	2
<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

3.4.2 Biofilm formation affected by CJC consumption

For the biofilm amount measured at each time point, the difference between the CJC group and the placebo group was determined using the Student’s t-test (Table 3.2). For all the strains except *E. coli* HB101, biofilm formation decreased within 24 hours after CJC consumption and slightly increased between 24 and 48 hours after CJC consumption (Table 3.2). The amount of biofilm formed by non-pathogenic *E. coli* HB101 after culturing in post-CJC

Table 3.2. Amount of biofilm formed after culturing in urine samples collected from volunteers drinking CJC or placebo. 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 \pm 0.101	0.021 \pm 0.193	0.030 \pm 0.170	0.157 \pm 0.410	0.213 \pm 0.427
	CJC	0.000 \pm 0.073	-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 \pm 0.097	0.028 \pm 0.268	0.063 \pm 0.305	0.082 \pm 0.321	0.087 \pm 0.214
	CJC	0.000 \pm 0.060	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 \pm 0.029	0.011 \pm 0.058	-0.019 \pm 0.073	0.023 \pm 0.067	0.051 \pm 0.073
	CJC	0.000 \pm 0.036	-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 \pm 0.083	0.245 \pm 0.398	0.263 \pm 0.363	0.256 \pm 0.375	0.428 \pm 0.307
	CJC	0.000 \pm 0.066	-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 \pm 0.015	0.026 \pm 0.028	0.005 \pm 0.027	0.087 \pm 0.026	0.089 \pm 0.023
	CJC	0.000 \pm 0.009	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).

urine stayed unchanged over the 48 hours and did not show a difference from the biofilm amount formed after culturing in post-placebo urine.

3.5 Discussion

The first step of biofilm formation is bacterial adhesion, which is facilitated by adhesins on the surface of bacterial cells [10]. In order to develop cranberry juice products as a preventive therapy for UTIs, we need more information on cranberry's role in biofilm formation. According to the results of the biofilm formation assay, after the volunteers drank CJC, their urine collected could a decrease biofilm formation in bacteria strains that have adhesins (*E. coli* CFT073, *E. coli* B37, *E. coli* BF1023, and *S. aureus*), whereas the strain that does not have adhesins, *E. coli* HB101, was not affected (Table 2). These results indicate that drinking CJC prevents biofilm formation. The extent to which biofilm formation was inhibited could differ based on the adhesin density and type (*E. coli* B37, CFT073, and BF1023 have P-fimbriae, *E. coli* HB101 do not have fimbriae, and *S. aureus* have surface protein adhesins that are smaller than *E. coli* fimbriae).

We also investigated the duration of CJC's effects on biofilm formation after oral consumption. A previous study using human cell agglutination assay showed anti-adhesion activity of urine lasted for 10 hours after CJC consumption [7]. In our 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.

In conclusion, our study demonstrated that cranberry juice is an effective method for preventing biofilm formation in the urine of healthy women. Our study suggests that the inhibitory action of CJC is due to its ability to prevent bacterial adhesion and biofilm formation.

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3.6 Acknowledgements

We are grateful to Ocean Spray for providing cranberry juice cocktail and the placebo, and Dr. James Johnson from VA Medical Center, Minneapolis, MN, who provided us the clinical *E. coli* strain B37. We thank Dr. Amy Howell from Rutgers University, NJ for helpful discussion.

We would also like to thank the National Center for Complementary and Alternative Medicine (NIH R15 AT003385-01A1), the Cranberry Institute, and the Wisconsin Cranberry Board for funding this study.

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Chapter 4: Research Summary and Future Work

4.1 Research Summary

Throughout this project, we have sought to better understand how cranberry juice interacts with the adhesion activity of uropathogenic bacteria. In particular, we have made emphasis on how the oral consumption of cranberry juice cocktail (CJC) induces anti-adhesion effects in urine. Two mechanisms of cranberry's effects on uropathogenic bacteria were studied, namely surface adhesion and biofilm formation.

In Chapter 2, we investigated the effects of oral consumption of cranberry juice cocktail (CJC) on the surface adhesion forces of uropathogenic *E. coli* that were isolated from UTI patients. Our results showed that urine collected after CJC consumption decreased the non-specific adhesion forces of uropathogenic *E. coli* measured by AFM, as well as the specific adhesion measured in a human red blood cell (HBRC) assay.

In Chapter 3, we evaluated the biofilm formation of uropathogenic bacteria affected by consumption of CJC and the duration of CJC's effects. We determined that biofilm formation of uropathogenic bacteria is reduced by urine collected after CJC consumption, and the inhibitory effects of CJC lasts 24-48 hours after consumption.

4.2 Future Work

It is important to understand how cranberry affects the interaction between bacteria and epithelial cells lining the urinary tract. To extend our work on surface adhesion forces of

uropathogenic *E. coli* to the investigation of *E. coli*-uroepithelium adhesion, we started collaborating with Dr. Amy Howell from Rutgers University in NJ, in an attempt to measure the adhesion forces between *E. coli* and uroepithelial cells.

Dr. Amy Howell provided us with urine samples that were collected from volunteers who drank CJC or placebo. The time points of sample collection were 0, 2, 4, and 6 hours after consumption. To bind bacteria cells to the AFM probe, the probe was coated with poly-L-lysine (PLL) by immersing in PLL for 30 minutes. After drying in air for 10 minutes, the probe was immersed in a concentrated bacteria suspension for 10 minutes to allow bacteria to bind on the probe. The success of binding was confirmed using scanning electronic microscopy (SEM). We have maintained a human uroepithelial cell culture, and the adhesion forces between epithelial cells and bacteria were measured using the same technique we used to measure the bare probe-bacteria adhesion. Using the same force data, the adhesion energy was also calculated by integrating force with separation distance using the MFP3D AFM software (detailed method and program script in the appendage). We have acquired some preliminary data of the bacteria-uroepithelium adhesion forces using urine samples from one volunteer, as shown in Figure 4.1. *E. coli* CFT073 is a strain we used in the AFM probe-bacteria adhesion measuring experiment, and it showed decreasing adhesion force with time after CJC consumption. However, the adhesion force between *E. coli* CFT073 and uroepithelial cells did not show a similar trend as we expected. To further investigate the adhesion, we calculated the adhesion energy using the force data. The result did not show a trend either (Figure 4.1). Similar results were acquired in experiments using *S. aureus*, where post-CJC did neither cause lower adhesion force or energy compared to post-placebo urine, or adhesion force or energy decreasing with time after CJC consumption. There may be multiple factors that caused the unexpected results. First, the surface of uroepithelial cells

was highly heterogeneous. Even though in each experiment, we took force curves at multiple locations on each cell and multiple cells were measured, the surface property variation might be still too large to be overcome by the number of measurements we did. Second, the bacteria lawn coated on the AFM probes may vary from one experiment to another. With the dipping method

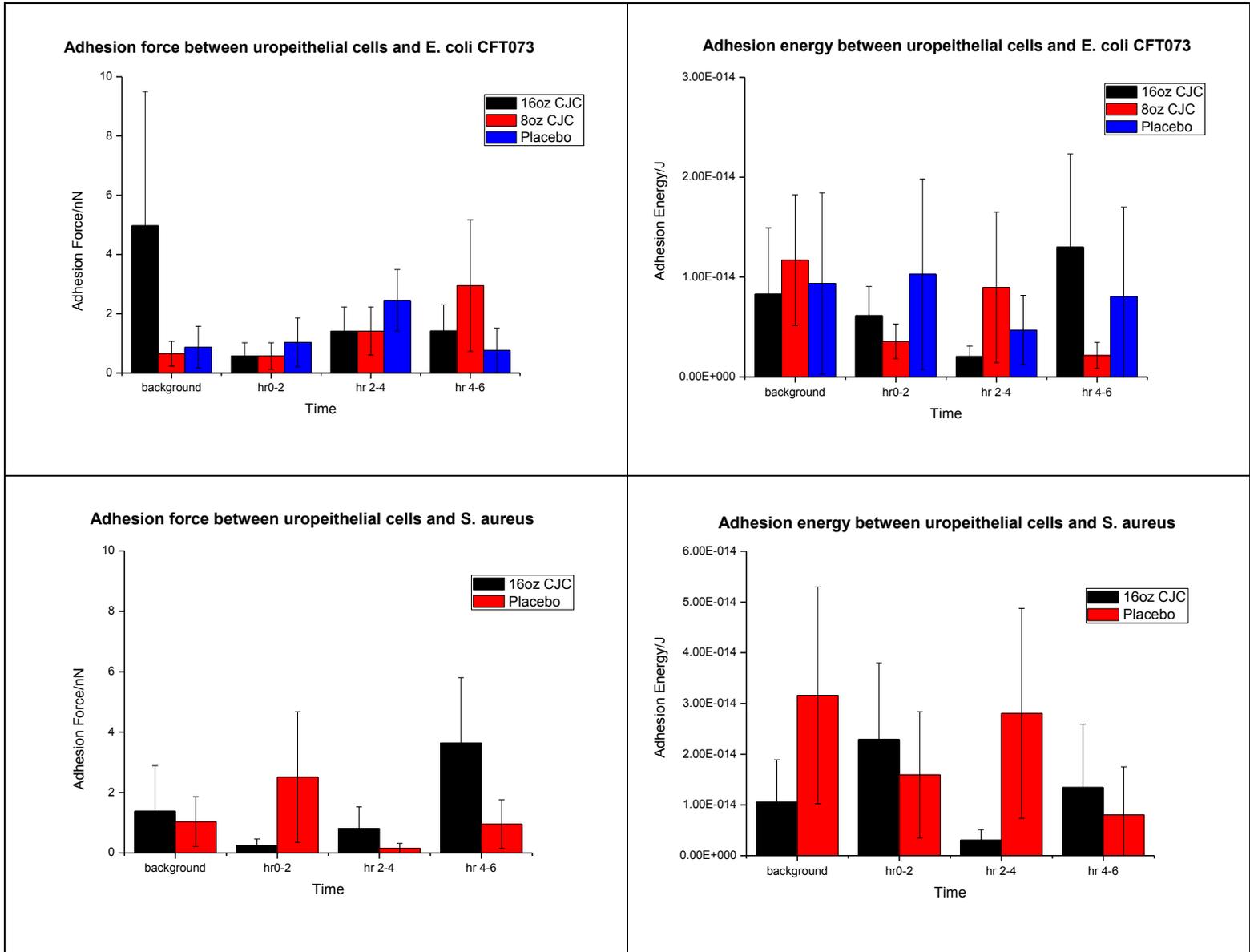


Figure 4.1 Adhesion force and energy between uroepithelial cells and bacteria (Volunteer 1)

we used to coat AFM probes, it is impossible to control the amount of bacteria attaching to the probe. When more bacteria are attached to the probe, the interaction between the probe and uroepithelial cells is stronger, and vice versa. Therefore, the accuracy of the measurements was impaired by the difference in the bacteria coating.

To overcome these artifacts, measurements could be done on more locations on epithelial cells to acquire a large enough sample size; or we could develop techniques to mount single bacteria cell onto AFM probes. If the accuracy could not be improved largely enough by these measures, we may pursue other assays to characterize interaction between uroepithelial cells and bacteria.

Appendage: Igor Script for Adhesion Energy Calculating

```
#pragma rtGlobals=1          // Use modern global access method.

#include ":AsylumResearch:Code3D:Initialization"

#include <ProcedureBrowser>

//Finding the integral of force curves, verision 2, 9-14-2010

// For Igor Pro 6.20, MFP3D 090909+0825

//Written by Anne Murdaugh (amurdaugh@ gmail.com)

//This code is still a little clunky when it comes to displaying and saving data.

// Got questions or improvements? Feel free to contact me at the email addy above.

// Updates from v1:

// Integral curves are now found between the zero intercept and the last point on the curve.

// It automatically opens tables now.

// Data is saved as a .txt file rather than as igor waves.

//To use this protocol to find the integral:

// 1. Select the force curves of interest. Make sure you're plotting force vs. Zsnsr

// 2. Place the cursors on the starting and ending points.

// 3. In the Command line (that box titled "Untitled") type SetFilepath ("the name of the file
folder containing the force curves")

// Usually that folder is named something like "X100827" unless you specified a different
save directory. Just look on the master force panel if you're
```

```

// not sure.

// 4. In the Command line type FindArea ("prefix",j,k,"results")

// "prefix" - the prefix to your file names. Default is "Image". Don't include the
numbers that go with all these files

// j - the first curve number to be analyzed; leave off the extra zeros (ie, 15, not 0015)

// k - the last curve number to be analyzed

// "results" - the name to save your data to

// If you get a bunch of blanks or 0s, check to see that you're using Force vs. Zsnsr. Also check
and make sure the curves you told it to analyze are

// displayed on the Force Review graph.

```

```

/*****
*****

```

Function SetFilepath (input1)

```

String input1 //The directory name where the force curves are
kept

string /g directory = input1 //makes directory a global variable

SVAR gdirectory = directory //Pulling up globla variable directory

String Filepath = "root:ForceCurves:Display:Subfolders:"+gdirectory
//creating needed path name

SetDataFolder Filepath //Setting path

```

End

```
/**  
**
```

Function FindArea (prefix,j,k,results)

```
String prefix //The first part of the file name, ie Image  
Variable j //First file number  
Variable k //Last file number  
String results // Name for the results file  
  
Variable n // internal counting variable  
String filename //File number part of file name, ie 0002  
String suffix // End part of filename, ie Force_Ret  
String xsuffix //End part of filename for X axis, ie Zsnsr_Ret  
String filename // Total filename put together, y axis  
String xfilename // Total filename for x axis  
Variable x1  
Variable x2
```

```
// Force curves have the name structure Image0000Force_Ext. We need to increment the  
middle part (0000), and get the area
```

// under the curve. Yes, that's an integral. I know. Just trust me here.

```
edit
Make /O /N=(k-j+1) energy //Make the wave to hold the adhesion energy
Variable i //Variable to increment adhesion energy
wave
    i = 0

suffix = "Force_Ret" //This is added on to designate the retraction curve
xsuffix = "LVDT_Ret" //This is added on to get us a Force vs Zsnr
curve
```

// Force curve names have extra zeros, ie 0001 instead of 1. We need to put in the right number.

```
String sigfig //String to create the proper amount of zeros in
filename
    sigfig = "" // We'll determine that number in the for loop.
```

// The for-loop follows. For every curve between j and k it will:

```
//      1. Get the number of needed zeros (ie, 0001)
//      2. Put the whole filename together (ie, Image0001Force_Ext)
```

```
// 3. Find the area under the zero line between the cursors.
```

```
for (n = j; n <= k ; n += 1)
```

```
    // 1. Get the number of needed zeros
```

```
        if (n<=9)
```

```
            sigfig = "000"
```

```
        elseif (10 <= n && n<= 99)
```

```
            sigfig = "00"
```

```
        elseif (100<= n && n <=999)
```

```
            sigfig = "0"
```

```
        else
```

```
            sigfig = ""
```

```
        endif
```

```
    //2. Create the filename
```

```
        filenumber = num2str (n)
```

```
        filename =prefix+sigfig+filenumber+suffix
```

```
        xfilename =prefix+sigfig+filenumber+xsuffix
```

```
    //3. Find the area:
```

```
        //3a) Find the last point on the curve:
```

```

variable lastpoint
Wavestats /Q /W $filename
    lastpoint = V_endRow
Duplicate /O $filename, tempcurve
Duplicate /O $filename, ytempcurve

x2 = tempcurve[lastpoint]
//print x2

//3b) Find the zero line
variable zeropoint
    zeropoint = 0
variable m

for (m = 0; m<100; m+=1)
    if (ytempcurve[m] > 0)
        zeropoint = m
    endif
endfor

x1 = tempcurve[zeropoint]
//print x1

```

```
Wave adhesion = $filename //This is just getting strings to  
turn into wave names.
```

```
Wave xadhesion = $xfilename
```

```
energy[i] = areaxy (xadhesion,adhesion,x2,x1) // The magic  
command that actually finds the integral. Hooray!
```

```
// Sanity Check. Uncomment to check and make sure you're not  
getting the integral from -infinity to infinity
```

```
//variable energy_check
```

```
//energy_check = areaxy (xadhesion,adhesion,-inf,inf)
```

```
//print energy_check
```

```
//print "*****"
```

```
i +=1
```

```
//Whee! Increment!!!
```

```
killwaves tempcurve, ytempcurve
```

```
endfor
```

```
// This next part is just so that we can save our data in a unique file. It too, could use  
some polishing.
```

```
Make /O /N=(k-j+1) $results  
results
```

```
//Make the waves to hold the
```

```
Duplicate /O energy, $results
```

appendtoTable \$results

Save /J \$results
the whales! But mostly your data!

//Save the data! And

End