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Role of Cranberry on Bacterial Adhesion Forces and Implications for *Escherichia coli*–Uroepithelial Cell Attachment

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ABSTRACT Previous clinical research has suggested that the consumption of cranberry products prevents the adhesion of *Escherichia coli* to uroepithelial cells by causing changes in bacterial fimbriae. Atomic force microscopy was used to probe the adhesion forces between *E. coli* (nonfimbriated strain HB101 and the P-fimbriated variant HB101pDC1) and a model surface (silicon nitride), to determine the effect of growth in cranberry products on bacterial adhesion. Bacteria were grown in tryptic soy broth supplemented with either light cranberry juice cocktail (L-CJC) or cranberry proanthocyanidins (PACs). Growth of *E. coli* HB101pDC1 and HB101 in L-CJC or PACs resulted in a decrease in adhesion forces with increasing number of cultures. In a macroscale bacteria–uroepithelial cell adhesion assay a decrease in bacterial attachment was observed for *E. coli* HB101pDC1 grown in L-CJC or PACs. This effect was reversible because bacteria that were regrown in cranberry-free medium regained their ability to attach to uroepithelial cells, and their adhesion forces reverted to the values observed in the control condition. Exposure to increasing concentrations of L-CJC resulted in a decrease of bacterial attachment to uroepithelial cells for the P-fimbriated strain after L-CJC treatment (27% by weight) and after PACs treatment (345.8 μg/mL). Cranberry products affect the surface properties, such as fimbriae and lipopolysaccharides, and adhesion of fimbriated and nonfimbriated *E. coli*. The concentration of cranberry products and the number of cultures the bacteria were exposed to cranberry determines how much the adhesion forces and attachment are altered.

KEY WORDS: • ***atomic force microscopy*** • ***fimbriae*** • ***proanthocyanidins*** • ***urinary tract infections*** • **Vaccinium macrocarpon**

INTRODUCTION

THE ADHESION OF BACTERIA to uroepithelial cells and urinary catheters is the first step in the development of a urinary tract infection (UTI).[1,2] The treatment to eradicate these infections results in medical expenditures that exceed $2 billion dollars each year.[3] Bacterial adhesion to biotic and abiotic surfaces is governed by nonspecific, long-range forces, including electrostatic and steric interactions, and by short-range forces, such as hydrophobicity, van der Waals forces, and surface charge. The presence of specific proteins on the bacterial surface can lead to stronger and more specific interactions.[4]

Escherichia coli predominates as the most common urinary pathogen, causing ~85% of UTIs; ~90% of acute pyelonephritis cases are caused by *E. coli* expressing type P-fimbriae.[5] Adhesins at the distal end of fimbriae can bind to receptors found on the surface of uroepithelial cells.[6,7] The Class I, II, and III G adhesins encoded by *pap* or *prs* gene clusters have been closely related to development of acute pyelonephritis (kidney infection) and cystitis (bladder infection) because they bind to receptors of the αGal(1 $\rightarrow$ 4)βGal glycolipid family on uroepithelial cells.[5,7] This binding step is then the first phase in the development of an infection. While cystitis is usually associated with the attachment of bacteria expressing type 1 fimbriae to bladder mucosa,[8] P-fimbriae with Class III adhesins are associated with 12% of cystitis cases in adult women,[5] 37% of cystitis cases in children,[9] and 13% of acute pyelonephritis incidents.[5] The majority of acute pyelonephritis cases (66%) are caused by P-fimbriated bacteria that contain Class II adhesins.[5]

Although bacteria present in the urinary tract are usually treated with common antimicrobial agents,[10,11] concerns about antibiotic resistance, side effects, and prevention of recurrent UTIs in susceptible populations have led to increased interest in using other remedies to prevent bacterial infections. UTI prevention through the use of the North American cranberry (*Vaccinium macrocarpon* Ait., Family Ericaceae) is being investigated.[12–14] Decades ago, it was believed that the acidity of cranberries was responsible for these benefits,[15] but more recent studies have shown that the

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ingestion of cranberry products does not alter the pH of urine significantly and that cranberry is not bacteriostatic.[16] Cranberry is now believed to prevent UTIs by hindering the ability of pathogenic bacteria to attach to the urinary tract.[12] A similar effect has been proposed for other infections that rely on bacterial adhesion to host tissue, such as the use of cranberry to prevent *Helicobacter pylori* infections in gastric lumen,[17–19] and inhibition of biofilm formation on tooth-supporting tissues and periodontal ligaments.[20–22]

Certain phytochemicals in cranberries, such as A-type proanthocyanidins (PACs), have been identified as the agents responsible for inhibiting the adhesion of *E. coli* to uroepithelial cells.[23] Exposure of bacteria to 60 μg/mL PACs was sufficient to prevent the agglutination of P-fimbriated *E. coli* to human red blood cells.[24] Using a direct force measurement technique of atomic force microscopy (AFM), exposure to increasing concentrations of cranberry juice cocktail resulted in a decrease in adhesion forces between P-fimbriated *E. coli* and a silicon nitride probe.[25] This study also showed through steric modeling that the length of bacterial fimbriae decreased with exposure to increasing concentrations of cranberry juice.

Few studies have addressed the effects of cranberry products on the growth of uropathogenic bacteria. Loss of fimbriae due to growth in cranberry juice has been reported, which led to the inability of bacteria to agglutinate P-specific receptor beads and also caused cellular elongation.[26] Changes in growth rate due to growth of bacteria in cranberry juice for 24 or 48 hours have also been reported.[16]

Traditional methods to study the adhesive properties of pathogens are by enumeration of bacteria attached to receptor-specific beads, epithelial cells, or erythrocytes through light microscopy.[6,27,28] While these methods can provide important information, they can only provide attachment data for a population of bacteria. In contrast, AFM can be used to examine properties of microbial surfaces at molecular resolution and for single cells.[29,30] Interaction forces between bacteria and different substrates can also be obtained through AFM in their natural environments where minimum sample preparation is required.[25,31,32] Using a model surface of a clean AFM tip, the atomic force microscope is used as a force probe to discriminate between molecular adhesion forces associated with different bacteria or different treatments. Our laboratory was the first to investigate the effects of cranberry juice on the adhesion forces of single *E. coli* cells using AFM.[25]

No previous research has investigated how prolonged culturing of *E. coli* in the presence of cranberry products affects bacterial adhesion forces. Patients with UTIs may be advised to consume cranberry juice for long periods to prevent recurrent episodes, which can allow bacteria to grow in an environment that contains metabolites derived from cranberries. We correlated the AFM adhesion data with results of macroscale experimental observations of the number of bacteria that attached to single uroepithelial cells after growth in cranberry products.

MATERIALS AND METHODS

Cranberry products

Ocean Spray light cranberry juice™ cocktail (referred to hereafter as "L-CJC") was purchased from Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA). The pH of cranberry juice was adjusted to 7.0 using sodium hydroxide (Sigma-Aldrich, St. Louis, MO), and the solution was passed through an Acrodisc® syringe filter (pore size, 0.2 μm; Pall Corp., East Hills, NY) for further purification. A 345.8 μg/mL stock solution of isolated cranberry PACs (Ocean Spray Cranberries) was prepared in ultrapure water (Milli-Q® water, Millipore Corp., Bedford, MA). The mixture was shaken overnight at room temperature, in the dark. The pH of the PACs solutions was not adjusted because the values were close to neutral (6.8–7.1).

Bacteria and growth conditions

E. coli HB101 (ATCC 33694) was purchased from the American Tissue Culture Collection (Manassas, VA). *E. coli* HB101 bacteria are plasmid-less and lack fimbriae.[33] The P-fimbriated *E. coli* variant HB101pDC1 was kindly provided by Dr. M. Svensson from the Department of Medical Microbiology, Lund University, Lund, Sweden. *E. coli* HB101pDC1 is a variant of HB101 that expresses P-fimbriae because of the insertion of a chloramphenicol resistance plasmid.[34]

Bacteria were grown at 37°C in 30 g/L tryptic soy broth (TSB) (Sigma-Aldrich). For strain HB101pDC1, medium was supplemented with 20 μg/mL chloramphenicol (Sigma-Aldrich) to ensure the expression of P-fimbriae. Growth rates and doubling times were analyzed by measuring the optical density of the solution at 600 nm (Thermo Spectronic, Rochester, NY), and bacteria were harvested in the late exponential growth phase (absorbance at 600 nm = 0.9).

Experiments were conducted as a function of cranberry product concentration or the number of times the bacteria were cultured in cranberry products. For the growth experiments, the concentration of cranberry product was held constant, at either 10% by weight L-CJC or the equivalent amount of PACs, calculated to be 128 μg/mL.[24] The cranberry product was added to the growth medium (30 g/L TSB), which was supplemented with chloramphenicol (20 μg/mL) for strain HB101pDC1. To simulate continuous culture of bacteria in cranberry juice, *E. coli* bacteria were grown in cranberry products with continuous exchange of the growth medium every time the late exponential growth phase was reached. After initial growth to an absorbance at 600 nm of 0.9, 100 μL of bacterial solution was transferred to a new flask with fresh medium to start the next culture in cranberry-rich medium. Twelve successive transfers of bacteria in TSB and L-CJC or PACs were made per strain. Samples were collected periodically, corresponding to the first, fourth, eighth, and 12th bacterial cultures, with the 12th

culture corresponding to ~10 days of growth. To evaluate if the effects of cranberry-rich medium on bacterial adhesion were permanent, 100 μL of culture 12 solution was placed in a flask of pure TSB (cranberry-free medium) and harvested at an absorbance at 600 nm of 0.9 (condition referred to hereafter as "reverse"). For each sample, collected bacteria were centrifuged for 10 minutes at 2,500 *g*, washed three times with ultrapure water to remove all the components of the growth medium,[29] and resuspended in ultrapure water for AFM experiments. Table 1 summarizes the different cranberry treatments used.

For the concentration experiments, bacteria were exposed to solutions of L-CJC at 0%, 5%, 10%, or 27% by weight or to PACs concentrations of 0, 64, 128, and 345.8 μg/mL. The L-CJC and PACs concentrations were estimated to be equivalent based on the amount of PACs reported to be found in cranberry juice.[24] When cranberry solution concentration was the main variable, bacteria were grown in TSB (no L-CJC or PACs present) and were exposed to the cranberry product for 3 hours.

AFM

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

Bacteria were attached to clean glass slides. Slides were treated with 35% aminosilane solution (3-aminopropyltrimethoxysilane; Sigma-Aldrich) in distillation-quality methanol (Sigma-Aldrich). The slides were kept in the aminosilane solution for 15 minutes and rinsed with methanol followed by 25 mL of ultrapure water. *E. coli* from cultures 1, 4, 8, and 12, control (no cranberry treatment), and the reverse condition were immobilized on acid-clean glass slides using a protein–protein cross-linking reaction that we have successfully used for bacterial attachment in other studies.[25,35] A 300-μL volume of a 100 m*M* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) HCl (Pierce, Rockford, IL) solution was added to previously washed bacteria with rotation at 18 rpm for 10 minutes at room temperature. A 600-μL volume of a 40 m*M* *N*-hydroxysulfosuccinimide (NHS) (Pierce) solution was added to the bacterial solution, followed by rotation at 18 rpm for 20 minutes. This mixture was added to the aminosilane glass slides and allowed to shake at 70 rpm for 6 hours. EDC/NHS-treated bacteria remained viable, and no cell damage occurred during this bonding process.[35] Viability was confirmed by placing the slide back into growth medium and evaluating growth of bacteria after the AFM experiment by measuring the growth rates and doubling times with a spectrophotometer (Thermo Spectronic).

TABLE 1. EXPOSURE PARAMETERS IN CRANBERRY PRODUCTS FOR *E. COLI*

	Culture number	*Time of exposure (hours)*	*Concentration*
L-CJC			
Short-term[a]	—	4.5	0
	—	4.5	5
	—	4.5	10
	—	4.5	27
Growth[b]	1	6	10
	4	66	10
	8	162	10
	12	258	10
Control (pure TSB)	1	0	0
Reverse (12th culture back into pure TSB)	1	0	0
PACs			
Short-term[a]	—	4.5	0
	—	4.5	64
	—	4.5	128
	—	4.5	345.8
Growth[b]	1	6	128
	4	66	128
	8	162	128
	12	258	128
Control (pure TSB)	1	0	0
Reverse (12th culture back into pure TSB)	1	0	0

The concentrations of L-CJC and PACs are % by weight and μg/mL, respectively.
[a]Bacteria grown in pure TSB.
[b]Bacteria grown in cranberry products.

AFM measurements were carried out by using a Dimension 3100 instrument with Nanoscope IIIa Controller (Veeco Metrology, Santa Barbara, CA), with all measurements performed in 0.01 *M* phosphate-buffered saline (PBS) solution. Silicon nitride AFM tips (DNPS, Veeco Metrology) were used to acquire images in fluid in tapping mode, which was performed at the resonant frequency of the cantilever. Images were obtained with 512×512 points at a scan rate of 1.0 Hz.

Before using the cantilevers, they were cleaned with ethanol and exposed to ultraviolet light to remove potential contaminants. Cantilever spring constants were measured using the thermal calibration method.[36] The average spring constant was 0.06 ± 0.01 N/m. Force measurements were carried out in PBS, and five individual cells were probed, where six force cycles were recorded per bacterium per condition. Data were converted to ASCII format and exported to a spreadsheet where the information was converted from deflection data to force, as discussed previously.[37]

AFM adhesion force analysis

Peaks observed in the retraction curve were identified as independent and random events that occur when the AFM tip retracts from the bacterial surface and fimbriae or other molecules are released from the probe (also known as pull-off events). Each peak represents the adhesion force exerted between each *E. coli* surface molecule and the bare silicon nitride AFM probe. The retraction peaks were combined, and histograms were created.

Since retraction curves can result in different number of adhesion peaks, the sample size of every condition was different. Data were analyzed using SigmaStat® (Systat Software, San Jose, CA) version 2.03. The Kruskal-Wallis one-way analysis of variance test on ranks was used to analyze all conditions with different sample sizes. Dunn's test was used to compare among treatment groups and to compare cranberry-treated groups against the control condition (pure TSB).

Uroepithelial cell culture

Human ureteral epithelial cells (CRL 9520 VA) were purchased from the American Type Culture Collection and maintained in liquid nitrogen vapor phase. The cells were grown in Kaighn's modification of Ham's F12 medium and supplemented with 10% fetal bovine serum (American Type Culture Collection). Culture flasks were placed in a 5% CO_2 in air atmosphere incubator at 37°C for 7 days, and the medium was replaced every 2 days. Uroepithelial cells were harvested by adding 0.25% (wt/vol) trypsin-0.03% (wt/vol) EDTA (Sigma-Aldrich) to detach the cells from the culture flasks. The low concentration of trypsin allowed us to detach the cells without compromising their viability or surface properties, as has been shown in prior studies.[28] The viability of the cells after trypsin treatment was assessed by regrowth to confluence in new tissue culture flasks. The cells were kept in trypsin-EDTA for no more than 10 minutes at 37°C, washed, centrifuged, and resuspended in uroepithelial cell growth medium.

Bacterial attachment to uroepithelial cells

Experiments were conducted as a function of cranberry product concentration or as a function of the number of growth cultures in cranberry-supplemented medium. Quantitative assessment of bacterial adhesion to uroepithelial cells was determined as described previously.[1,28,38,39]

Concentration experiments. Bacteria cultured in pure TSB (no cranberry in the medium) were suspended in aqueous solutions, diluted in PBS, containing 0%, 5%, 10%, and 27% by weight L-CJC or 0, 64, 128, and 345.8 μg/mL PACs for 3 hours at 37°C with slow shaking. Uroepithelial cells were incubated with equal volumes of the different concentrations of L-CJC or PACs for 3 hours at 37°C.

Growth experiments. The first, fourth, eighth, and 12th cultures of both strains cultured in cranberry-rich medium and the reverse culture were harvested in the late exponential growth phase and washed three times in PBS.

After cranberry treatment or growth in cranberry, bacterial suspensions of 1×10^9 colony-forming units/mL and uroepithelial cell suspensions of 1×10^6 cells/mL were placed in tissue culture flasks and incubated for 90 minutes at 37°C with rotation at 18 rpm. After incubation, loosely attached bacteria were removed by gentle centrifugation (100 *g*) for 10 minutes, and the uroepithelial cell–bacteria solution was resuspended in 0.01 *M* PBS.[27]

Bacteria attached to uroepithelial cells were counted at a magnification of ×1,000 under oil immersion, using phase contrast (Eclipse E400 microscope, Nikon, Tokyo, Japan). All images were collected using a camera and stored with SPOT version 4.6 advanced software (Diagnostic Instruments, Inc., Sterling Heights, MI). The number of bacteria attached to 20 uroepithelial cells was determined per sample condition.

Attachment data were analyzed using SAS® (SAS Institute, Cary, NC) and SigmaStat version 2.03 statistical software. Statistical analysis was performed by two-way analysis of variance for repeated measurements. Tukey's test was used for multiple comparisons among each treatment group, whereas Dunnett's and Duncan's tests were used for comparisons between treatment and control groups. A difference was considered significant if $P < .05$.

RESULTS

Analysis of AFM retraction force data after growth of E. coli *in cranberry products*

For either *E. coli* strain grown in L-CJC or PACs, the retraction cycles showed multiple large adhesive peaks with the silicon nitride probe, which indicated that the tip was

making contact with more than one surface polymer per measurement. Growing bacteria in the presence of cranberry products resulted in a decrease in the adhesive forces with the model probe (Figs. 1 and 2). While there was a broad distribution in the adhesion forces for either bacterial strain grown in TSB, the distribution narrowed and became more skewed towards lower adhesion force values as the number of culture periods in L-CJC or PACs increased (L-CJC, Fig. 1B and D; PACs, Fig. 2B and D).

E. coli HB101pDC1 cultured in pure TSB showed strong adhesion forces with an absolute average of 1.60 ± 0.71 nN. After the first culture of *E. coli* HB101pDC1 in 10% by weight L-CJC the adhesion forces decreased to 1.13 ± 0.6 nN. Culturing bacteria for the first time in the presence of 128 μg/mL PACs resulted in average adhesion forces that were slightly increased (1.9 ± 1.1 nN); however, this effect was not significant according to Dunn's test ($P > .05$). A continuous decrease in adhesion forces was observed after the fourth culture for HB101pDC1 cultured in L-CJC or PACs. By the 12th culture of bacteria in cranberry products, the adhesion forces had decreased to 0.56 ± 0.3 nN (L-CJC) and 0.42 ± 0.2 nN (PACs). There was a statistically significant difference between the control group (bacteria grown in pure TSB) and *E. coli* HB101pDC1 grown in the presence of L-CJC or PACs for all culture periods ($P < .001$).

Regrowth of cranberry-treated bacteria in pure TSB resulted in a reversion of adhesion forces. *E. coli* HB101pDC1 from the 12th L-CJC culture regrown in TSB exhibited adhesion forces of 1.33 ± 0.6 nN, and bacteria from the 12th PACs culture regrown in TSB showed adhesion forces of 1.24 ± 0.77 nN, which were not statistically different from the control case.

The trends for HB101pDC1 and HB101 were very similar. Even though HB101 does not express fimbriae, we observed a decrease in adhesion forces with increasing number of cultures in L-CJC or PACs (Figs. 1D and 2D). For

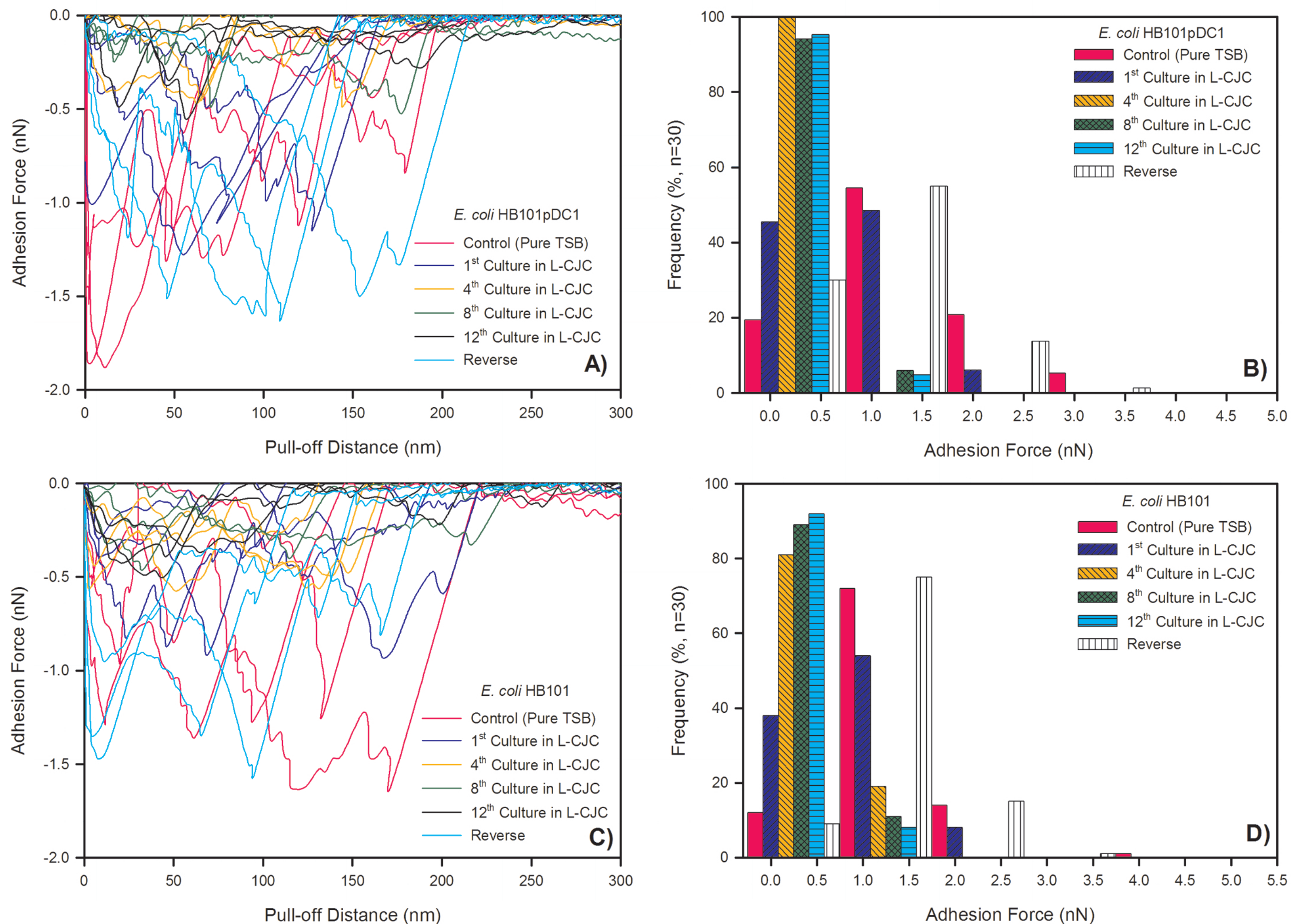

FIG. 1. AFM adhesion data of *E. coli* cultured in the presence of 10% by weight L-CJC: (**A** and **B**) *E. coli* HB101pDC1 and (**C** and **D**) *E. coli* HB101. (A and C) Representative AFM retraction curves. (B and D) Distribution parameters from AFM retraction curves. Five individual cells were probed six times by a clean silicon nitride AFM tip in 0.01 *M* PBS solution ($n = 30$).

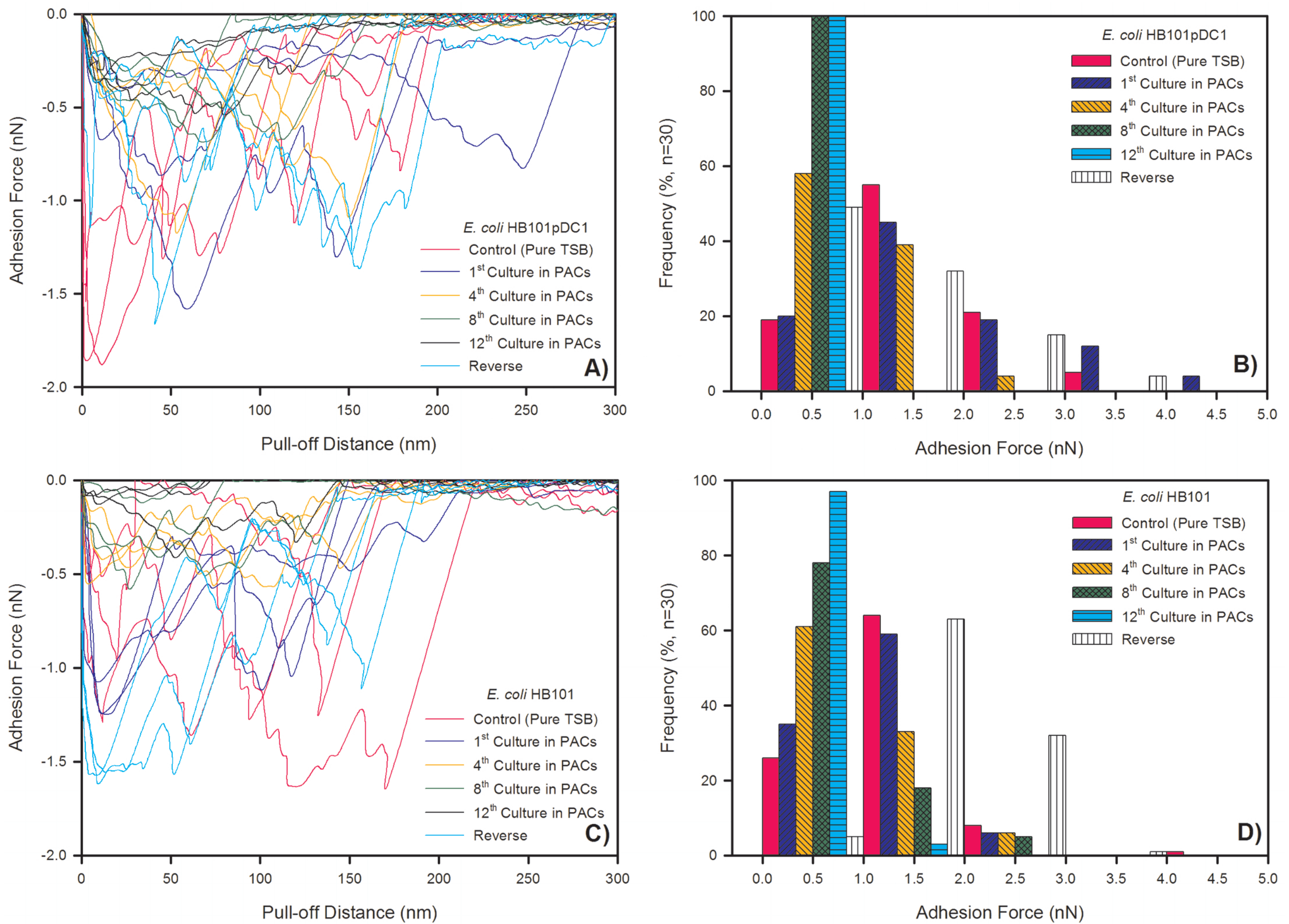

FIG. 2. AFM adhesion data of *E. coli* cultured in the presence of 128 μg/mL PACs: (**A** and **B**) *E. coli* HB101pDC1 and (**C** and **D**) *E. coli* HB101. (A and C) Representative AFM retraction curves. (B and D) Distribution parameters from AFM retraction curves. Five individual cells were probed six times by a clean silicon nitride AFM tip in 0.01 *M* PBS solution ($n = 30$).

the nonfimbriated HB101 strain, Dunn's test showed that there was a statistically significant difference between the adhesive forces of bacteria grown in pure TSB and all L-CJC- and PACs-treated bacteria ($P < .05$), which suggests that cranberry products may also affect nonspecific adhesion. Similarly to the P-fimbriated HB101pDC1 strain, regrowth of HB101 cranberry-treated bacteria in pure TSB resulted in a reversion of adhesive force values (control vs. reverse; $P > .05$).

The pull-off distances did not show any trend as a function of bacterial strain or growth condition and were distributed over a broad range for all cases.

Bacterial attachment to uroepithelial cells after continuous incubation of bacteria in cranberry products

The role of continuous growth of *E. coli* in L-CJC or PACs on bacterial attachment was evaluated. *E. coli* HB101pDC1 cultured in TSB attached readily to uroepithelial cells, with 50.2 ± 22.9 bacteria per cell (Fig. 3A). Culturing of the same strain in the presence of L-CJC or isolated PACs resulted in significant decreases in attachment, with 7.9 ± 3.0 and 7.1 ± 2.8 bacteria per cell, respectively, after the first growth period in cranberry-supplemented medium ($P < .001$). For *E. coli* HB101pDC1 grown in L-CJC or PACs, there were no significant differences among the means of the different cultures within each cranberry treatment, according to Tukey's test ($P > .9$). For HB101, attachment was low even without growth in cranberry, and attachment remained low for all L-CJC and PACs cultures tested (Fig. 3B).

After L-CJC or PACs treatment, bacteria from culture 12 were recultured in pure TSB, and *E. coli* organisms regained their ability to attach to uroepithelial cells (Fig. 3A). *E. coli* HB101pDC1 bacteria treated with L-CJC or PACs that were regrown in pure TSB medium resulted in an attachment of 48.75 ± 16.38 bacteria per cell and 38.7 ± 19.1 bacteria per

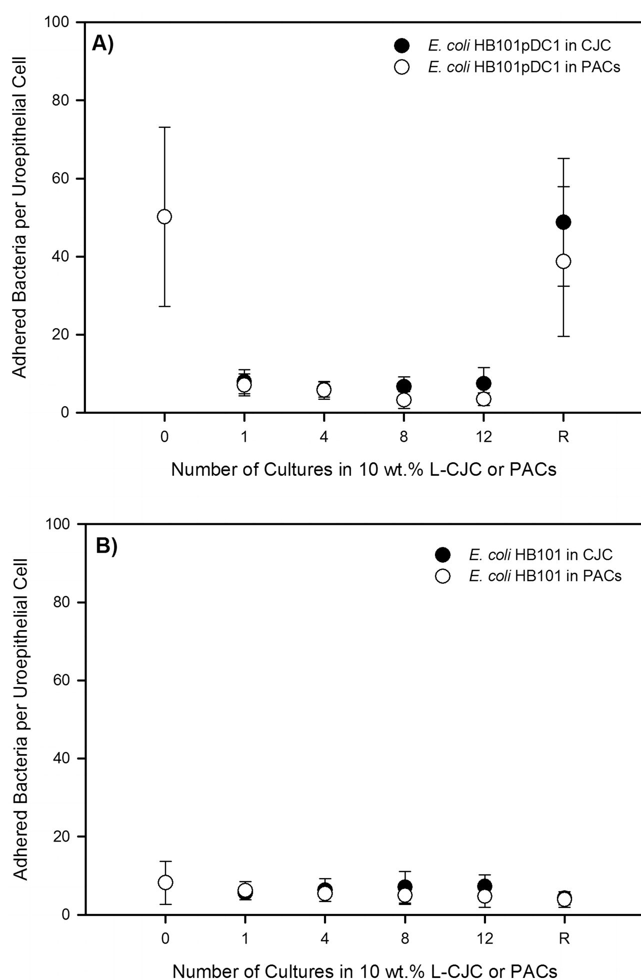

FIG. 3. Effects of continuous incubation of bacteria in 10% by weight L-CJC or 128 μg/mL PACs on bacterial attachment ($P < .001$) for (**A**) *E. coli* HB101pDC1 and (**B**) *E. coli* HB101. Reverse experiment denoted as "R." Data are mean ± SD values.

cell, respectively, which were not statistically different from the control group.

Effects of increasing concentrations of cranberry constituents on bacterial attachment to uroepithelial cells

Exposure of *E. coli* HB101pDC1 to increasing concentrations of L-CJC resulted in a decrease in the number of attached bacteria per uroepithelial cell when compared to our control sample of bacteria exposed to PBS (Fig. 4A), and this difference was statistically significant, according to Dunnett's and Duncan's tests ($P < .001$). After exposure to 5% by weight L-CJC, the number of attached bacteria per uroepithelial cell decreased from 50.2 ± 22.9 to 13.6 ± 5.7. Treatment with L-CJC at 10% and 27% by weight resulted in further decreases in attachment, to 9.3 ± 4.1 and 2.9 ± 1.5 bacteria per cell, respectively.

Exposure to isolated PACs also caused a decrease in bacterial attachment to uroepithelial cells for the P-fimbriated *E. coli* strain (Fig. 4A). For the different concentrations of PACs tested, Dunnett's and Duncan's tests showed significant differences between our control group (exposed to PBS) and each sample of HB101pDC1 exposed to PACs ($P <$ .001). Exposure of HB101pDC1 to 64 μg/mL PACs resulted in 32.5 ± 13.9 bacteria attached per cell. *E. coli* HB101pDC1 exposed to higher concentrations of PACs (128 μg/mL and 345.8 μg/mL) resulted in a further decrease in attachment, with 28.9 ± 12.2 and 10.6 ± 2.4 attached bacteria per cell, respectively.

Using Tukey's test to compare the statistical significance of the different concentrations of L-CJC used to treat bacteria and uroepithelial cells, we found a significant difference between *E. coli* HB101pDC1 treated with 5% and 27% by weight L-CJC ($P = .001$), but no statistical difference was found between the mean numbers of bacteria treated

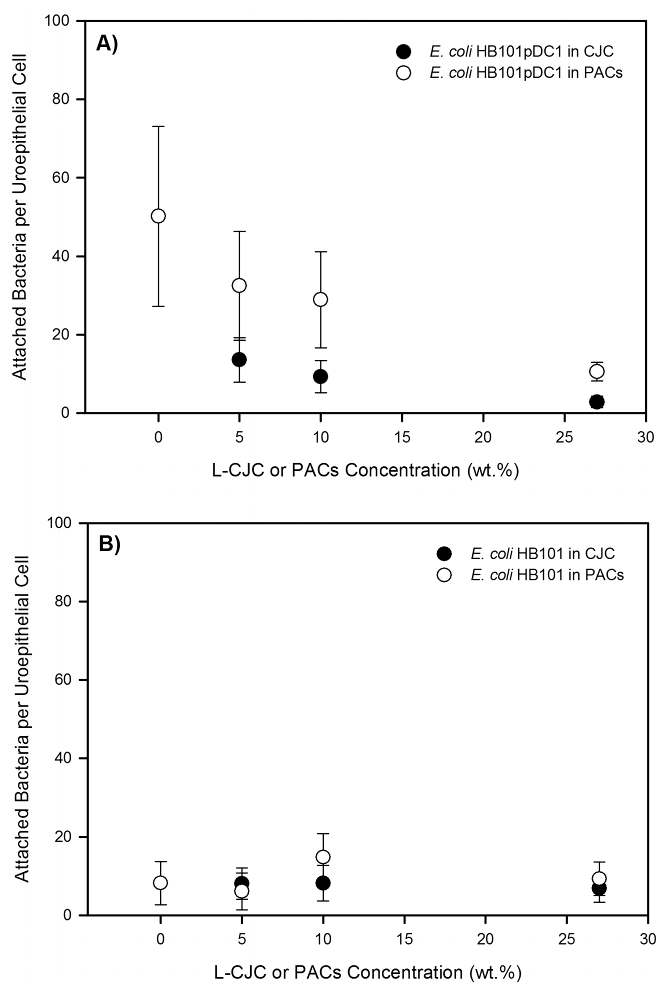

FIG. 4. Effects of concentration of L-CJC and PACs on bacterial attachment for (**A**) *E. coli* HB101pDC1 and (**B**) *E. coli* HB101. Data are average number of adhered bacteria per uroepithelial cell ± SD. By Tukey's test $P < .001$.

with 5% and 10% by weight or between 10% and 27% by weight cranberry juice ($P = .436$ and $P = .109$, respectively). For the different concentrations of PACs, Tukey's test showed that all concentrations resulted in statistically significant differences among the mean numbers of bacteria attached, with the exception of 5% versus 10% by weight PACs ($P = .734$).

The attachment of *E. coli* HB101 to uroepithelial cells was not significantly different for any of the L-CJC or PAC treatments or for the control ($P > .2$; Fig. 4B).

DISCUSSION

Correlating growth in cranberry products and adhesive forces of E. coli

Although some studies have addressed the effects of cranberry on bacterial adhesion and prevention of UTIs,[12,25,40,41] few studies have examined cranberry's effects on growing bacteria over long periods of time. Patients who are prone to UTIs are advised to consume cranberry juice for long periods, which may result in the growth of bacteria in an environment rich in cranberry metabolites. Cranberry metabolites were found in the urine of volunteers who consumed 240 mL of cranberry juice, and these metabolites had a bacterial anti-adhesive activity that lasted up to 8 hours post-consumption.[24] Thus, growth of bacteria in cranberry products offers the advantage of maximizing the effect that cranberry juice or isolated cranberry PACs can have on bacterial adhesion. We investigated the ability of PACs and L-CJC to reduce the adhesion forces between a silicon nitride AFM probe and *E. coli* HB101 and HB101pDC1 after growth in L-CJC and PACs. AFM is a sensitive tool for analysis of interactions between single bacteria and other surfaces.[25,31,42] While attachment or retention assays provide information on how a population of bacteria attaches to a specific cell line, they cannot provide mechanistic interpretations of the interaction forces that govern bacterial adhesion.

Determining the interaction forces of the AFM tip with Gram-negative bacteria like *E. coli* is possible because of the thick and rigid peptidoglycan layer in the cell wall that protects the cell from elastic deformation by the AFM probe.[43] The AFM tip interacted with several polymers on the surface of bacteria as indicated by the numerous adhesion peaks in the retraction force portions of the force measurement cycle, independent of growth conditions (Fig. 1A).

After growth in cranberry products, adhesion forces were reduced, but we still saw long-range interactions between the *E. coli* and the silicon nitride probes (Figs. 1 and 2). The fact that we observed such interactions for both strains, along with their large magnitudes, suggests that we were not only probing the fimbriae in these experiments, but other surface molecules. We suggest that *E. coli* lipopolysaccharides (LPSs) were responsible for these long-range interactions.

Previous studies with *E. coli* JM109 and silicon nitride probes showed pull-off distances of up to 600 nm,[44] which were also attributed to LPS. In a recent study, *E. coli* tethers of LPS were found to be up to 4 μm in length when they were measured using optical tweezers.[45] Two recent studies have shown that PACs can bind directly to bacterial LPS, most likely targeting the lipid A region.[46,47] Thus, these studies suggest that in addition to the well-known specific actions of cranberry compounds on bacterial fimbriae, cranberry products can also lead to nonspecific interactions with bacterial LPS.

Previous work focused more on the effects on bacterial fimbriae. For example, our prior work with *E. coli* HB101pDC1 grown in TSB (with no cranberry) and exposed to ultrapure water showed that the fimbriae were ~148 nm, and their length was compressed to ~48 nm when exposed to 27% by weight cranberry juice cocktail.[25] In addition, Ahuja *et al.*[26] grew *E. coli* JR1 and DS17 in cranberry-rich agar and could not detect any fimbriae when imaging the cells with transmission electron microscopy.

While loss of fimbriae was observed after continuous culturing in cranberry-rich medium, our previous AFM studies and steric modeling indicated that cranberry juice cocktail does not inhibit the expression of P-fimbriae but, instead, results in a conformational change where there is a decrease in the polymer length.[25] The results of the current study indicated that culturing of *E. coli* in cranberry products results in a decrease in adhesion forces, which may be due to conformational changes of these surface polymers and not to loss of fimbriae. However, this change in adhesion was transient and could be easily reversed by reculturing bacteria in cranberry-free medium. Furthermore, the strain used during this study is a variant of the HB101 strain where a chloramphenicol resistance plasmid has been inserted into the cell.[34] Growth of this strain in the presence of the antibiotic ensures that the plasmid is not lost and the bacteria will express P-fimbriae as long as chloramphenicol is not removed from the medium.

Correlating growth in cranberry products and attachment of bacteria to uroepithelial cells

While several studies evaluated the effects of short-term exposure of bacteria to cranberry constituents on attachment to epithelial cells or human red blood cells,[23,48,49] this was the first study to investigate the effects that long-term growth of *E. coli* in the presence of L-CJC and PACs have on adhesive activity to uroepithelial cells. These *in vitro* experiments were aimed to maximize the effects that L-CJC or PACs can have on bacterial adhesion by using different concentrations and exposure times. Furthermore, we seek to understand how this effect can explain the beneficial properties that have been observed in clinical trials of volunteers who consumed cranberry products for long periods.[13,14] The seminal clinical study of cranberry's effects on UTIs was conducted in 1994 by Avorn *et al.*[14] In this placebo-controlled, double-blinded study, elderly women with recurrent UTIs consumed 300 mL of cranberry juice daily for 6 months,[14] allowing bacteria in the urinary tract to grow and

multiply in a cranberry- or PACs-rich environment. During *in vivo* experiments there are many unknown and uncontrollable factors that can make it difficult to understand the mechanisms of cranberry juice in inhibiting bacterial adhesion. Thus, we designed these *in vitro* experiments to allow bacteria to grow in the culture medium with L-CJC or PACs to obtain a better understanding of changes in bacterial attachment ability after undergoing repeated culture in cranberry.

We found that growing *E. coli* HB101pDC1 for 6 hours in TSB with 10% by weight L-CJC (first culture) resulted in an 84% decrease in attachment to uroepithelial cells. It was previously reported that growth of *E. coli* JR1 in cranberry-rich agar resulted in an inability to agglutinate P-receptor specific beads, which was attributed to ~90% of bacteria having absent or nonfunctional P-fimbriae.[26] Our results suggest that in addition to affecting fimbriae, cranberry products can affect other molecules on *E. coli*, probably LPS. This was concluded because both *E. coli* HB101pDC1 and HB101 were affected by growth in cranberry according to our AFM results. However, while the AFM experiments were sensitive enough to detect these changes, the macroscopic cell attachment studies could not.

Growth of *E. coli* in the presence of 128 μg/mL PACs resulted in an inhibition of bacterial adherence similar to what was seen for bacteria grown in L-CJC. Growth of *E. coli* HB101pDC1 for 6 hours in the presence of PACs resulted in ~86% decrease in attachment to uroepithelial cells. An exponential decay relationship was observed between attached bacteria and number of cultures in cranberry products. This nonlinear relationship has been previously observed by other researchers.[23,42,50] The results of our research suggest that continuous exposure of *E. coli* to cranberry juice will result in an inability of bacteria to adhere to uroepithelial cells because bacteria would be growing in a L-CJC or PACs-rich environment.

Reversibility of effects of cranberry juice and PACs

Bacteria from reverse conditions (cranberry-treated bacteria recultured in TSB) regained their ability to attach to uroepithelial cells, and the adhesion forces reverted to the values observed in the control condition. Cranberry products lowered the adhesion of bacteria to uroepithelial cells as long as they were constantly exposed to cranberry-rich medium. A similar effect was observed for *E. coli* JR1 and DS17 grown in cranberry-rich agar, which lost their ability to agglutinate P-specific receptor beads but regained this ability once bacteria were plated back into cranberry-free medium.[26] Clinical studies have also showed that removal of cranberry juice from the dietary regimen results in recurrence of UTIs for most patients.[51] For instance, Prodromos *et al.*[51] placed 60 patients with recurrent UTIs in a treatment where 16 ounces (~470 mL) of cranberry juice was consumed daily for 21 days. Although ~73% of the patients showed a positive improvement during the time that the juice was being consumed, more than 60% of the patients had recurrent UTIs 6 weeks after the therapy was discontinued.[51] In another study, 150 women with UTIs were asked to consume either 50 mL of cranberry-lingonberry concentrate, 100 mL of a *Lactobacillus* GG drink, or no intervention (open control group) daily for 6 months, and recurrences of UTIs were evaluated during a 12-month follow-up.[52] In the follow-up, there were 98 episodes of UTIs, of which 21% of the cases occurred in the cranberry group. While the number of episodes in the cranberry group was considerable, the number of recurrent UTIs after cranberry treatment was significantly lower for this group than for the *Lactobacillus* group (40%) or the control group (39%).[52]

Based on the ability of cranberry to lower adhesion forces of *E. coli* HB101, which do not express fimbriae, we suggest that cranberry compounds are also binding reversibly to LPS molecules. Previous molecular explanations of how the effect of cranberry on adhesion is reversible focused on fimbriae. Liu *et al.*[25] proposed that the adhesion forces between *E. coli* bacteria and an AFM probe decreased after exposure to cranberry juice because specific compounds in cranberry bound to the adhesins on P-fimbriae. They speculated that compounds in cranberry juice bind to P-fimbriae and block their binding to receptors on uroepithelial cells.

Correlating concentration of cranberry products and attachment of bacteria to uroepithelial cells

Exposing bacteria and uroepithelial cells to 64 μg/mL PACs decreased bacterial attachment by 35%. Prior work has reported a bioactivity detection threshold of 60 μg/mL PACs to inhibit the agglutination of P-fimbriated bacteria to human red blood cells.[24] While agglutination tests using human red blood cells offer valuable information, the use of uroepithelial cells as the adhesion target has more significance because these are the cells that are going to be encountered by *E. coli* in the kidneys, better simulating the development of acute pyelonephritis. *In vivo*, the lining of the urinary tract is covered by layers of uroepithelial cells where bacteria would first adhere to the apical side.[53] The ideal *in vitro* conditions that would simulate the *in vivo* multicellular layer structure in the urinary tract would include the controlled orientation of uroepithelial cells by growing them as adherent monolayers. However, this was not possible because bacteria could not be clearly observed and enumerated when they were incubated with flask-attached uroepithelial cells. Several other researchers have used epithelial cells in solution to study the attachment of bacteria.[28,38] This setting does not simulate physiological conditions, but it does help establish the mechanisms by which cranberry products act against bacterial adhesion. In our studies, higher concentrations of PACs resulted in less time to inhibit bacterial attachment.

Exposure of bacteria and uroepithelial cells to a L-CJC concentration of 5% by weight, which contains about 64 μg/mL PACs as previously reported,[24] decreased bacterial attachment to uroepithelial cells by 75%. Although the trends were the same and the overall effects similar, L-CJC

had a slightly better ability to inhibit bacterial attachment compared to isolated PACs. One possible explanation is that some of the other compounds in CJC might interact with PACs. Zeta potentials of *E. coli* HB101 and HB101pDC1 exposed to different concentrations of L-CJC did not show significant changes in surface charge,[54] but bacteria exposed to isolated cranberry PACs showed decreased zeta potentials.

The process of isolating PACs from cranberries may change the chemical properties of the compound, by deactivating it or breaking down some of the molecules, as well as changing the shelf life of the active compound. The isolation of PACs is a difficult process because there is a structural heterogeneity between different monomer units of these cranberry constituents.[55] In raw cranberries, there are at least six different chemical structures of cranberry anthocyanidins that are composed of cyanidins and peonidin aglycons linked to sugars arabinose, glucose, and galactose.[56] The process of extraction of anthocyanidin compounds has been well established,[24,57] but there is a lack of analytical standards to assess the percentage of A versus B type PACs present in cranberries.[57,58] Although it was not possible to determine the exact amount of A type PACs in our sample, we were able to verify through high-performance liquid chromatography that the PACs peak in our sample was not diminished after storing the sample in the refrigerator (in the dark) for over 1 year (data not shown).

The mechanism of action of L-CJC or PACs is associated with an interference of bacterial attachment to uroepithelial cells by modification of surface properties of bacteria. Long-term growth of *E. coli* in cranberry products decreases the adhesion of bacteria to uroepithelial cells, and cranberry can modify the properties of bacterial surfaces because a decrease in adhesion was observed for both fimbriated and nonfimbriated *E. coli*. L-CJC and cranberry PACs may affect small molecules on the surface of *E. coli* HB101, such as LPS, changing the attachment through nonspecific interactions. Finally, the effect of L-CJC and PACs on bacterial adhesion is transient and can be reversed by removal of the cranberry compound from the medium. Future studies are aimed at analyzing the antibacterial activity of urine from volunteers who have consumed different amounts of cranberry juice for different periods of time. These studies will help determine how long the cranberry metabolites found in urine have this anti-adherence effect and what is the minimum amount of cranberry juice that needs to be consumed to have a beneficial effect.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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