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Ratio of "A-type" to "B-type" Proanthocyanidin Interflavan Bonds Affects Extra-intestinal Pathogenic *Escherichia coli* Invasion of Gut Epithelial Cells

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ABSTRACT: Gut colonization by extra-intestinal pathogenic *Escherichia coli* (ExPEC) increases the risk of subsequent infections, including urinary tract infection and septicemia. Previous work suggests that cranberry proanthocyanidins (PAC) interact with bacterial surface factors, altering bacterial interaction with host cells. Methods were developed to determine if ratios of "A-type" to "B-type" interflavan bonds in PAC affect ExPEC agglutination and invasion of enterocytes. In cranberries, 94.5% of PAC contain one or more "A-type" bonds, whereas in apples, 88.3% of PAC contain exclusively "B-type" bonds. Results show that cranberry "A-type" PAC have greater bioactivity than apple "B-type" PAC for increasing ExPEC agglutination and decreasing ExPEC epithelial cell invasion.

KEYWORDS: *cranberry, apple, proanthocyanidins, "A-type" bonds, "B-type" bonds, ExPEC, agglutination, invasion, Caco-2*

INTRODUCTION

Extra-intestinal pathogenic *Escherichia coli* (ExPEC) cause many extra-intestinal infections, including urinary tract infections (UTI), bacteremia, septicemia, and neonatal meningitis. ExPEC have a large economic impact on public health, with a conservatively estimated cost of several billion dollars annually in the United States.[1] ExPEC may be part of the gut microbiota in healthy individuals.[2] However, once they get access to niches outside the gut, they are able to efficiently colonize these niches and cause diseases such as UTI.[2,3]

Previous research indicates that proanthocyanidins (PAC) from cranberries interact with ExPEC surface virulence factors such as P fimbriae that inhibit adhesion and invasion. In vitro studies demonstrate that cranberry PAC,[4] particularly those with higher degree of polymerization (DP), inhibit the adherence of the pathogen to uroepithelial cells.[5−7] Ironically, PAC, especially those with DP >2, are not absorbed into circulation and do not reach the urinary tract.[8] Although this paradox is readily acknowledged, in vitro mechanistic studies continue to explore the effects of PAC on the interaction between pathogens and uroepithelial and kidney cells.[9] The impact of "A-type" PAC on host cell morphology was described using HeLa cells, a cervical cancer cell line,[10] which are not representative of the polarized cells that line the intestines. On the other hand, Caco-2 cells, which were used in the research reported in this paper, form polarized apical and basolateral surfaces that mimic enterocytes. ExPEC virulence factors that are involved in the colonization of uroepithelial cells are also important in the ability of ExPEC to invade enterocytes.[11] Because the gut is the likely origin of *E. coli* that cause UTI[12,13] and PAC do not appear in the urine at sufficiently high concentrations to inhibit *E. coli* adhesion and invasion of uroepithelial cells, we believe a new paradigm is needed to advance the mechanistic and structure−function understanding of how "A-type" PAC affect UTI in vivo by inhibition of transient gut colonization. As part of this paradigm shift, we report on the development of two methods to determine the bioactivity of PAC that are relevant to inhibition of transient gut colonization: ExPEC agglutination and enterocyte cell invasion. We also use a newly developed method for structural elucidation to determine how relative ratios of "A-type" to "B-type" interflavan bonds affect how PAC agglutinate ExPEC and inhibit ExPEC invasion of gut epithelial cells.

MATERIALS AND METHODS

Chemicals and Reagents. Water, methanol, acetone (HPLC grade), sodium carbonate, sodium chloride, Luria broth (LB) Miller, agar, Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (Pen/Strep) mixture (10000 units of each antibiotic per mL), gentamicin sulfate (50 mg/mL), nonessential amino acid (NEAA) solution (100×), GlutaGRO supplement (200 mM L-alanyl-L-glutamine), and Dulbecco's phosphate-buffered saline solution 10× (PBS) with calcium and magnesium (PBS + Ca^{2+}/Mg^{2+} − 0.1 g/L of $CaCl_2$ and $MgCl_2$) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol (200 proof) was obtained from Decon Laboratories Inc. (King of Prussia, PA, USA). Sterilized water, thyamin hydrochloride, 2,5-dihydroxybenzoic acid (DHB), gallic acid, Folin−Ciocalteu reagent, and Triton 100X were purchased from Sigma-

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Aldrich, (St. Louis, MO, USA). Sephadex LH-20 was purchased from GE Healthcare (Uppsala, Sweden). Tryptose and dextrose were obtained from BD (Sparks, MD, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). HyClone Dulbecco's PBS without calcium and magnesium, Hyclone Roswell Park Memorial Institute medium (RPMI-1640), and Hyclone trypsin 0.25% in 0.1% ETDA were obtained from Thermo Scientific (South Logan, UT, USA). Caco-2 cells ATCC HTB-37 were obtained from ATCC (Manassas, VA, USA).

Samples. Cranberry fruits (*Vaccinium macrocarpon* Ait. cv. 'Stevens') were obtained from the United Cranberry Growers Cooperative (Wausau, WI, USA) and apple fruits (*Malus pumila* cv. 'Golden Delicious') were obtained from a privately owned fruit tree in October 2011. The fresh fruits were homogenized to a fine powder by blending with liquid nitrogen. The fruit powders were stored at −80 °C until extraction.

Isolation of Proanthocyanidins. *Extraction Procedure.* The proanthocyanidin extraction procedure was adapted from our previous work.[14,15] Cranberry powder (100 g) was extracted with 70% aqueous acetone (v/v; 400 mL) in an ultrasonic bath for 15 min. The extract was centrifuged at 400*g* at 15 °C for 10 min, and the supernatant was collected. The extraction was repeated two additional times, and the supernatants were combined. After filtration with cellulose paper, acetone was removed by rotary evaporation under vacuum at 35 °C, and the remaining suspension was solubilized in ethanol. The extract was then centrifuged at 13416*g* at 0 °C for 10 min to eliminate insoluble material. The apple extract was produced in the same manner, adding the 70% aqueous acetone (v/v) to the frozen apple powder immediately to prevent long thawing processes, which favor polyphenol oxidase activity.[16] The supernatants were used to isolate proanthocyanidin fractions by chromatography on Sephadex LH-20 as described below.

Proanthocyanidin Isolation. The ethanolic proanthocyanidin extracts were loaded on glass columns (2.5 cm i.d. × 60 cm length, Kontes, Chromaflex) packed with Sephadex LH-20 that was previously swollen and washed in water and equilibrated with ethanol for 45 min at a flow rate of 4 mL/min. Preliminary work had shown that a combination of three solvents was sufficient to achieve a proanthocyanidin fraction that was devoid of other classes of polyphenols. The resin bed was consecutively eluted with ethanol, ethanol/methanol (1:1), and 80% aqueous acetone (v/v). The 80% aqueous acetone (v/v) fraction that contained PAC was evaporated as described above and solubilized in methanol.

Proanthocyanidin Characterization. The proanthocyanidin content of the cranberry and apple fractions was quantified by using the Folin−Ciocalteu method,[17] and proanthocyanidin concentrations are expressed as gallic acid equivalents (GAE). Previously developed matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) methods were applied to characterize the cranberry and apple PAC.[18] An aliquot of each sample was evaporated to dryness and suspended in 20 μL of ethanol to a final concentration of 20 μg GAE/mL. An aliquot (0.5 μL) of each sample was then mixed with 1 μL of DHB (50.0 mg/mL in ethanol) in three different wells on the stainless steel MALDI target. Mass spectra were collected on a Bruker ULTRAFLEX-III MALDI-TOF/TOF mass spectrometer (Billerica, MA, USA). All analyses were performed in positive reflectron mode. Spectra were the sum of different locations in each well, accumulating a total of 500 shots to minimize intrawell variability, and deflection was set at 800 Da. After analysis, the deconvolution method based on the relative intensity of proanthocyanidin isotope patterns was used to calculate the ratios of "A-type" to "B-type" bonds in cranberry and apple PAC.[18]

Bacterial Culture. P fimbriae expression is an important virulence factor for ExPEC colonization of the gut[19] and is critical for attachment of *E. coli* to epithelial cells in the urinary tract, colon, and ileum.[20,21] The strain of ExPEC (strain 5011) used in this study was isolated from a woman with recurrent UTI at the University of Wisconsin Hospital and Clinics. The selected strain was found in both the gastrointestinal and urinary tracts and expresses fimH and papA, the adhesins of type 1 and P fimbriae, respectively.

Strain 5011 was cultured from frozen stock under static culture conditions in tryptose broth (10 g of tryptose, 2.5 g of sodium chloride, 0.5 g of dextrose, and 0.0025 g of thyamin hydrochloride in 500 mL of deionized water) at 37 °C and washed twice with 1× PBS + Ca^{2+}/Mg^{2+} by centrifugation at 1840*g* for 10 min. The optical density of the inoculum suspension at 450 nm was used to calculate and adjust the bacterial cell density using a previously established bacterial density−absorbance curve.

Bacterial Agglutination Assay. The agglutination assay was conducted in 3.0 mL microcuvettes using 1.0 mL total reaction volume. First, ExPEC stock solutions (50 μL) were added to the cuvettes, resulting in a final ExPEC concentration of 5.0×10^8 colony forming units (CFU)/mL. Next, proanthocyanidin solutions of either apple (13.6 mg GAE/mL) or cranberry (121.2 mg GAE/mL) were diluted 1/10 and 1/100, respectively, and added to each bacterial inoculum to reach final concentrations of 0, 5, 10, 25, 50, and 100 μg GAE/mL in a total volume of 1 mL with 1× PBS + Ca^{2+}/Mg^{2+}. The cuvettes were sealed and shaken vigorously for 20 s. Absorbances were read at 450 nm every 5 min for 300 min on a Beckman DU 640 spectrophotometer equipped with a six-position cuvette holder (Schaumburg, IL, USA), and the absolute values were converted to transmittance (%). The area under the transmittance curve (AUC) for each concentration level was calculated as a function of the ability of PAC to agglutinate ExPEC from 0 to 300 min.

Gut Epithelial Cell (Enterocyte) Culture. Caco-2 (HTB-37) cells were cultured in DMEM supplemented with 10% FBS (v/v), 1% nonessential amino acids (v/v), 1% L-alanyl-L-glutamine (v/v), and 1% penicillin/streptomycin (v/v) at 37 °C with 5% CO_2. For use in experiments, cells at approximately 80% confluence, based on surface area coverage, were split 1:1.6 into 24-well cell culture treated plates. Once the cells reached confluence (approximately 8.0×10^5 cells/well), they were allowed to differentiate for 8−10 days to become phenotypic polarized enterocytes with medium changes every other day. For maintenance purposes, stock cells were passaged biweekly using 0.25% trypsin.

Invasion Assay. Bacteria were cultured as described previously in this section and were added to 5 mL tubes to reach a multiplicity of infection of 100 (8.0×10^7 CFU/well) relative to the number of Caco-2 cells (8.0×10^5 cells/well). 1× PBS + Ca^{2+}/Mg^{2+} and proanthocyanidin treatments (0, 5, 10, 25, 50, and 100 μg GAE/mL) were added to a total volume of 75 μL, gently mixed, and then incubated for 10 min. RPMI medium, supplemented with 5% FBS (v/v), was added to bring the final volume to 2.5 mL and incubated for 5 min. Caco-2 cell plates were washed twice with 1× PBS + Ca^{2+}/Mg^{2+}, and 0.5 mL of each treatment was added to the plate in quadruplicate. After 1 h at 37 °C to allow for bacterial invasion, Caco-2 cells were washed twice with 1× PBS + Ca^{2+}/Mg^{2+}, and extracellular bacteria were killed by incubating 1 mL of RMPI medium supplemented with 5% FBS (v/v) and gentamicin at a final concentration of 100 μg/mL for 1 h. Caco-2 cells were washed twice with PBS (without Ca^{2+} and Mg^{2+}) and lysed with 1 mL/well Triton 1X for 30 min on a shaker. After intracellular bacteria were released from Caco-2 cells, each well content was homogenized via pipetting, vortexed, and diluted 1:10 in PBS without Ca^{2+} and Mg^{2+}, and 100 μL was plated in duplicate on LB plates (25 g LB Miller, 15 g of agar in 1 L of deionized water). All plates were incubated for 18 h at 37 °C, and the number of CFU/plate was counted. The results were expressed in percentage of enterocyte invasion using the following formula: (CFU sample/CFU control) × 100.

Data and Statistical Analysis. All data were reported as the mean ± standard deviation of at least three replicates. mMass version 3.9.0[22] was used for mass spectra analysis, and SAS was used for AUC calculation using the trapezoidal method (version 9.3; SAS Institute Inc., Cary, NC, USA). Statistical analysis was done using JMP Pro (version 10.0.0; SAS Institute Inc.), setting $\alpha = 0.05$. Results were analyzed with two-way ANOVA models with interaction between the independent variables "sample" and "concentration" to assess significant differences. The dependent variables were AUC and $\arcsin[\sqrt{(\%\ \text{invasion}/100)}]$ for the agglutination and invasion assays, respectively.

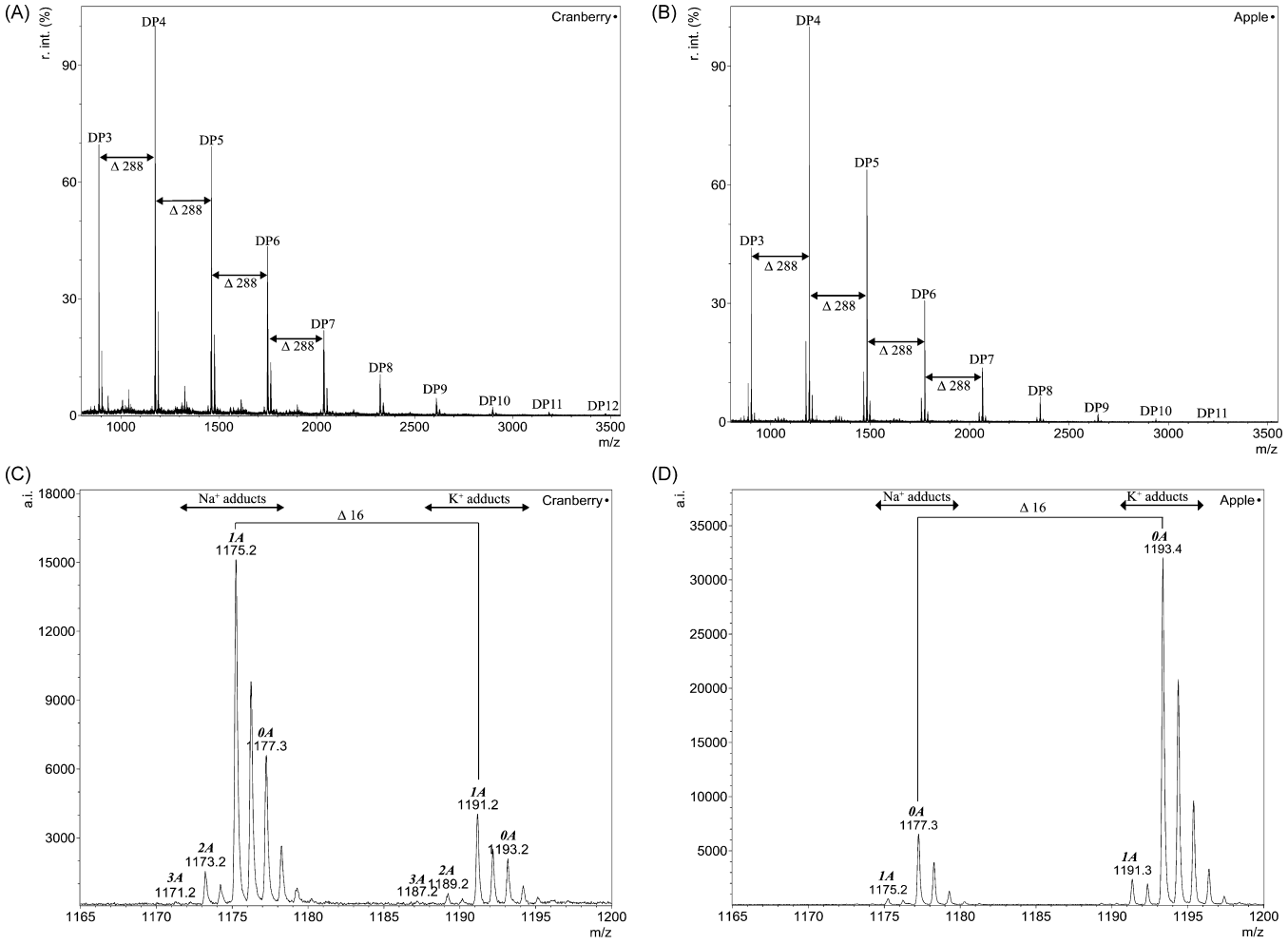

Figure 1. (A, B) Mass spectra obtained with MALDI-TOF MS in positive reflectron mode for cranberry (A) and apple (B) proanthocyanidins (PAC). PAC with different degrees of polymerization (DP) have a 288 mass difference. "r.i." corresponds to relative intensity. (C, D) Mass spectra obtained with MALDI-TOF MS in positive reflectron mode for cranberry (C) and apple (D) proanthocyanidins (PAC) tetramers displaying sodium and potassium adducts showing a 16 units mass difference. The number of "A-type" bonds is assigned to each peak. "a.i." corresponds to absolute intensity.

RESULTS

MALDI-TOF MS Characterization of PAC. Chromatography on Sephadex LH-20 produces fractions from cranberries and apples that contain only PAC.[15] Analysis by MALDI-TOF MS showed that the predominant mass at each DP (Δ288 amu) for cranberry (Figure 1A) and apple (Figure 1B) samples are representative of proanthocyanidin structures that consist of multiple linked catechin/epicatechin units with predominantly either "A-type" or "B-type" bonds for cranberries and apples, respectively. These observed masses and mass distribution are consistent with previously described proanthocyanidin composition of cranberry[23] and apple.[27] Mass spectral analysis indicated that cranberries contain an oligomeric distribution of PAC with a DP from 3 (trimer) to 12 (dodecamer) (Figure 1A) and that apples contain a distribution of PAC with DP from 3 (trimer) to 11 (undecamer) (Figure 1B).

PAC associate with sodium $[M + Na]^+$ and potassium $[M + K]^+$ forming alkali metal adducts,[24] thereby splitting the signal unevenly, leading to Δ16 amu between $[M + Na]^+$ and $[M + K]^+$ in both cranberry (Figure 1C) and apple PAC (Figure 1D). Cranberry PAC predominantly ionized as sodium adducts (Figure 1C), whereas apple PAC predominantly ionized as potassium adducts (Figure 1D). In cranberry PAC, the MALDI-TOF MS spectrum shows that the most predominant linkage type corresponds to 1 "A-type" but peaks corresponding to 2 and 3 "A-type" bonds have enough intensity to resolve above baseline noise (Figure 1C). For each additional "A-type" bond there is a 2 amu decrease due to the loss of two hydrogen atoms that are involved in forming the ether bond typical of this type of interflavan linkage (Figure 2). For example, in tetramers the sodium adduct series shows four distinct peaks: m/z 1177.3 (0 "A-type" and 3 "B-type" bonds), m/z 1175.2 (1 "A-type" and 2 "B-type" bonds), m/z 1173.2 (2 "A-type" and 2 "B-type" bonds), and m/z 1171.2 (3 "A-type" and 0 "B-type" bonds) (Figure 1C). The intermediary peaks that are present between each DP in cranberry PAC correspond to anthocyanins that are covalently bonded to PAC and have been previously described.[15]

In spectra from apple PAC, the peaks with the highest absolute intensity correspond to 0 "A-type" bonds. For example, in tetramers the sodium adduct series shows only two distinct peaks: a predominant peak at m/z 1177.3 (0 "A-type" and 3 "B-type" bonds) and a peak at m/z 1175.2 (1 "A-type" and 2 "B-type" bonds) with only 6% of the intensity of the all "B-type" tetramer (Figure 1D).

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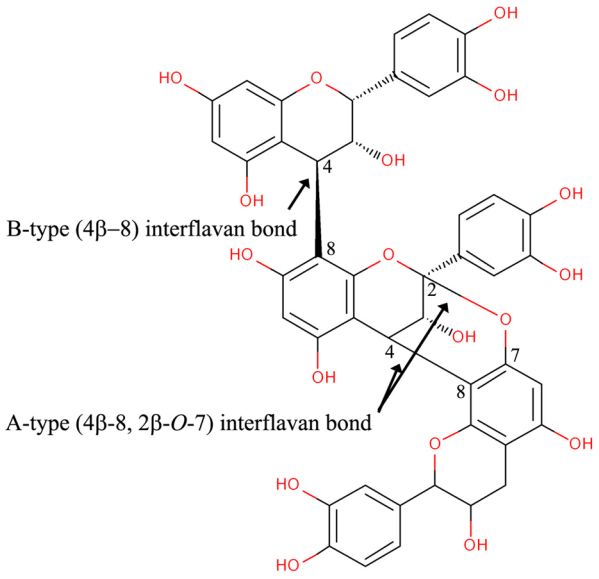

Figure 2. Representative structure of a cranberry proanthocyanidin trimer (epicatechin-(4β-8)-epicatechin-(4β-8, 2β-*O*-7)-epicatechin).[39] Variation in degree of polymerization, position, and number of "A-type" versus "B-type" interflavan bonds leads to large structural heterogeneity among cranberry PAC.[23,29]

When our deconvolution method[18] was applied to the MALDI-TOF MS spectra, median values showed that 94.5% of cranberry PAC were "A-type" and 88.3% of apple PAC were "B-type" (Table 1). Our results indicate that apple PAC contain

Table 1. Percentage of Proanthocyanidins (PAC) with at Least One or Two "A-type" Bonds per Degree of Polymerization (DP) in Cranberry and Apple[a]

	PAC with at least one "A-type" bond		PAC with at least two "A-type" bonds	
DP	apple	cranberry	apple	cranberry
3	3.1 ± 5.4	93.5 ± 2.4	0.0 ± 0.0	1.7 ± 0.7
4	10.1 ± 1.9	88.0 ± 3.2	0.0 ± 0.0	5.4 ± 0.7
5	12.3 ± 1.4	90.1 ± 1.8	1.7 ± 1.3	13.1 ± 3.3
6	12.1 ± 1.9	95.4 ± 1.8	0.7 ± 0.7	39.0 ± 3.5
7	11.3 ± 2.9	99.6 ± 0.7	0.0 ± 0.0	51.7 ± 1.9
8	12.4 ± 3.4	97.4 ± 4.4	0.0 ± 0.0	60.1 ± 0.6
median	11.7	94.5	0.0	26.1

[a]The results are expressed as the average of three independent replicates ± standard deviation.

a median value of 11.7% "A-type" PAC as determined by predictive equations.[25] Furthermore, median values showed that 26.1% of cranberry PAC had 2 or more "A-type" interflavan bonds, whereas none of the apple PAC had 2 or more "A-type" bonds (Table 1). However, mass spectrometry alone does not provide sufficient information for the structural elucidation of "A-type" PAC. To our knowledge, the confirmation of "A-type" interflavan bonds in apple PAC has not yet been described. Thus, whereas our interpretation of the data is that the detected masses represent "A-type" PAC in apples, reactions resulting from polyphenol oxidase activity may also alter PAC oligomer structure, resulting in modifications that involve the loss of two hydrogen molecules. This observation has also been reported for MALDI-TOF MS analysis of apple juices.[26]

The absence of PAC "A-type" to "B-type" interflavan linkage ratios for proanthocyanidin oligomers greater than an octamer (DP >8) is a result of the MALDI-TOF MS instrument parameters. The nature of the MALDI-TOF MS analysis requires the sample to be mixed with a matrix (DHB) to afford ionization and subsequent detection of charged compounds. This process produces numerous low molecular weight (<800 Da) matrix adducts resulting in background noise (unwanted signals). To improve the quality of the PAC spectra and allow for quantification of relative "A-type" to "B-type" interflavan bonds, the MALDI-TOF MS instrument parameters are set to gate (deflect) masses below a 800 Da threshold. As a result of this gating process, dimers (m/z 599 and 601) are also deflected and not detected. In the case of proanthocyanidin oligomers with DP >8, spectra are excluded from analysis when one or more of the peaks included in the deconvolution of isotope patterns have signal-to-noise ratios of <3.0.[18] On the basis of these predefined criteria, the MALDI-TOF MS instrument parameters are adjusted to provide high-resolution spectra for PAC trimers through octamers (DP 3–8). These parameters allow the relative proportions of "A-type" to "B-type" interflavan bonds for 23 individual proanthocyanidin masses to be compared across samples.

Bacterial Agglutination Assay. In our study design, we compared "A-type" to "B-type" PAC for their ability to increase ExPEC agglutination. We monitored the increase in transmittance of light (450 nm) that results when PAC interact with ExPEC, causing agglutination and precipitation from suspension. To correct for baseline transmittance, all values were normalized to zero at the beginning of the experiment. The AUC for each concentration level was calculated as a function of the ability of PAC to agglutinate ExPEC. Results showed that both "A-type" and "B-type" PAC significantly increased agglutination in a dose-dependent manner when compared to control. Figure 3 shows an example of an agglutination experiment using the "B-type" PAC from apples. Bacterial agglutination was 77, 58, 28, 27, and 32% higher for 5, 10, 25, 50, and 100 μg GAE/mL, respectively, when "A-type" were compared to "B-type" PAC. "A-type" PAC were significantly more effective (p values < 0.05) at increasing agglutination

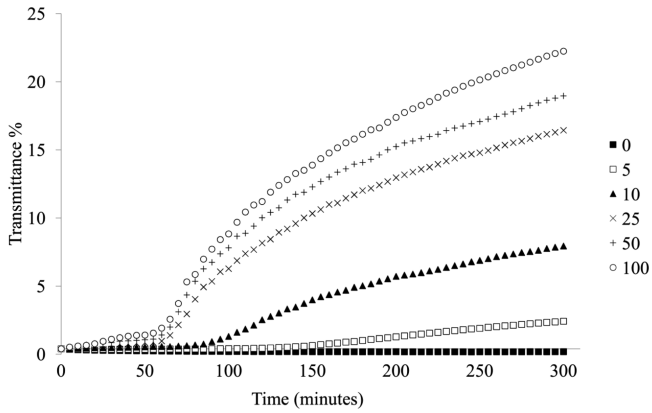

Figure 3. Relationship between transmittance (%) at 450 nm and time over 300 min of the agglutination assay with ExPEC in PBS and six levels of apple proanthocyanidins concentration (0, 5, 10, 25, 50, and 100 μg GAE/mL). The results represent the average of three independent replicates.

when compared to "B-type" PAC at all concentrations, except for the lowest concentration (Figure 4A).

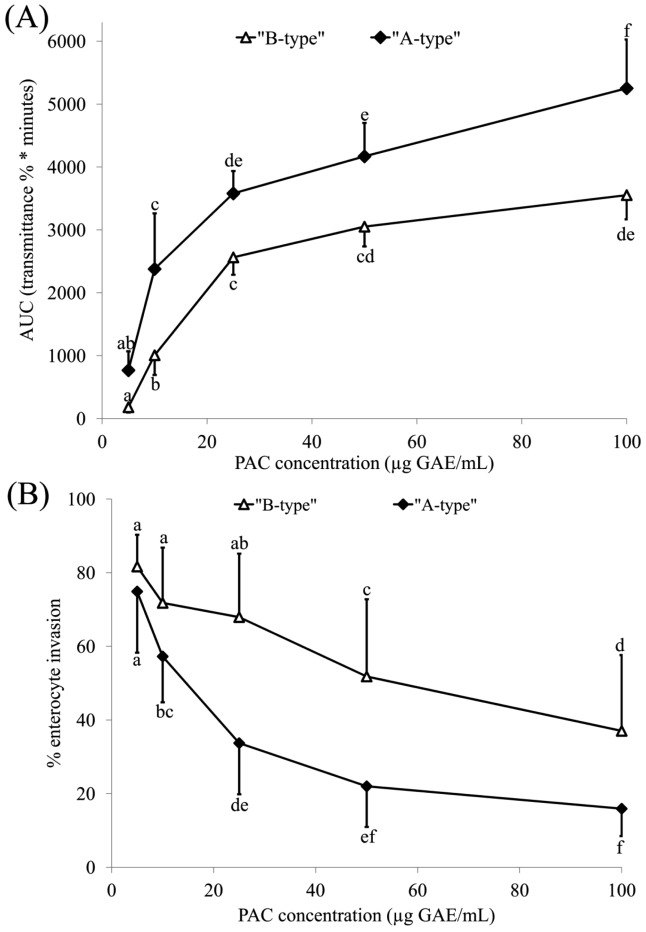

Figure 4. (A) Area under the curve obtained for the agglutination assay of *E. coli* 5011 for increasing concentrations of apple and cranberry PAC, expressed in μg GAE/mL. The results are expressed as the average of three independent assays ± standard deviation. Different letters correspond to significant differences at $p < 0.05$. (B) Dose−response curves on the effect of PAC extracted from apples and cranberries on the ability of *E. coli* to invade Caco-2 cells, expressed in μg GAE/mL, as quantified by the Folin−Ciocalteu method. Different letters correspond to significant differences at $p < 0.05$.

Invasion Assay. In the current study, we compared the ability of "A-type" and "B-type" PAC to inhibit invasion of gut epithelial cells by ExPEC. The results showed that both "A-type" and "B-type" PAC were able to significantly inhibit ExPEC invasion of Caco-2 cells in a dose-dependent manner (Figure 4B). However, "A-type" PAC produced significantly greater inhibition in comparison to "B-type" PAC. ExPEC invasion was reduced by 7, 15, 34, 30, and 21% for 5, 10, 25, 50, and 100 μg GAE/mL, respectively, when "A-type" were compared to "B-type" PAC.

The shape of the dose−response curve indicated that for "A-type" PAC, a plateau is attained at concentrations of 50 μg GAE/mL (Figure 4B). The curve for "B-type" PAC did not plateau, suggesting that this class of PAC required a higher concentration to reach a level of inhibition similar to that of "A-type" PAC.

The results of the two-way ANOVA model showed that the greater inhibitory effect of "A-type" PAC was statistically significant except for the lowest concentration (Figure 4B). Because every assay was run on a different day, we tested the effect of day within PAC treatment, and no significant differences of the dose−response curves were found.

DISCUSSION

MALDI-TOF MS Characterization of PAC. The goal of this work was to compare the effect of "A-type" with "B-type" PAC on ExPEC agglutination and invasion of gut epithelial cells. To determine the difference between "A-type" and "B-type" PAC we chose apples because this fruit contains only "B-type" PAC,[27] with catechin/epicatechin monomers, which yielded a MALDI-TOF MS spectrum with excellent baseline resolution.

PAC are oligomers of flavan-3-ols that have variable chain lengths.[23] There are two common series of procyanidin dimers. The "B-type" series are dimers linked in either the C4−C6 or C4−C8 position,[28] whereas the "A-type" series are dimers linked in the C4−C8 position with an additional C2−*O*−C7 ether linkage (Figure 2). PAC oligomers with a DP >2 may contain both "A-type" and "B-type" interflavan bonds. Therefore, for the purpose of discussing differences between PAC oligomers from cranberries and apples, cranberry PAC that contain one or more "A-type" interflavan bonds in their structure are referred to as "A-type" PAC, whereas apple PAC that contain only "B-type" interflavan bonds are referred to as "B-type" PAC.[28]

Analytical methods such as MALDI-TOF MS are routinely used to characterize unique structural features of PAC.[23−26,28−32] An understanding of the natural abundance of C, H, and O isotopes within proanthocyanidin oligomers allowed us to develop a novel MALDI-TOF-MS isotope deconvolution method.[18] We were able to quantify ratios of "A-type" to "B-type" interflavan bonds on the basis of theoretical isotope distributions by applying matrix algebra to the experimental spectra. In the context of this publication, MALDI-TOF MS methods were applied to provide both quantitative and qualitative assessment of proanthocyanidin structures.

Cranberry fruit (*Vaccinium macrocarpon* Ait.) has previously been shown to contain PAC that predominately include one or more "A-type" interflavan bonds.[23,33] Apple fruit (*Malus pumila* cv. 'Fuji') conversely has been shown to contain PAC that predominately contain all "B-type" interflavan bonds.[27]

Agglutination and Inhibition of Enterocyte Invasion. Bacterial agglutination, a bacterium-to-bacterium interaction, corresponds to an increase in concentration of bacteria in a limited area of the human body. This phenomenon can inhibit bacterial growth, cause structural damage to bacteria,[34] and increase bacterial clearance by macrophages and other phagocytic cells, potentially leading to a decrease in enteroctye invasion by ExPEC. We developed an agglutination assay to compare cranberry "A-type" PAC to apple "B-type" PAC. This assay is fundamentally different from the antiadherence assay that evaluates the ability of bacteria to adhere to uroepithelial cells based on the similarity of receptors on the uroepithelium with P blood group antigens.[26] Cranberry PAC inhibit this specific adhesion at low concentrations. Our new method allowed us to measure the effect of PAC with different structural characteristics on ExPEC agglutination over time and at low concentrations and demonstrated that cranberry "A-

type" PAC have greater agglutination activity than apple "B-type" PAC.

The results suggest that the direct effect of PAC on ExPEC agglutination is related to the specific inhibition in the red blood cell antiadherence assay because "A-type" PAC appear to have greater activity than "B-type" PAC. The ExPEC strain used in our agglutination study expresses both type 1 and P fimbriae. The red blood cell antiadhesion assay is based on the specific interaction of P-fimbriated *E. coli* with the Gal-Gal receptor on red blood cells that express the P blood group antigens.[26] Cranberry "A-type" PAC inhibit the adhesion of P-fimbriated *E. coli* to these red blood cells and thus prevent agglutination. The "B-type" PAC do not have activity in this assay. In contrast, both types of PAC have bioactivity in our agglutination assay, although cranberry "A-type" PAC showed greater activity than apple "B-type" PAC. Because strain 5011 expresses both type 1 and P fimbriae along with several other virulence factors, our results suggest that interactions of PAC with ExPEC surface virulence factors that lead to agglutination may result from specific binding of "A-type" PAC to P fimbriae and nonspecific binding to other proteins on the bacterial surface that can be mediated by both "A-type" and "B-type" PAC.

In vivo, bacterial invasion of gut epithelial cells provides a mechanism by which pathogens are protected from complements, antibodies, and other immune defense molecules, which in turn allows the pathogens to colonize and persist in the gut.[35,36] We developed an in vitro cell culture assay of bacterial invasion to assess the ability of compounds and immune factors to interfere with this mechanism of gut colonization. Proanthocyanidin concentrations used in this work are physiologically relevant because these concentrations in the gut are obtainable after the ingestion of a single serving of cranberry juice, which contains approximately 36 mg of PAC. If there was instantaneous mixing in the gut, then PAC concentration would be 7.2 μg/mL, assuming that gut volume is approximately 5000 mL (36 mg/5000 mL = 7.2 μg/mL). However, instantaneous mixing is impossible and PAC are poorly or not absorbed. Therefore, the concentration of PAC in the chyme would be much higher following ingestion and passage.

In summary, the results indicate that exposure of ExPEC to PAC consisting of either "A-type" or "B-type" interflavan linkages significantly produces bacterial agglutination and inhibits invasion of gut epithelial cells in a physiologically relevant dose-dependent manner. "A-type" PAC were observed to be significantly more effective in causing agglutination and reducing invasion when compared to "B-type" PAC at equivalent concentrations. Furthermore, it is known that the exposure of "A-type" PAC from cranberries to *E. coli* CFT073, a highly virulent uropathogenic strain, down-regulates *fliC* gene expression, which is responsible for flagellin expression, the major protein in bacterial flagellum, reducing bacterial motility[37] and compromising adherence to enterocytes. Our results suggest that "A-type" PAC act in a manner similar to opsonization of pathogens by secretory immunoglobulin A, which may also agglutinate bacteria, inhibit invasion of enterocytes, increase intestinal clearance, and promote killing by immune cells.[38]

This work provides a new insight on the specificity of the interaction PAC interflavan bond type with bacterial virulence factors highlighting the importance of this area of research in the context of understanding the mechanism by which "A-type" PAC prevent UTI by inhibiting gut colonization by ExPEC. This new understanding of proanthocyanidin structure/bioactivity relationships can be applied to develop a clinically relevant hypothesis of how proanthocyanidin-containing fruits, juices, botanicals, and dietary supplements can be used to develop therapeutic strategies against ExPEC.

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Notes

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There were errors in Figures 3 and 4, and the first paragraph of the Bacterial Agglutination Assay section of the version of this paper published November 18, 2013. The correct version published February 21, 2014.