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Reduction of *Escherichia coli* adherence to uroepithelial bladder cells after consumption of cranberry juice: a double-blind randomized placebo-controlled cross-over trial

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Abstract To determine the efficacy of the consumption of cranberry juice versus placebo with regard to the presence of in vitro bacterial anti-adherence activity in the urine of healthy volunteers. Twenty healthy volunteers, 10 men and 10 women, were included. The study was a double-blind, randomized, placebo-controlled, and cross-over study. In addition to normal diet, each volunteer received at dinner a single dose of 750 ml of a total drink composed of: (1) 250 ml of the placebo and 500 ml of mineral water, or (2) 750 ml of the placebo, or (3) 250 ml of the cranberry juice and 500 ml of mineral water, or (4) 750 ml of the cranberry juice. Each volunteer took the four regimens successively in a randomly order, with a washout period of at least 6 days between every change in regimen. The first urine of the morning following cranberry or placebo consumption was collected and used to support bacterial growth. Six uropathogenic *Escherichia coli* strains (all expressing type 1 pili; three positive for the gene marker for P-fimbriae *papC* and three negative for *papC*), previously isolated from patients with symptomatic urinary tract infections, were grown in urine samples and tested for their ability to adhere to the T24 bladder cell line in vitro. There were

no significant differences in the pH or specific gravity between the urine samples collected after cranberry or placebo consumption. We observed a dose dependent significant decrease in bacterial adherence associated with cranberry consumption. Adherence inhibition was observed independently from the presence of genes encoding type P pili and antibiotic resistance phenotypes. Cranberry juice consumption provides significant anti-adherence activity against different *E. coli* uropathogenic strains in the urine compared with placebo.

Keywords Urinary tract infection · Cranberry · *Escherichia coli* · Adherence · Colonization · Pili · Trial

Introduction

Urinary tract infections (UTIs) are common community-acquired and nosocomial diseases. *Escherichia coli* is by far the most predominant pathogen causing UTI: *E. coli* causes 85–95% of the episodes of uncomplicated cystitis and over 90% of the cases of uncomplicated pyelonephritis in premenopausal women [1]. Several virulence factors enable *E. coli* cells to colonize selectively the mucosal uroepithelium, evoke an inflammatory reaction and eventually proceed from the lower urinary tract to the renal cavities and tissues [2]. Amongst virulence factors, colonization factors, termed adhesins, are essential in the infectious process. *E. coli* adhesins, mainly fimbrial in nature, promote bacterial adhesion and growth on the surface of the urinary epithelium. Virtually all uropathogenic *E. coli* (UPEC) isolates are capable of expressing a mannose-specific lectin associated with type 1 fimbriae, which mediates the adherence of the bacteria to uroepithelial cells [2]. There is no difference in the presence of type 1 pili at the bacterial surface between isolates causing cystitis or pyelonephritis [3]. In addition to type 1 fimbriae, 23% of cystitis and most pyelonephritis isolates of *E. coli* express an α -Gal(1–4) β -Gal-specific lectin associated with P-fimbriae, which also mediates the adherence of the

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bacteria to uroepithelial cells [4]. There is experimental and clinical evidence for the pathogenic role of P-fimbriae and type 1 pili of *E. coli* strains in both persistent bladder colonization and recruitment of inflammatory response [5, 6].

The consumption of antibiotics is directly correlated to resistance in uropathogens emphasizing the importance of controlling antibiotic usage and developing alternative preventive and curative treatments for UTIs [7]. Dietary consumption of cranberries (*Vaccinium macrocarpon*, *V. m.*) has long been associated with the maintenance of urinary tract health. A number of clinical studies have demonstrated a positive link between cranberry (*V. m.*) consumption and the prevention of UTIs [8, 9]. Cranberry juice is thought to affect the adhesion of the UPEC to uroepithelial cells at least in part by interfering with specific receptor-ligand modes of bacterial adhesion. Cranberry juice (*V. m.*) and cranberry juice constituents (i.e. proanthocyanidins and fructose) have been shown to inhibit pili mediated adhesion to uroepithelial cells in vitro [10]. Fructose, a constituent of many fruit juices, has been implicated in inhibiting type 1 pili mediated adherence. Proanthocyanidins, specifically present in cranberries, have been demonstrated to inhibit type P pili mediated adherence. Anti-adherence activities of proanthocyanidins have also been demonstrated by human erythrocyte and P-receptor resin-coated bead agglutination suppression when incubated with P-fimbriated *E. coli* [11]. The effects of the consumption of cranberry juice on *E. coli* adherence to uroepithelial cells after bacterial growth in corresponding urine samples have not yet been documented. This is an important trait since during cystitis, UPEC are constantly in contact with urine in the bladder, and *V. macrocarpon*, the American cranberry, has been shown to inhibit the expression of P-fimbriae of *E. coli* [12]. Habash et al. [13] have demonstrated that the urine obtained after cranberry supplementation reduced the initial deposition rates and numbers of adherent *E. coli* to silicone rubber in a parallel plate flow chamber. This study included only 10 healthy male volunteers and one UPEC strain, the fimbriae harbored by the bacterial strain were unknown, *E. coli* adherence was not tested onto human uroepithelial cells, and this was not a placebo-controlled trial.

The present study was designed as a randomized, double-blind, placebo-controlled trial to test for the presence of an inhibitory activity onto *E. coli* adherence to human uroepithelial cells in the urine of healthy subjects after consumption of cranberry juice (*V. m.*).

Subjects and methods

Twenty healthy volunteers, 10 women and 10 men, ranging in age from 21 to 25 years, from the student population of the University of Cergy-Pontoise, France, were included. The study was a double-blind, randomized, placebo-controlled, and cross-over study.

Exclusion criteria included antibiotic treatment 2 weeks prior to and all along the study and pregnancy. All volunteers were asked to sign their informed consent. During all the trials, the volunteers maintained their usual unrestricted diet.

The double-blind study was carried out on the volunteers using a pasteurized low calorie juice of the *V. macrocarpon* cranberry, and placebo beverage. The cranberry juice is similar to the product consumers can purchase in grocery stores. Ingredients include filtered water, cranberry juice concentrate (27%), sucrose, aspartame, and ascorbic acid. The placebo beverage is an isocaloric formulation that mimics the flavor and color of the cranberry beverage. Fructose is present in the two beverages at similar concentrations (1.1 g and 1.4 g per 250 ml in the cranberry and placebo beverages, respectively). There are no cranberry ingredients or proanthocyanidins in the placebo beverage. Both beverage formulations are the proprietary formulas of Ocean Spray Cranberries, Inc., USA. Table 1 provides compositional information for the cranberry drink and placebo beverage. They are provided on a 250 ml basis, since the study was conducted using 250 ml cartons of the beverages.

In addition to normal diet, each volunteer received at dinner a single dose of 750 ml of a total drink composed of: (1) 250 ml of the placebo and 500 ml of mineral water, or (2) 750 ml of the placebo, or (3) 250 ml of the cranberry juice and 500 ml of mineral water, or (4) 750 ml of the cranberry juice. Ten to twelve hours after cranberry or placebo consumption, the first urines of the morning were collected. Different biological and physical-chemical parameters of the urine samples were checked with the Multistix 8 SG system (Bayer Diagnostics, Bridgend, UK). Urine samples were centrifuged at 4,000g for 15 min, and were sterilized by filtration (0.45 µm). After sterilization, the urine samples were stored at -20°C. Each volunteer received the four regimens successively in a random order, with a washout period of at least 6 days between every change in regimen. During these washout periods, the volunteers did not receive any cranberry juice.

The properties of the six UPEC strains are presented in Table 2. These strains have been previously isolated from patients with symptomatic UTIs at the hospital

Table 1 Compositional information for the cranberry drink and placebo beverage

	Cranberry juice	Placebo
Calories (Kcal)	41.6	42.7
Total carbohydrates (g)	10.4	10.7
Ascorbic acid (mg)	63	63
Total organic acids (g)	1.8	1.6
Total phenolics (mg)	179	161
Total anthocyanidins (mg)	9.3	42
Proanthocyanidins (mg)	40	ND

The compositional information is provided on a 250 ml basis
ND none detected

Table 2 Properties of the *E. coli* uropathogenic strains

Strains	Mannose sensitive adherence	<i>papC</i>	Antibiotic resistance
G1473	+	+	None
G1487	+	+	SSS, SXT, TE, C
G1722	+	+	AMX, AMC, TIC, TCC, PIP, CF, TE
G1926	+	–	AMX, AMC, TIC, TCC, PIP, CF, FOX, SSS, SXT, TE, C
G4117	+	–	None
G4790	+	–	TE

The presence of the gene marker for P-fimbriae *papC* was determined by PCR amplification experiments. The expression of type 1 pili was checked by the inhibition of bacterial adherence to T24 bladder cells in the presence of 2% D-mannose. Antibiotic resistance phenotypes were checked by agar disk diffusion susceptibility tests. *AMX* amoxicillin; *AMC* amoxicillin-clavulanic acid; *TIC* ticarcillin; *TCC* ticarcillin-clavulanic acid; *PIP* piperacillin; *CF* cefalothin; *FOX* cefoxitin; *SSS* sulfonamides; *SXT* trimethoprim-sulfamethoxazole; *TE* tetracycline; *C* chloramphenicol

Raymond Poincaré, Garches, France. The presence of the gene marker for P-fimbriae *papC* was established by using the PCR conditions described by Le Bouguenec et al. [14]. The expression of type 1 pili was checked by inhibition of the bacterial adherence to T24 bladder cells in the presence of 2% D-mannose. Agar disk diffusion susceptibility tests were performed on Mueller–Hinton agar with the disks purchased from Diagnostics Pasteur, France. The following antibiotics were tested and resistance was measured as zone diameters according to the NCCLS system [15]: amoxicillin, amoxicillin-clavulanic acid, ticarcillin, ticarcillin-clavulanic acid, piperacillin, piperacillin-tazobactam, cefoxitin, cefalothin, moxalactam, cefotaxime, ceftazidime, aztreonam, imipenem, kanamycin, gentamicin, tobramycin, netilmycin, amikacin, pefloxacin, ofloxacin, ciprofloxacin, sulphonamide, trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, colistine, fosfomycine (+25 mg/l Glucose-6-Phosphate).

Bacterial adherence experiments were carried out with the human T24 epithelial cell line (ATCC HTB-4) as previously described [16]. T24 cells are urinary bladder epithelial cells previously shown to be an efficient model to study UPEC strains adherence in vitro [17]. Monolayers of epithelial cells were grown at 37°C in McCoy's 5a medium containing 10% (v/v) fetal calf serum, 1.5 mM glutamine, and antibiotics (200 U of penicillin and 50 mg of streptomycin per liter, respectively), on 24-wells Falcon tissue culture plates (Becton Dickinson Labware, Oxnard, California). Before the adhesion tests, the cells were washed with phosphate-buffered saline (PBS; pH 7.2). Bacteria were grown for 36 h in human urine containing 5% (v/v) LB. Bacterial cells were harvested by centrifugation, and were resuspended at 10⁸ bacteria per ml in McCoy's medium, added to the tissue culture and incubated for 3 h at 37°C. After five washes with PBS, the cells were fixed in ethanol, stained with 20% Giemsa (v/v), and examined

microscopically under oil immersion. An adhesion index representing the average number of bacteria per cell was determined by examining 100 cells. Each adhesion result represents the mean of at least three independent experiments.

The statistical significance of differences was evaluated with the equal-variance Student's *t* test, following the variance test with Fisher *F* statistics. *P* values below 0.05 were considered significant.

Results

The pH and specific gravity of the urine samples collected after the consumption of the four different beverage regimens were not statistically different (Table 3). The means for the six UPEC strains of the adherence indices obtained with bacteria grown in urine samples collected after the consumption of different beverage regimens are presented in Table 4. The higher adherence index was obtained with bacteria grown in urine samples collected after drinking 250 ml of placebo. Cranberry juice intake caused a very highly significant reduction in bacterial adherence to T24 cells compared to equivalent volumes of placebo (*P* < 0.001). There was a dose dependent decrease in bacterial adherence with the

Table 3 pH and specific gravity variations of the urine samples between the different regimens, means for the 20 subjects

Regimens	pH ± SD	SG ± SD
250 ml placebo +		
500 ml mineral water	5.63 ± 0.74	1.021 ± 0.007
750 ml placebo	5.42 ± 0.56	1.022 ± 0.005
250 ml cranberry +		
500 ml mineral water	5.42 ± 0.73	1.021 ± 0.007
750 ml cranberry	5.43 ± 0.61	1.022 ± 0.005

pH and specific gravity (SG) of the urine samples were checked with the Multistix 8 SG system (Bayer Diagnostics, Bridgend, UK) *SD* standard deviation

Table 4 Anti-adherence activity of the cranberry juice: comparison of the regimens, means for the 20 volunteers and 6 bacterial strains

Regimens	Adherence indices ± SD	Decrease in bacterial adherence (%)	<i>P</i> Student's <i>t</i> test
250 ml placebo +			
500 ml mineral water	7.04 ± 5.91	–	–
750 ml placebo	6.19 ± 4.92	12	0.049
250 ml cranberry +			
500 ml mineral water	3.90 ± 3.33	45	4.9 × 10 ⁻¹²
750 ml cranberry	2.70 ± 2.44	62	2.6 × 10 ⁻¹⁸

Adherence indices represent the average number of bacteria per cell determined by examining 100 cells. The decrease in bacterial adherence is related to the higher adherence index obtained after consumption of 250 ml placebo + 500 ml mineral water *P* values below 0.05 were considered significant *SD* standard deviation

Table 5 Anti-adherence activity of cranberry juice: comparison of the regimens and of the bacterial strains, means for the 20 volunteers, significance of the differences

Strains	Student's <i>t</i> test (<i>P</i>)			
	Comparison of the regimens		Placebo ^c 250 versus 750 ml	Cranberry ^d 250 versus 750 ml
	Placebo versus cranberry			
	250 ml ^a	750 ml ^b		
G1473	0.018	5×10 ⁻⁴	0.27 (NS)	0.02
G1487	4×10 ⁻⁴	6.6×10 ⁻⁶	0.28 (NS)	0.025
G1722	5×10 ⁻⁵	8×10 ⁻⁵	0.31 (NS)	0.32 (NS)
G1926	0.026	1×10 ⁻⁵	0.32 (NS)	0.01
G4117	0.005	5×10 ⁻⁴	0.11 (NS)	0.019
G4790	0.001	2×10 ⁻⁴	0.017	0.005

Comparison of the adherence indices observed after consumption

^a 250 ml of the placebo and 500 ml of mineral water versus 250 ml of the cranberry juice and 500 ml of mineral water

^b 750 ml of the placebo versus 750 ml of the cranberry juice

^c 250 ml of the placebo and 500 ml of mineral water versus 750 ml of the placebo

^d 250 ml of the cranberry juice versus 750 ml of the cranberry juice

P values below 0.05 were considered significant

NS not significant.

cranberry juice. The adherence index obtained with bacteria grown in urine samples collected after the intake of 750 ml of cranberry juice was lower than the adherence index obtained with urine samples collected after the intake of 250 ml of cranberry juice ($P=7.9\times 10^{-6}$). Placebo intake was also associated with a slight decrease in bacterial adherence when a dose of 750 ml was drunk ($P=0.049$). The statistical analysis of the differences in *E. coli* adherence between the six bacterial strains is presented in Table 5. Bacterial adherence to T24 cells was weaker for each of the six strains tested when bacteria were grown in urine samples collected after the consumption of cranberry juice compared with bacteria grown in urine samples collected after the intake of equivalent volumes of placebo. Again, except for strain G1722, the anti-adherence effect associated with cranberry consumption increased with the dose drunk. A placebo decrease effect on *E. coli* adherence was only observed with strain G4790 ($P=0.017$). The anti-adherence activity associated with cranberry consumption was not related to the antibiotic sensitivity or resistance (Table 2, 5). The bacterial G1473 strain, which was sensitive to the totality of the antibiotics tested, and the bacterial G1926 strain which was resistant to β -lactams, sulfonamide, trimethoprim-sulfamethoxazole, tetracycline, and chloramphenicol were both sensitive to the effect of the consumption of the cranberry juice on the adherence to uroepithelial cells. The comparison of the cranberry effects on *E. coli* adherence in relation with the presence or absence of the gene marker for P-fimbriae *papC* is presented in Table 6. The three strains harboring the *papC* gene adhered more efficiently to the T24 cells than strains which did not harbor this gene after bacterial growth in urines collected after placebo but not cranberry consumption. The difference in adherence between the two groups of strains was not significant with the lower dose of placebo

($P=0.09$) but was highly significant with 750 ml of placebo ($P=0.004$). An example of the anti-adherence effect of the cranberry juice on *E. coli* adherence to T24 cells is presented in Fig. 1.

Discussion

The presumed efficacy of cranberry (*V. m.*) in preventing UTI is related to its anti-adherent properties. Few studies have demonstrated that cranberry juice drinking was associated with the appearance of an anti-adherence activity in the human urine after the consumption of the beverage [18–21]. None of these studies were double-blind placebo-controlled trials, and the adherence assays used consisted of short incubation time contacts between bacteria and urine. During cystitis, UPEC are constantly in contact with urine in the bladder, urine representing a growth medium for bacteria. To break away from the

Table 6 Anti-adherence activity of cranberry juice: comparison of the regimens and of the *papC*⁺ and *papC*⁻ strains, means for the 20 volunteers

Regimens	Adherence indices \pm SD		<i>P</i> Student's <i>t</i> test
	<i>papC</i> ⁺ strains	<i>papC</i> ⁻ strains	
250 ml placebo + 500 ml mineral water	7.59 \pm 6.43	6.56 \pm 5.34	0.09 (NS)
750 ml placebo	7.40 \pm 5.92	5.50 \pm 4.29	0.004
250 ml cranberry + 500 ml mineral water	3.84 \pm 3.13	3.96 \pm 3.53	0.38 (NS)
750 ml cranberry	2.87 \pm 2.63	2.59 \pm 2.28	0.21 (NS)

The presence of the gene marker for P-fimbriae *papC* was determined by PCR amplification experiments. *Pap*⁺ strains: G1473, 1487, and 1722. *Pap*⁻ strains: G1926, G4117, and G4790

P values below 0.05 were considered significant

NS not significant

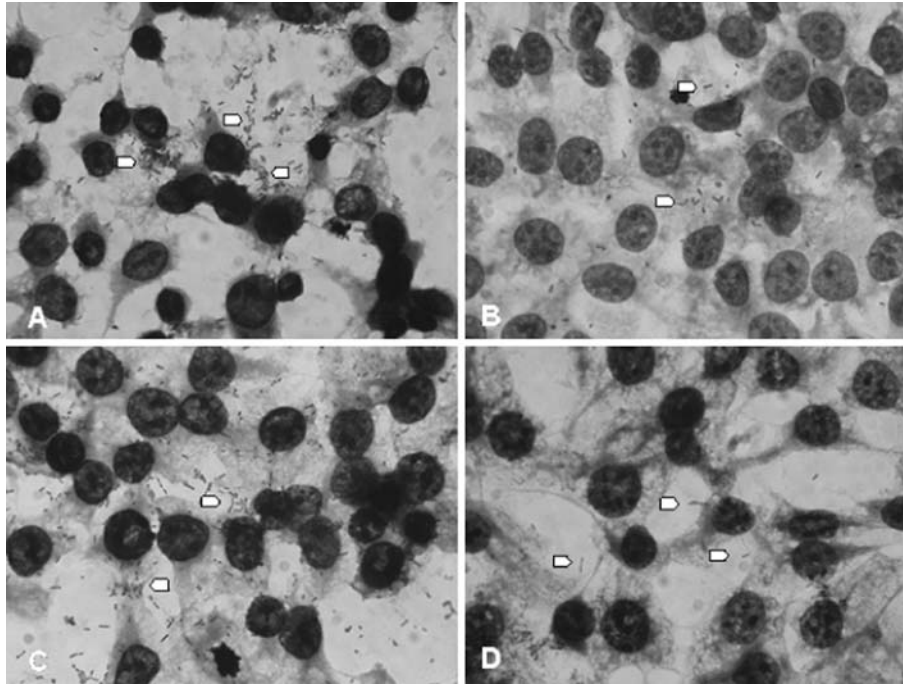


Fig. 1 Anti-adherence effect of cranberry juice consumption on the *E. coli* adherence to bladder epithelial T24 cells. Micrographs of Giemsa-stained preparations showing the uropathogenic *E. coli* G1722 strain adhering to T24 bladder epithelial cells after growth in urines collected after the intake of cranberry juice or placebo. **a** 250 ml of the placebo and 500 ml of mineral water, **b** 250 ml of the cranberry juice and 500 ml of mineral water, **c** 750 ml of the placebo, **d** 750 ml of the cranberry juice. Magnification: $\times 630$.

Adherent bacteria are indicated by arrows. A lot of adherent bacteria were observed at the surface of T24 cells with the bacteria grown in urines collected after the intake of 250 or 750 ml of placebo (**a** and **c**, respectively). Less amount of adherent bacteria were present after consumption of 250 ml of cranberry juice (**b**), and only few bacteria were associated with T24 cells after drinking 750 ml of cranberry juice (**c**).

possible bias associated with a non controlled trial, and to take into account the reality of bacterial growth in urine during UTI, we developed a bioassay to test the adhesion to the T24 bladder epithelial cell line of bacteria grown in urine samples collected after placebo or cranberry juice drinking in a double-blind procedure. Moreover, the use of a bladder epithelial cell line increases the reproducibility of the adherence test by comparison with uroepithelial desquamated cells coming from volunteers or patients. Our results clearly show that the consumption of cranberry juice is associated with a dose dependent decrease in *E. coli* adherence to bladder epithelial cells in vitro. This anti-adherence effect is not restricted to a particular group of strains: cranberry intake inhibited the adherence of *papC* positive and negative, antibiotic resistant with different resistance phenotypes and antibiotic sensitive strains. The only common phenotype shared by the six *E. coli* uropathogenic strains included in this study was the expression of the mannose sensitive adherence revealing the expression of type 1 pili. Zafriri et al. [10] have previously studied the direct effect of cranberry juice constituents on the adherence of *E. coli* expressing surface lectins of defined specificity to yeast, tissue culture cells, red blood cells, and mouse peritoneal macrophages. Cranberry juice or fructose alone inhibited the

mannose-specific activity of *E. coli*. The inhibitory effect of the dialyzed cranberry juice on hemagglutination by P-fimbriated bacteria was both time and concentration dependent. In the present study, the dose dependent adherence effect observed is consistent with the presence in urines, after cranberry consumption, of some compounds effective in decreasing *E. coli* adherence mediated by both type 1 and type P pili. This effect could result from the interference with specific receptor–ligand modes of bacterial adhesion or from the inhibition of expression of the bacterial pili [12, 22]. The active molecules could be proanthocyanidins and fructose but these compounds may be metabolized, at least in part, in the gastrointestinal tract. Nevertheless, since similar fructose concentrations are present in cranberry and placebo beverages, fructose cannot be responsible for the specific anti-adherence activity associated with cranberry juice. Fructose and the other additive to cranberry juice (vitamin C) have been previously evaluated for their independent effect on bacterial adherence [19]. Fructose was found to inhibit adherence at least minimally and vitamin C tested as ascorbic acid and sodium ascorbate had no observable effect on bacterial adherence. Thus, the dose dependent effect observed in our study with cranberry, and to some extent with placebo, could be in part the result of an increased intake of

fructose. Nevertheless, contrary to proanthocyanidins, there is no clinical evidence linking dietary fructose intake with maintenance of urinary tract health. Few pharmacokinetics studies have been carried out on proanthocyanidins due to the structural complexities of the molecules as well as the lack of commercial standards. Proanthocyanidins have been detected in the plasma of rats fed orally with proanthocyanidin-rich extracts [23]. Following the oral delivery of ^{14}C -labeled grape proanthocyanidins to rats, 19% of the dose was excreted in the urine [24], but the presence of proanthocyanidins in human urines has never been reported. Proanthocyanidins alone may be responsible for the anti-adherence activity against P-fimbriated *E. coli* since mice fed isolated cranberry proanthocyanidins produced urine with anti-adherence activity [25]. The nature of the active compounds involved in the type P pili independent inhibition of bacterial adherence is still unknown. These molecules may be present in cranberry juice but not in placebo or some molecules specifically present in cranberry juice may modify the properties of the human urine such that adhesion is inhibited. For a long period of time, the usefulness of cranberry juice was thought to be based on the urinary excretion of hippuric acid, which is a bacteriostatic agent and has the potential to acidify urine. Today, it is known that ingestion of large amounts of cranberry juice is required to slightly reduce the pH of urine and modestly increase hippuric acid excretion, changes that do not confer significant antibacterial activity to urine [26, 27]. In the present study, the drinking of cranberry juice was not associated with a reduction in the urine pH with regard to placebo consumption, and the increase in cranberry consumption did not conduct to a pH decrease. Specific gravity of urine was not affected either by cranberry or by placebo consumption. The universal antibacterial activity of beverages can be due to increased fluid intake and the subsequent bladder washout of bacteria [28]. Because our subjects drank the same liquid volume either containing or not containing a proportion of cranberry juice or placebo beverage, a washout effect of the increased fluid intake associated with the beverage consumption could not be observed. Besides, the specific gravity was not affected by the different beverage regimens used in the study.

In conclusion, the present study shows that cranberry (*V. m.*) supplementation can provide a degree of protection against adhesion to epithelial bladder cells by different UPEC strains. This protective effect is independent from antibiotic resistance and the type P pili genetic determinants presence. Further studies are needed to determine the mechanism of this anti-adherence effect and the nature of the anti-adherence compounds.

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