DOI:10.1021/jf901931b

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

The Effect of Pomegranate (*Punica granatum* L.) Byproducts and Ellagitannins on the Growth of Human Gut Bacteria

Dobroslawa Bialonska,[†,∥] Sashi G. Kasimsetty,[†] Kevin K. Schrader,[§] and Daneel Ferreira*[,†,‡]

[†]Department of Pharmacognosy, School of Pharmacy and [‡]National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, The University of Mississippi and [§]NPURU, Agricultural Research Service, U.S. Department of Agriculture, University, Mississippi 38677, and [∥]Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland

The consumption of pomegranate products leads to a significant accumulation of ellagitannins in the large intestines, where they interact with complex gut microflora. This study investigated the effect of pomegranate tannin constituents on the growth of various species of human gut bacteria. Our results showed that pomegranate byproducts and punicalagins inhibited the growth of pathogenic clostridia and *Staphyloccocus aureus*. Probiotic lactobacilli and bifidobacteria were generally not affected by ellagitannins, while relatively small growth inhibition by ellagic acid likely resulted from decreasing media quality due to the formation of tannin−protein complexes. The effect of pomegranate ellagitannins on bifidobacteria was species- and tannin-dependent. The growth of *Bifidobacterium animalis* ssp. *lactis* was slightly inhibited by punicalagins, punicalins, and ellagic acid. POMx supplementation significantly enhanced the growth of *Bifidobacterium breve* and *Bifidobacterium infantis*.

KEYWORDS: ***Punica granatum*** **L.; byproduct; punicalagins; punicalins; ellagic acid; gallic acid; intestinal pathogens; probiotics**

INTRODUCTION

A growing body of scientific evidence points to the contribution of human gut microbiota toward health improvement and the genesis of various diseases. Beneficial gut bacteria, known as probiotics (e.g., *Bifidobacterium* and *Lactobacillus*) provide the following benefits: they function as a "barrier" against pathogens, degrade undigested food components, stimulate the host immune system, prevent food allergies and tumors, produce vitamins, metabolize cholesterol and other lipids, and enhance mineral bioavailability (*1*−*3*). Conversely, the overgrowth of external and internal pathogenic species (e.g., proteolytic *Bacteroides* and *Clostridium* and *Staphylococcus aureus*) causes chronic and acute bowel diseases and has been associated with aging, cancer, obesity, and Alzheimer's disease (*4*, *5*).

Dietary substrates influence gut microbiota by either enhancing the growth of beneficial bacteria or causing their depletion and imbalance. Plant-derived polyphenols such as hydrolyzable tannins, condensed tannins, and flavonoids constitute on average 1 g of the daily human diet (*6*). Phenolic components of common foods (e.g., fruits, vegetables, and tea) and dietary supplements readily contribute to gut bacteria modulation. They are likely to selectively inhibit the growth of intestinal pathogens (*7*−*13*). Relatively little is known about the interaction between tannins and human intestinal bacteria. Most previous studies have been focused on the microflora of ruminant grazing animals that are exposed to high doses of tannins in feeds (*14*). The antimicrobial action of tannins is associated with their prevailing capability to form stable complexes with proteins, starch, and physiological metals, thereby disturbing the metabolic activity of bacterial enzymes, nutrient availability, and functionality of biological membranes (*14*, *15*).

Pomegranate fruits are rich in polyphenols and have been used for centuries for nutritional and medicinal purposes. In recent years, antioxidant, antimicrobial, anticancer, anticarcinogenic, and anti-inflammatory activities of pomegranate have been attributed to ellagitannins, mainly punicalagins (we prefer to use the plurals punicalins and punicalagins, since these compounds exist in solution as the α- and β-anomers as well as the acyclic hydroxyaldehyde analogue), ellagic acid, and punicalins (**Figure 1**) (*16*−*25*). During industrial hydrostatic processing of the whole fruits, ellagitannins are extracted in significant amounts, subsequently enriching pomegranate juice with at least 2 g/L of punicalagins (*21*). After the first squeezing of pomegranate fruits leading to juice production, the residual material is additionally pressed and extracted with water to afford a byproduct POMx, which is commercially available as a dietary supplement. Clinical reports specified that after human consumption the majority of ellagitannins remain in the colon for up to 56 h before excretion (*25*, *26*). This considerable amount of time permits the induction of biological interactions between tannins and colonic bacteria. Despite increased attention by researchers, industry, and consumers toward the beneficial health properties of pomegranate, the impact of pomegranate tannin constituents on human gut microbiota has not been elucidated.

*To whom correspondence should be addressed. Tel: 001 662 915-7026. Fax: 001 662 915-6975. E-mail: dferreir@olemiss.edu.

 Published on Web 08/25/2009

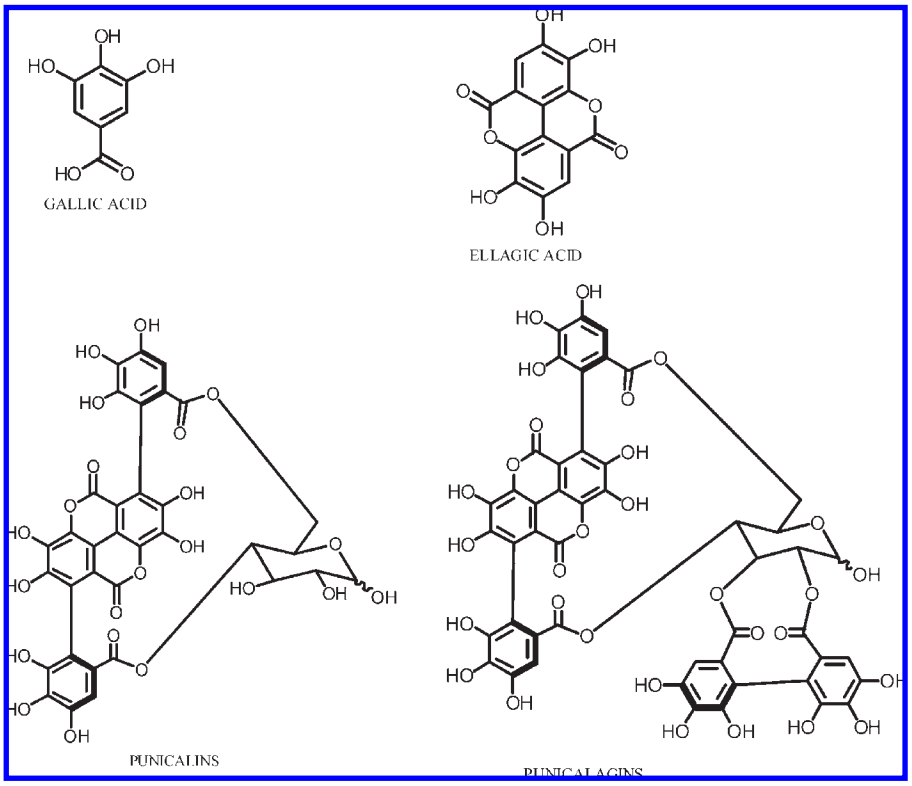

Figure 1. Chemical structures of pomegranate tannins.

In this study, we evaluated the effects of various pomegranate tannin constituents on the growth of a variety of human gut bacteria. In addition, in vitro studies using *S. aureus* were performed to determine the effects of pomegranate tannins toward common human pathogenic bacteria.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. Bacterial strains evaluated in the assay were as follows: *Lactobacillus casei* ssp. *casei* NRRL B-1922, *Lactobacillus paracasei* ssp. *paracasei* NRRL B-4560, *Lactobacillus rhamnosus* NRRL B-442, *Lactobacillus pentosus* NRRL B-227, *Lactobacillus acidophilus* NRRL B-4495, *Bifidobacterium animalis* ssp. *lactis* NRRL B-41405, *Bifidobacterium bifidum* NRRL B-41410, *Bifidobacterium breve* NRRL B-41408, *Bifidobacterium infantis* NRRL B-41661, *Bifidobacterium longum* NRRL B-41409, *Clostridium perfringens* NRRL B-23584, *Clostridium ramosum* NRRL B-23617, *Clostridium clostridioforme* NRRL B-23589, *Bacteroides fragilis* NRRL B-23622, and *S. aureus* ATCC 29213. Bacteria were kindly provided by the U.S. Department of Agriculture, Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research (Peoria, IL).

Lactobacilli were cultured aerobically at 37 °C in De Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Cambridge, United Kingdom). Bifidobacteria and *B. fragilis* were cultured anaerobically at 37 °C in brain heart infusion (BHI) broth (BD Difco, Becton, Dickinson and Co., Sparks, MD). *Clostridium* spp. were cultured anaerobically at 37 °C in reinforced clostridial medium (Oxoid). Anaerobic conditions were maintained using an anaerobic jar (BBL Gaspak 150 Anaerobic System, Becton, Dickinson and Co.) with BBL GasPak Plus (anaerobic system envelopes with palladium catalyst). Cultures of *S. aureus* were grown aerobically at 29 °C in Mueller−Hinton (MH) broth and agar (BD Difco, Becton, Dickinson and Co.).

Frozen stock cultures were stored at −80 °C. Before experimental use, cultures were transferred on solid media and incubated for 1−2 days. For growth studies, inocula were prepared from subculturing these cultures in liquid media.

Isolation and Characterization of Tannins. The commercial extract of pomegranate byproduct (POMx) was provided by POM Wonderful (Los Angeles, CA) in January, 2007. The approximate percent distribution of pomegranate polyphenols in POMx was as follows: 19% ellagitannins as punicalagins and punicalins, 4% free ellagic acid, and 77% oligomers composed of 2−10 repeating units of gallic acid, ellagic acid, and glucose in different combinations. Extraction of punicalagins and punicalins was performed by the procedure described previously by use of a step gradient consisting of increasing amounts of methanol in water (*20*). POMx (100 mL) was diluted to 500 mL with Millipore high-purity water and successively partitioned with EtOAc (3 × 200 mL) and *n*-BuOH (3 × 200 mL). The *n*-BuOH extract (2.0 g) was concentrated and subjected to Amberlite XAD-16 column chromatography (500 g, 6 × 35 cm) and eluted with H_2O (2.0 L) and MeOH (2.0 L) successively. The MeOH fraction on removal of solvent under reduced pressure afforded a tannin fraction (XAD-*n*-BuOH) (1.3 g). This was further purified on Sephadex LH-20 CC (6 cm × 55 cm) and eluted with H_2O:MeOH (2:8) (350 mL), H_2O:MeOH (1:9) (500 mL), MeOH (450 mL), and MeOH:Me_2CO (1:1) (600 mL) to give nine fractions. Subfractions 5 and 6 were combined and rechromatographed on Sephadex LH-20 with a H_2O:MeOH gradient (3:7) (250 mL), H_2O:MeOH (1:9) (500 mL), and MeOH (300 mL) to yield punicalins (19 mg). Subfractions 8 and 9 were combined and further purified on Sephadex LH-20 with MeOH (350 mL) and MeOH:Me_2CO (1:1) (400 mL) as eluents to yield punicalagins (35 mg). The purification process was monitored by one- (1D) and two-dimensional (2D) thin-layer chromatography (TLC). An one-dimensional TLC system was developed with MeOH:H_2O:HOAc (8.5:1.5:1) on silica gel 60 F254-coated aluminum plates (Sigma Aldrich, St. Louis, MO). Two-dimensional TLC was developed with *s*-BuOH:H_2O:HOAc (4:6:1) on cellulose-coated polyester plates, 20 cm × 20 cm, with an UV indicator (Sigma Aldrich). After it was treated with $FeCl_3$ (10% EtOH solution), the chromatogram permitted estimation of the ellagitannins present. Compounds were identified using liquid chromatography−mass spectrometry (LC-MS) based on their retention times, UV absorption patterns, molecular masses, and ^1H NMR spectra in relation to standards isolated previously (*20*). The LC-MS system consisted of a Waters Micromass ZQ mass Spectrometer, Waters 2695 Separation Module, and Waters 996 Photodiode Array Detector. Mass spectra were recorded in the negative mode. The capillary voltage was 4000/3500 V, and the gas temperature was 300 °C. The column used was a 150 mm × 3.0 mm i.d., 5 μm, Luna C18 100 Å (Phenomenex, Torrance, CA). The analyses were performed in the gradient system A, 2.5% HOAc, and B, 2.5% HOAc in MeOH, starting from 100% A for

5 min, 0−60% B for 15 min, and 60−100% B for 15 min. The flow rate was 0.3 mL/min, and the pressure was 900−1500 mmHg. The elution of metabolites was monitored at 254 nm. Ellagic acid and gallic acid were purchased from Sigma-Aldrich.

Microbiological Assay. The effect of pomegranate constituents on the growth of intestinal bacteria was evaluated in liquid cultures by measuring the optical density (OD) of culture media. In the assay, we included pure compounds (punicalagins, punicalins, ellagic acid, and gallic acid) as well as POMx. Prior to conducting the experiments, test compounds and POMx were dissolved separately in distilled water and sterilized by filtration through 0.22 μm filters (Millex GP, Millipore Corp., Bedford, MA). Because of poor water solubility, ellagic acid was dissolved in dimethyl sulfoxide (DMSO). The final volume of DMSO in culture media was 3%. The same concentration of DMSO was also added to tubes with control media.

Media (12 mL in sterile test tubes) containing 0.05% (v/v) of respective individual compounds or 0.01% (v/v) of POMx were inoculated with 1×10^8 colony-forming units/mL (CFU) of various test bacteria. The CFU of test bacteria were determined by measuring the absorbance (630 nm) of the inoculum and then using the absorbance reading to interpolate the CFU from a standardized curve developed previously (absorbance reading vs CFU from spread plate technique). The OD of medium at 630 nm was measured using a Multiskan Spectrum spectrophotometer (Thermo Electron Corp., Vantau, Finland). In each case, an OD of media supplemented with treatments only (e.g., individual compounds or POMx) was subtracted from an OD of media containing treatments and bacteria, to eliminate the background effect of a tannin−media components precipitation. Growth of bacteria in treatments was compared to growth of bacteria in media not supplemented with tannins (control).

For the growth assay, facultative anaerobes (*Lactobacillus* spp. and *S. aureus*) were cultured in MRS and MH media, respectively, under aerobic conditions at 37 °C, and OD readings of cultures were measured at 0, 24, and 72 h. Obligatory anaerobes (bifidobacteria, clostridia, and bacteroides) were grown in BHI medium at 37 °C in the anaerobic jar, and OD readings were taken at 0 and 72 h.

To determine the effect of the supplementation of medium with peptone extract on the growth of lactobacilli, *L. acidophilus* was grown in MRS medium containing 0.01% of POMx and additional peptone (2%). The same culture conditions and measurement readings were used as discussed previously.

In another study, the effect of punicalagins and POMx concentration on the growth of *S. aureus* was determined. In this assay, bacteria were grown in 96-well microplates in MH broth. Media (190 μL) containing a bacterial suspension equivalent to an OD of 0.082 (1.2×10^7 CFU) was added to wells containing 10 μL of appropriate dilutions of punicalagins to obtain final test concentrations of 0.01, 0.1, 1.0, 10.0, 100.0, and 1000.0 μM. For POMx, final test concentrations were 0.00000005, 0.0000005, 0.000005, 0.00005, 0.0005, and 0.005% (v/v). The growth of bacteria in treatment wells was compared to controls. The absorbance was measured at 630 nm using a microplate photometer (SpectraCount; Packard Instrument Co., Meriden, CT). Means and standard deviations of absorbance measurements of treatment and control wells were determined.

To evaluate the effect of POMx supplementation on the pH of culture media, BHI media in a gradient of different pH (5.5, 6.1, 6.6, 7.2, and 7.4) were treated with 0.01% (v/v) of POMx. The final pH of the media was measured after 72 h of incubation at 37 °C using a mini lab ISFET pH meter (IQ Scientific Instruments, Inc., San Diego, CA).

Statistical Analysis. All experiments were performed in triplicate. The differences in the growth of the control bacteria and bacteria treated with pomegranate tannins were calculated using Kruskal−Wallis analysis of variance (ANOVA) by ranks with multiple comparisons (p value test) in studies with lactobacilli, clostridia, and *S. aureus*. ANOVA with Tukey HSD test was used to evaluate differences in the growth of bifidobacteria and *B. fragilis*.

RESULTS AND DISCUSSION

Most health advantages of pomegranate products are attributed to the potent biological activity of water-soluble hydrolyzable ellagitannins, mainly punicalagins, which comprise 70% of the polyphenols in commercial juice. It is generally known that the intestinal absorption of phenolics is highly variable, often slow, and largely incomplete (*27*, *28*). Consequently, ellagitannins remain unabsorbed in the gut lumen and accumulate in the colon, where they can interact with complex intestinal bacteria. In the present study, we tested the growth of human gut bacteria in the presence of pomegranate ellagitannins (punicalagins and punicalins), as well as their structural constituents (ellagic acid and gallic acid), which can be released from ellagitannins due to hydrolysis facilitated by physiological pH and/or gut microflora action (*29*). By assuming that the mean volume capacity of the adult human colon is approximately 2 L (*30*) and pomegranate juice contains at least 2 g/L of punicalagins, it could be estimated that the consumption of two 250 mL glasses of juice provides around 0.05% of punicalagins in the colon. In addition, we tested the commercially available mixture of pomegranate tannins contained in the dietary supplement POMx, at the concentration 0.01% (v/v), which is equivalent to one pill or one tablespoon. In general, the mixture of pomegranate tannins as well as individual molecules inhibited the growth of pathogenic bacteria without adverse effects on beneficial bacteria tested.

Table 1. Growth (% Compared to Control) of Human Gut Bacteria in the Presence of Pomegranate Tannins[a]

	POMx (0.01%)	punicalagins (0.05%)	punicalins (0.05%)	ellagic acid (0.05%)	gallic acid (0.05%)
L. acidophilus	83*	95	131	66*	126
L. casei ssp. *casei*	81	70	101	67*	102
L. paracasei ssp. *paracasei*	90	81	107	79*	110
L. pentosus	88*	86	109	74*	107
L. rhamnosus	79*	82	108	74*	105
B. breve	275*	130*	121	81	112
B. infantis	241*	106	106	122	99
B. longum	99	68	121	93	96
B. bifidum	83*	86	96	114	83*
B. animalis ssp. *lactis*	112	78*	78*	52*	109
Bacterioides fragilis	73	83	117	24*	107
C. perfringens	−13*	−26*	90	0*	46
Clostridium clostriidoforme	58	0*	103	0*	114
C. ramosum	0*	−16*	65	26*	70
S. aureus	−3*	−27*	86	75	97

[a] The asterisk denotes statistically significant differences between means of treated and control groups ($p \leq 0.05$).

Punicalagins and ellagic acid showed the most potent growth inhibition among studied compounds. In contrast, punicalins and gallic acid generally enhanced or did not affect the growth of most test bacteria. Gram-positive intestinal pathogenic species of *Clostridium* were the most sensitive to pomegranate constituents (**Table 1**). Punicalagins inhibited the growth of all test species, and ellagic acid completely inhibited the growth of *C. perfringens* and *C. clostridioforme*, while partially inhibiting the growth of *C. ramosum* (only 26% as compared to control) ($p < 0.05$) (**Table 1**). POMx inhibited the growth of *C. perfringens* and *C. ramosum* and reduced the growth of *C. clostridioforme* to approximately 60% (**Table 1**). Gallic acid was partially inhibitory to the growth of *C. perfringens* and *C. ramnosum*. However, the reduction was not statistically significant ($p \geq 0.05$) (**Table 1**). The growth of Gram-positive potentially harmful *B. fragilis* was inhibited only in the presence of ellagic acid ($p < 0.05$)

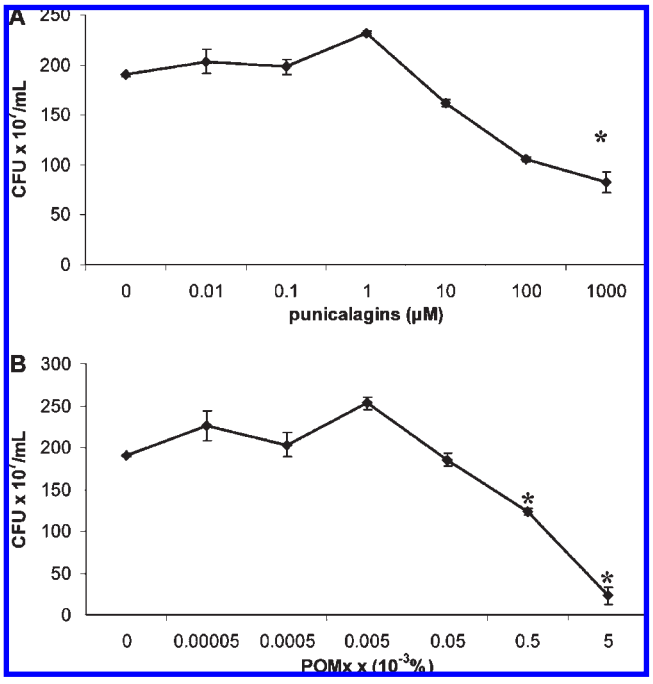

Figure 2. Effect of different concentrations of punicalagins (**A**) and POMx (**B**) on the growth of *S. aureus* after 24 h of incubation. The asterisk denotes statistically significant differences between means of the experimental and the control group ($p \leq 0.05$).

(**Table 1**). Both POMx and punicalagins inhibited the growth of the Gram-positive enteropathogen *S. aureus* (**Table 1**). Graphed data indicated a 50% lethal concentration (24 h LC_{50}) of 100 μM for punicalagins toward *S. aureus* (**Figure 2A**). The addition of 0.01% POMx to the medium completely inhibited growth (**Table 1**), 0.005% added POMx reduced the growth to 12% ($p < 0.001$) as compared to controls, and 0.0005% POMx reduced the growth to 65% ($p < 0.05$) (**Figure 2B**).

In previous research, punicalagins inhibited the growth of the pathogenic Gram-negative bacteria *Escherichia coli* (IC_{50} = 9.2 μM) and *Pseudomonas aeruginosa* (IC_{50} = 3.2 μM) (*20*). Although the inhibition of *S. aureus* occurred at much higher concentrations of punicalagins in our study (IC_{50} = 100 μM) than in the previous case of the above pathogens, it is likely that sufficient and effective concentrations of punicalagins can occur in the colon after the consumption of pomegranate products. It is also likely that ellagitannins can be combined with other antimicrobial agents to enhance their action against undesirable bacteria. For instance, pomegranate extract has been found to increase the activity of antibiotics against 30 clinical isolates of methicillin-resistant *S. aureus* (*31*, *32*).

To maintain the proper balance of bacteria in humans, it would be desirable to inhibit the growth of potential pathogens without decreasing the abundance of probiotics in human guts. Therefore, we also evaluated the growth of several species of lactobacilli and bifidobacteria in the presence of pomegranate constituents. Probiotic lactobacilli were relatively unaffected by pomegranate chemical constituents (**Table 1**). Ellagic acid slightly reduced their growth, by 66% for *L. acidophilus* and by 74% for both *L. pentosus* and *L. ramnosus* ($p < 0.05$). POMx inhibited lactobacilli growth to approximately 10−20% of controls ($p < 0.05$). However, the growth of *L. acidophilus* in media containing POMx and additionally supplemented with 2% peptone was not statistically different from the control ($p \geq 0.05$) (**Figure 3**). Therefore, the detected growth inhibition was likely due to a decrease in media quality after tannin complexation with nutritional components. Punicalins and gallic acid were not inhibitory toward the growth of lactobacilli and actually stimulated bacterial growth, although differences with the control were not statistically significant ($p \geq 0.05$) (**Table 1**). The effect of pomegranate constituents on the growth of bifidobacteria was species-specific. Punicalagins, punicalins, and ellagic acid partially inhibited the growth of *B. animalis lactis* by 78, 78, and 52%, respectively, and both POMx and gallic acid inhibited the growth of *B. bifidum* by 83% as compared to controls. Conversely, the growth of *B. breve* and *B. infantis* was significantly enhanced ($p < 0.05$) by POMx to 275 and 241%, respectively (**Table 1**). Because probiotic growth was relatively unaffected or even enhanced, their colonization in the intestines should continue in the presence of pomegranate ellagitannins. This suggests that pomegranate products may help regulate pathogens without adverse effects on beneficial bacteria.

Evidence from our study supports earlier reports showing that polyphenols inhibit the growth of pathogens without affecting probiotics and helps to explain the epidemiological evidence indicating that diets rich in fruits and vegetables are associated with a decreased risk of bowel diseases. For example, Puupponen-Pimiä et al. (*11*−*13*) measured the growth of human gut bacteria under the influence of various berries (e.g., cloudberry, strawberry, bilberry, raspberry, blackcurrant, loganberry, cranberry, and buckthorn berry) and their phenolic extracts, and they detected the selective growth inhibition of *Salmonella enterica*, *E. coli*, and *S. aureus*, while Gram-positive probiotic lactobacilli were not affected. The most potent antimicrobial action occurred with extracts of cloudberry and raspberry, both of which are characterized by possessing 10× higher concentrations of ellagitannins than other berries. An antimicrobial effect of individual cloudberry and raspberry ellagitannins was not investigated. However, structural components of ellagitannins (ellagic acid and gallic acid) were tested for antimicrobial activity against *S. enterica*; ellagic acid did not inhibit the growth, but gallic acid caused strong inhibition (*12*). In another study, different patterns of inhibition by ellagitannin berry extracts were noticed for *S. aureus* and *S. enterica*. The growth of *S. aureus* was clearly inhibited, and inhibition was maintained throughout the incubation period, with no viable bacterial cells detected after 24 h. Conversely, *S. enterica* was slightly inhibited at the beginning of the incubation period, and inhibition weakened over time (i.e., bacteriostatic with no complete growth inhibition) (*12*).

In another study, tea polyphenols (0.1% of media) inhibited the growth of human gut bacteria, with strong inhibition of *E. coli*, moderate inhibition of clostridia and *B. fragilis*, and minimal effect on the growth of probiotic lactobacilli and bifidobacteria (*9*). Among individual compounds, 3-*O*-methylgallic acid and gallic acid exhibited strong inhibition of *C. perfringens* (*9*). Our results showed that gallic acid decreased the growth of *C. perfringens* to 46% as compared to the control. However, we tested lower concentrations of phenolics (0.05%) in media. These results are similar to growth experiments by Ahn et al. (*8*) in which gallic acid (isolated from *Galla rhois*) exhibited lower activity than methyl gallate, moderately inhibiting *C. perfringens*, *C. parapurficatum*, *B. fragilis*, and *S. aureus*, with no effect on lactobacilli and bifidobacteria. However, methyl gallate significantly inhibited the growth of *C. perfringens*, *C. parapurficatum*, *B. fragilis*, *S. aureus*, and *E. coli*, with no inhibition of *Bifidobacterium adolescentis* and *B. longum* and weak inhibition of *B. bifidum*, *B. breve*, *B. infantis*, *B. animalis*, *B. tremophilum*, *L. acidophilus*, and *L. plantarum* (*8*). Conversely, our experiments revealed no growth inhibition of *B. fragilis* grown in the presence of gallic acid, possibly due to lower test concentrations used in our study.

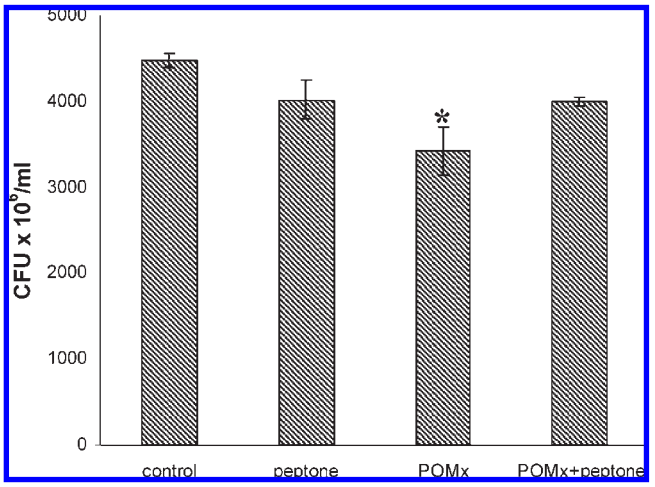

Figure 3. Growth of *L. acidophilus* in the presence of 0.01% POMx in media with and without 2% peptone. The asterisk denotes statistically significant differences of means between the experimental and the control group ($p \leq 0.05$).

In general, tannins are assumed to be toxic to gut microflora, and a few mechanisms of antimicrobial action have been proposed. When in solution, tannins create stable complexes, mainly with proteins and to a lesser extent with carbohydrates or physiological metal ions (e.g., Fe and Cu) (*14*). The complexation of tannins with enzymes changes their structural conformation, thereby inhibiting enzymatic activity. The formation of complexes with cell wall proteins decreases cell wall permeability and reduces the transport of substrates into the cell. In addition, tannins decrease metal ion availability to bacteria when forming stable complexes with these metal ions. Subsequently, metal depletion may adversely affect the activity of metalloenzymes in microbial cells (*15*). Polyphenols may also significantly affect intestinal bacterial population by decreasing the pH of the intestinal environment. Generally, lower pH favors probiotic bacteria as compared to pathogenic bacteria, which are highly variable in their tolerance to acids. The antimicrobial activity of certain berries and their phenolic extracts has been attributed to the resulting lower pH of the media by Puupponen-Pimiä et al. (*12*). Berries and their phenolic extracts contain organic acids, and their addition to culture media decreased the pH to ≤5. For example, critical pH values for the growth of *S. aureus* were 5−5.5 when tested in the presence inorganic acids in the same study (*12*). To evaluate if POMx supplementation modified the pH of the culture media, POMx was added to MRS media of different initial pH. POMx incubation decreased the media pH by 1 unit, when the initial pH was in the range 6.1−7.4 and by 0.5 unit when the initial pH was 5.5. Therefore, the growth inhibition toward pathogenic bacteria by POMx may partially be due to lower media pH and could also provide an explanation as to the higher efficiency of *S. aureus* growth inhibition by punicalagins in BHI media (pH 6.5) as compared to MH broth (pH 7.4) used during the growth assay.

Because our in vitro results demonstrated that POMx and punicalagins were selectively toxic toward intestinal pathogenic bacteria as compared to probiotic bacteria, the consumption of tannin-rich pomegranate products by humans may have the potential to improve the imbalance of intestinal bacteria caused by stress and other factors, thereby promoting gut health and general well-being. However, interactions between tannins and gut bacteria are far more complex in the gut environment, dependent upon the abundance and type of bacterial species present and the quantity and variety of phenolics consumed by the host. In addition, human gut bacteria are capable of metabolizing polyphenols, and metabolites released from one bacterial species may influence the growth of metabolite-producing bacteria as well as neighboring microbiota species (*9*). For example, punicalagins and ellagic acid are metabolized to urolithins by colonic bacteria (*26*). Therefore, the results of growth effects on individual bacteria species obtained in the present in vitro study should be verified in additional studies using human fecal microbiota.

ACKNOWLEDGMENT

We acknowledge the U.S. Department of Agriculture, Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research, Peoria, IL, for donation bacterial strains. The technical assistance of Dewayne Harries and Phaedra Page is greatly appreciated.

LITERATURE CITED

(1) Gibson, G. R. Prebiotics as gut microflora management tools. *J. Clin. Gastroenterol.* **2008**, *42*, S75–S79.
(2) Hord, N. G. Eukaryotic-microbiota crosstalk: potential mechanisms for health benefits of prebiotics and probiotics. *Annu. Rev. Nutr.* **2008**, *28*, 215–231.
(3) Vanderhoof, J. A. Probiotics in allergy management. *J. Pediatr. Gastroenterol. Nutr.* **2008**, *47*, S38–S40.
(4) Onoue, M.; Kado, S.; Sakaitani, Y.; Uchida, K.; Morotomi, M. Specific species of intestinal bacteria influence the induction of aberrant crypt foci by 1,2-dimethylhydrazine in rats. *Cancer Lett.* **1997**, *113*, 179–186.
(5) Rastall, R. A.; Gibson, G. R.; Gill, H. S.; Guarner, F.; Klaenhammer, T. R.; Pot, B.; Reid, G.; Rowland, I. R.; Sanders, M. E. Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: an overview of enabling science and potential applications. *FEMS Microbiol. Ecol.* **2005**, *52*, 145–152.
(6) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S.
(7) Ahn, Y. J.; Kawamura, T.; Kim, M.; Yamamoto, T.; Mitsuoka, T. Tea polyphenols: Selective growth inhibitors of *Clostridium* spp. *Agric. Biol. Chem.* **1991**, *55*, 1425–1426.
(8) Ahn, Y. J.; Lee, C. O.; Kweon, J. H.; Ahn, J. W.; Park, J. H. Growth-inhibitory effects of *Galla rhois*-derived tannins on intestinal bacteria. *J. Appl. Microbiol.* **1998**, *84*, 439–443.
(9) Lee, H. C.; Jenner, A. M.; Low, C. S.; Lee, Y. K. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Res. Microbiol.* **2006**, *157*, 876–884.
(10) Parkar, S. G.; Stevenson, D. E.; Skinner, M. A. The potential influence of fruit polyphenols on colonic microflora and human gut health. *Int. J. Food Microbiol.* **2008**, *124*, 295–298.
(11) Puupponen-Pimia, R.; Nohynek, L.; Alakomi, H. L.; Oksman-Caldentey, K. M. Bioactive berry compounds-novel tools against human pathogens. *Appl. Microbiol. Biotechnol.* **2005**, *67*, 8–18.
(12) Puupponen-Pimia, R.; Nohynek, L.; Hartmann-Schmidlin, S.; Kahkonen, M.; Heinonen, M.; Maatta-Riihinen, K.; Oksman-Caldentey, K. M. Berry phenolics selectively inhibit the growth of intestinal pathogens. *J. Appl. Microbiol.* **2005**, *98*, 991–1000.
(13) Puupponen-Pimia, R.; Nohynek, L.; Meier, C.; Kahkonen, M.; Heinonen, M.; Hopia, A.; Oksman-Caldentey, K. M. Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol.* **2001**, *90*, 494–507.
(14) Chung, K. T.; Lu, Z.; Chou, M. W. Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria. *Food Chem. Toxicol.* **1998**, *36*, 1053–1060.
(15) Goel, G.; Puniya, A. K.; Aguilar, C. N.; Singh, K. Interaction of gut microflora with tannins in feeds. *Naturwissenschaften* **2005**, *92*, 497–503.
(16) Adams, L. S.; Seeram, N. P.; Aggarwal, B. B.; Takada, Y.; Sand, D.; Heber, D. Pomegranate juice, total pomegranate ellagitannins, and

punicalagin suppress inflammatory cell signaling in colon cancer cells. *J. Agric. Food Chem.* **2006**, *54*, 980–985.
(17) Aviram, M.; Rosenblat, M.; Gaitini, D.; Nitecki, S.; Hoffman, A.; Dornfeld, L.; Volkova, N.; Presser, D.; Attias, J.; Liker, H.; Hayek, T. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. *Clin. Nutr.* **2004**, *23*, 423–433.
(18) Aviram, M.; Volkova, N.; Coleman, R.; Dreher, M.; Reddy, M. K.; Ferreira, D.; Rosenblat, M. Pomegranate phenolics from the peels, arils, and flowers are antiatherogenic: studies in vivo in atherosclerotic apolipoprotein e-deficient (E 0) mice and in vitro in cultured macrophages and lipoproteins. *J. Agric. Food Chem.* **2008**, *56*, 1148–1157.
(19) Gil, M. I.; Tomas-Barberan, F. A.; Hess-Pierce, B.; Holcroft, D. M.; Kader, A. A. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J. Agric. Food Chem.* **2000**, *48*, 4581–4589.
(20) Reddy, M. K.; Gupta, S. K.; Jacob, M. R.; Khan, S. I.; Ferreira, D. Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* L. *Planta Med.* **2007**, *73*, 461–467.
(21) Seeram, N. P.; Adams, L. S.; Henning, S. M.; Niu, Y.; Zhang, Y.; Nair, M. G.; Heber, D. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J. Nutr. Biochem.* **2005**, *16*, 360–367.
(22) Shiner, M.; Fuhrman, B.; Aviram, M. Macrophage paraoxonase 2 (PON2) expression is up-regulated by pomegranate juice phenolic anti-oxidants via PPAR gamma and AP-1 pathway activation. *Atherosclerosis* **2007**, *195*, 313–321.
(23) Aviram, M.; Dornfeld, L.; Rosenblat, M.; Volkova, N.; Kaplan, M.; Coleman, R.; Hayek, T.; Presser, D.; Fuhrman, B. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *Am. J. Clin. Nutr.* **2000**, *71*, 1062–1076.
(24) Aviram, M.; Dornfeld, L.; Kaplan, M.; Coleman, R.; Gaitini, D.; Nitecki, S.; Hofman, A.; Rosenblat, M.; Volkova, N.; Presser, D.; Attias, J.; Hayek, T.; Fuhrman, B. Pomegranate juice flavonoids inhibit low-density lipoprotein oxidation and cardiovascular diseases: studies in atherosclerotic mice and in humans. *Drugs Exp. Clin. Res.* **2002**, *28*, 49–62.
(25) Seeram, N. P.; Henning, S. M.; Zhang, Y.; Suchard, M.; Li, Z.; Heber, D. Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 h. *J. Nutr.* **2006**, *136*, 2481–2485.
(26) Cerda, B.; Espin, J. C.; Parra, S.; Martinez, P.; Tomas-Barberan, F. A. The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxyl-6H-dibezopyran-6-one derivatives by the colonic microflora of healthy humans. *Eur. J. Nutr.* **2004**, *43*, 205–220.
(27) Gee, J. M.; Johnson, I. T. Polyphenolic compounds: interactions with the gut and implications for human health. *Curr. Med. Chem.* **2001**, *8*, 1245–1255.
(28) Scalbert, A.; Morand, C.; Manach, C.; Remesy, C. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed. Pharmacother.* **2002**, *56*, 276–282.
(29) Seeram, N. P.; Lee, R.; Heber, D. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. *Clin. Chim. Acta* **2004**, *348*, 63–68.
(30) Edmonds, C. J. Absorption of sodium and water by human rectum measured by a dialysis method. *Gut* **1971**, *12*, 356–362.
(31) Braga, L. C.; Leite, A. A.; Xavier, K. G.; Takahashi, J. A.; Bemquerer, M. P.; Chartone-Souza, E.; Nascimento, A. M. Synergic interaction between pomegranate extract and antibiotics against *Staphylococcus aureus*. *Can. J. Microbiol.* **2005**, *51*, 541–547.
(32) Braga, L. C.; Shupp, J. W.; Cummings, C.; Jett, M.; Takahashi, J. A.; Carmo, L. S.; Chartone-Souza, E.; Nascimento, A. M. Pomegranate extract inhibits *Staphylococcus aureus* growth and subsequent enterotoxin production. *J. Ethnopharmacol.* **2005**, *96*, 335–339.

Received June 5, 2009. Revised manuscript received July 31, 2009. Accepted August 5, 2009. We thank POM Wonderful, Los Angeles, for financial support.