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Pomegranate ellagitannins stimulate growth of gut bacteria in vitro: Implications for prebiotic and metabolic effects

Zhaoping Li [a, b, d, e, *], Paula H. Summanen [b], Tomoe Komoriya [b, f], Susanne M. Henning [e], Ru-Po Lee [e], Eliisa Carlson [e], David Heber [e], Sydney M. Finegold [a, b, c, d]

[a] Department of Medicine, VA Medical Center West Los Angeles, 11301 Wilshire Blvd., Los Angeles, CA 90073, USA
[b] Department of Research Service, VA Medical Center West Los Angeles, 11301 Wilshire Blvd., Los Angeles, CA 90073, USA
[c] Department of Microbiology, Immunology and Molecular Genetics, UCLA School of Medicine, 405 Hilgard Ave., Los Angeles, CA 90095, USA
[d] Department of Medicine, UCLA School of Medicine, 405 Hilgard Ave., Los Angeles, CA 90095, USA
[e] UCLA Center for Human Nutrition, UCLA School of Medicine, 405 Hilgard Ave., Los Angeles, CA 90095, USA
[f] Department of Sustainable Engineering, College of Industrial Technology, Nihon University, Japan

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ABSTRACT

The present study investigated the effect of pomegranate extract (POMx) and pomegranate juice (POM juice) on the growth of major groups of intestinal bacteria: *Enterobacteriaceae*, *Bacteroides fragilis* group, clostridia, bifidobacteria, and lactobacilli, and the utilization of pomegranate polyphenols by *Bifidobacterium* and *Lactobacillus*. The total phenolic content of the pomegranate extract and juice was determined using the Folin-Ciocalteau colorimetric method and reported as gallic acid equivalent (GAE). The polyphenol composition was determined by HPLC. Stool specimens were incubated with 400, 100, and 25 μg/ml GAE POMx and POM juice and subjected to selective culture. *Bifidobacterium* and *Lactobacillus* strains were incubated with 400 μg/ml GAE POMx and POM juice and metabolites were analyzed. POMx and POM juice increased the mean counts of *Bifidobacterium* and *Lactobacillus* and significantly inhibited the growth of *B. fragilis* group, clostridia, and *Enterobacteriaceae* in a dose–response manner. *Bifidobacterium* and *Lactobacillus* utilized ellagic acid and glycosyl ellagic acid but little or no punicalin was utilized. Neither POMx nor POM juice was converted to urolithins by the test bacteria or the in vitro stool cultures. The effect of pomegranate on the gut bacteria considered to be beneficial (*Bifidobacterium* and *Lactobacillus*) suggests that pomegranate may potentially work as a prebiotic. The concept that polyphenols such as those in pomegranate impact gut microbiota populations may establish a new role for polyphenols in human health.

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1. Introduction

Growing evidence suggests that the gut microbiota can contribute to the development of metabolic dysregulation and inflammation both in the intestine locally and systemically in peripheral tissues and organs [1,2]. Diet can significantly alter gut microbiota [3]. Preclinical human and animal studies have demonstrated the health benefit of pomegranate fruit (*Punica granatum* L., Family *Lythraceae*) [4–8]. Pomegranate health benefits have been attributed to hydrolyzable tannins including ellagitannins (punicalagin and punicalin) and gallotannins and other phenolic compounds including phenolic acids, flavonols, and anthocyanins [9]. Pomegranate ellagitannins are not absorbed intact into the blood stream but are hydrolyzed to smaller phenolics such as ellagic acid by gut microbiota. While ellagic acid is absorbed into the blood stream, unabsorbed ellagitannins and ellagic acid are metabolized into urolithins by gut microbiota. While the ultimate evidence for prebiotic effects must be obtained in clinical studies, pomegranate polyphenols can alter bacterial populations in mixed cultures while also being metabolized by the bacteria to smaller metabolites such as urolithin A [10]. A number of in vitro studies have investigated the effects of pomegranate polyphenols on the growth of individual bacterial species. Pomegranate by-products and punicalagins significantly inhibited the growth of pathogenic *Escherichia coli*, *Pseudomonas aeruginosa*, clostridia and *Staphylococcus aureus* [11,12] and toxigenic *Clostridium difficile* [13].

* Corresponding author. David Geffen School of Medicine, UCLA, Warren Hall, 900 Weyburn Place, Suite 14-171, Los Angeles, CA 90095, USA.
E-mail address: ZLi@mednet.ucla.edu (Z. Li).

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Similarly, in vitro studies have demonstrated that pomegranate polyphenols may promote the growth of beneficial bacteria including *Bifidobacterium* spp. and *Lactobacillus* spp. [10,11]. The aim of the present study was to investigate the effect of pomegranate extract and pomegranate juice on the growth of major groups of intestinal bacteria from stool specimens: *Enterobacteriaceae*, *Bacteroides fragilis* group, clostridia, bifidobacteria, and lactobacilli. We also investigated the utilization of pomegranate polyphenols by *Bifidobacterium* and *Lactobacillus*. These studies contribute to the concept that pomegranate polyphenols have prebiotic activity and support the evidence being developed in clinical studies.

2. Material and methods

2.1. Phytochemical preparations

The pomegranate extract (POMx) and pomegranate juice (POM juice) were from POM Wonderful, Inc., Los Angeles, CA. The POMx consists of extract prepared from the whole fruit including the husks, seeds and peels remaining after juice production (POM Wonderful). POMx was prepared by dissolving 350 mg of POMx (capsule powder) in 50 ml boiling water, placed in the refrigerator (4 °C) until room temperature was reached and then centrifuged for 10 min at 2800 × g. The supernatant was filtered (MillexGP, PES membrane filter 0.22 μm, EMD Millipore, Billerica, MA) and frozen in aliquots. POM juice was centrifuged, filtered and aliquoted in the same way as the POMx extract. The total phenolics content of the POMx and POM juice was determined using the Folin-Ciocalteau colorimetric method [14] and reported as gallic acid equivalent (GAE). The polyphenol composition of the POMx solution and POM juice was determined by HPLC as previously described [15,16].

2.2. Stool specimens

The specimens were obtained from volunteers who were recruited into a POMx intervention study described elsewhere (Li, 2015, submitted). For the intervention study, any subjects with a history of cigarette smoking in the past 5 years, history of bleeding disorders, inflammatory bowel diseases (Crohn's/ulcerative colitis), irritable bowel syndrome, gastrointestinal surgery within the past 2 years, diabetes, cardiovascular disease, hypertension, regular intake of NSAIDs, steroids, or other anti-inflammatory medications, use of antibiotics (other than topical) in the past 2 months and current use of dietary supplements, including probiotics, prebiotics, and synbiotics were excluded. In this in vitro study we used the pre-treatment samples from 8 healthy subjects. The stool specimens were collected in a large, zip-lock freezer bag and all air was pushed out of the bag as the zip lock was closed and double-bagged in a clean zip-lock freezer bag. The specimens were immediately stored at - 20 °C and delivered on ice to the laboratory within 24 h of collection. The specimens were weighed and placed into an anaerobic chamber for processing. The stool samples were diluted in pre-reduced phosphate buffered saline (PBS) in appropriate amounts to allow mixing and then homogenized in a Waring blender.

2.3. Stool microbiology

Aliquots of 10 μl of the homogenized stool specimens were inoculated into seven different test broths: Thioglycollate (Thio, Anaerobe Systems, Morgan Hill, CA) broth (5 ml) as baseline, Thio broths supplemented with 400, 100, and 25 μg/ml GAE POMx and Thio broths supplemented with 400, 100, and 25 μg/ml GAE POM juice. The test tubes were incubated at 37 °C for 6 days. Selective and differential media were used as indicated to grow and enumerate *Enterobacteriaceae*, *B. fragilis* group, *Clostridium*, *Bifidobacterium* and *Lactobacillus*. After incubation, an aliquot from the test tubes was diluted 1:10 in pre-reduced PBS and plated (10 μl and 1 μl/plate using calibrated loops) onto Brucella blood agar (Anaerobe Systems) plates for total anaerobic count, Trypticase Soy blood agar (TSA, BD BBL, Sparks, MD) for total aerobic count, Bacteroides-bile-esculin agar (BBE; BD BBL) to enumerate *B. fragilis* group and MacConkey agar plates (BD BBL) to enumerate *Enterobacteriaceae*. The *Bifidobacterium* medium consisted of 42.5 g Columbia agar base (BD BBL), 5 g glucose, 0.4 g cysteine, 0.01 g riboflavin, and 0.8 ml propionic acid in 1 L dH_2O [17]. The *Lactobacillus* medium consisted of 84 g LBS agar (BD BBL) and 1.32 ml glacial acetic acid in 1 L dH_2O [18]. For the selective isolation of *Clostridium*, diluted aliquots of the stool specimens were incubated in 50% ethanol for 30 min, and subsequently plated onto Brucella agar plates. Brucella agar plates for total anaerobic count and the *Bifidobacterium* medium and *Lactobacillus* medium plates were incubated under anaerobic conditions at 37 °C for 7 days; BBE and Brucella plates for *Clostridium* enrichment were incubated under anaerobic conditions at 37 °C for 5 days. TSA blood agar plates were incubated under 10% CO_2 for 72 h and MacConkey agar plates aerobically for 24 h. Characteristic colony types on various selective media were counted. The identity of all colony types on the *Lactobacillus* and *Bifidobacterium* selective media were confirmed by Gram stain. Details of the culture techniques are described in the Wadsworth-KTL Anaerobic Bacteriology Manual [19].

2.4. Transformation of POMx by bifidobacteria and lactobacilli

Pure cultures of bifidobacteria and lactobacilli were used to examine urolithins and other metabolites formed from POMx (Table 2). The bacteria included were stool isolates from patients in the Greater Los Angeles VA Healthcare Center and reference strains from the American Type Culture Collection (ATCC). Altogether 29 *Bifidobacterium* strains including 7 ATCC strains and 4 *Lactobacillus* species were studied. Bacteria were identified by 16S rRNA sequence analysis. The *Bifidobacterium* and *Lactobacillus* strains were grown on Brucella agar plates under anaerobic conditions at 37 °C for 48 h. Anaerobic conditions consisted of a gas mixture of 5% CO_2, 5% H_2, and 90% N_2; the residual oxygen was removed by palladium catalysts. After incubation, an inoculum was prepared by making a suspension equal to MacFarland 0.5 standard (~1×10^8 cfu/ml) in 1 ml of phosphate buffered saline. 50 μl of each suspension was inoculated in 5 ml test broths yielding ~1×10^6 cfu/ml inoculum. The test broths consisted of a Thio as control, a Thio supplemented with 400 μg/ml GAE POMx, and a Thio supplemented with 400 μg/ml GAE POM juice. The test tubes were incubated at 37 °C for 48 h. A 10 μl aliquot was subcultured onto Brucella and TSA blood agar plates and incubated as described above to verify growth and viability. We also assayed for the presence of urolithins and other metabolites from stool in vitro culture tubes. Altogether 3 stool samples were tested (Table 3). As described above, 10 μl of the homogenized stool specimens were inoculated into Thio broth (5 ml) as control and Thio broths supplemented with 400 μg/ml GAE POMx and 400 μg/ml GAE POM juice. The test tubes were incubated at 37 °C for and assayed at 48 h and 6 days.

2.5. POMx conversion assay

The 48 h culture test tubes were centrifuged for 5 min at 3000× g. The supernatant was then dripped through solid phase extraction filter and dried. The dried sample was reconstituted with 200 μl DMSO and sonicated until completely dissolved. The