RESEARCH ARTICLE

Effect of daily intake of pomegranate juice on fecal microbiota and feces metabolites from healthy volunteers

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Scope: The purpose of the study was to evaluate the effect, regarding the metabolic and microbial profile of feces, of diet supplementation of healthy adults with pomegranate juice (PJ).

Methods and results: Twelve healthy adults were recruited to the study, which consisted of the intake of 200 mL/day of PJ during 4 weeks. Feces were collected before and after the supplementation with PJ. Metabolites (phenolic catabolites, short-chain fatty acids, and fecal steroids) and microbial profile were analyzed at baseline and at 4 weeks. Fecal phenolic metabolites, 3-phenylpropionic acid, catechol, hydroxytyrosol, and urolithin A, showed a significant increase in their concentration after supplementation with PJ. Among fecal steroids, parallel to the significant increase of cholesterol concentration, a significant decrease of coprostanol was observed. Although no significant changes in the microbiota profile were observed, different relationships between initial microbiota and the metabolites produced were found. Catechol showed positive and negative correlation with *Oscillospora* and *Paraprevotella* genera, respectively, and 3-phenylpropionic acid was positively correlated with *Odoribacter* genus.

Conclusion: Inclusion of PJ in the diet did not significantly alter the gut microbiota composition in healthy adults, but the individual bacterial composition could contribute to the generation of potential health-promoting phenolic metabolites.

Keywords:
Fecal steroids / Microbiota / Phenolic compounds / Pomegranate / Short-chain fatty acids

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1 Introduction

Gut microbiota represents the largest microbial environment of the human organism and its composition and interplay with the host are closely connected to healthy conditions [1].

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Abbreviations: **BAs**, bile acids; **FID**, flame ionization detector; **IBD**, inflammatory bowel disease; **NF-kB**, nuclear factor-kappa B; **PJ**, pomegranate juice; **SCFAs**, short-chain fatty acids

Colour Online: See the article online to view Fig. 2 in colour.

Intestinal microbiota markedly depends on diet and numerous nutritional interventions have been shown to selectively modify specific bacterial groups [2, 3]. Although some studies have been conducted on the effect of dietetic fiber as prebiotic [3], little is known about the ability of other components of fresh fruit, such as phenolic compounds, which are also important contributors to modulate the gut microbiota composition. Some studies have revealed that gut microbiota is able to transform intact phenolic compounds into bioactive metabolites and the accumulation of these microbial metabolites may be a key factor to promote changes in the gut ecosystem, including modifications of microbial population [4–6]. In general, human studies evidence that dietary polyphenols may contribute to the maintenance of intestinal health by preserving the gut microbial balance through the stimulation of the growth of beneficial bacteria (i.e. *Lactobacilli* and *Bifidobacteria*) and the inhibition of pathogenic bacteria, exerting prebiotic-like effects [5, 7]. Therefore, the analysis of fecal composition not only provides valuable information regarding the unabsorbed diet components, but also clarifies whether the functional stability of the gut ecosystem could undergo modifications after dietary interventions.

Apart from the study of the gut microbiota modulation through diet, recent studies have also revealed that fecal steroids might exert a much wider range of biological activities than initially recognized [8]. In this sense, it has been established that secondary metabolites of fecal steroids produced by the gut microbiota may promote health or favor disease development depending on the quantity and type produced. Specifically, increased concentrations of secondary bile acids (BAs) and cholesterol microbial metabolites in feces are involved in colorectal carcinogenesis [8, 9].

Therefore, it is interesting to study whether the fecal excretion of these metabolites can be modulated into a healthier profile through dietary intervention, as has already been demonstrated for phenolic compounds in rats [10]. On the other hand, the presence of other microbial products formed from nondigestible carbohydrates (dietary fiber), such as short-chain fatty acids (SCFAs) [11], could serve as a protector factor of gastrointestinal disease, limiting the generation of pathogenic bacteria as well as promoters of the growth of beneficial bacteria [12], and maintain optimal bowel balance [11, 13].

Pomegranate is one of the most polyphenol-rich fruits with many associated health benefits such as anti-inflammatory [14], anticarcinogenic [15] and cardioprotective effects [16] and are mainly attributed to its phenolic content [17]. Ellagitannins and anthocyanins are the most representative phenolic groups, followed by variable amounts of lignans, flavonoids, and phenolic acids [18, 19]. Most of these compounds are poorly absorbed in the small intestine and reach the large intestine, where they are subjected to catabolic transformations, being the urolithins derived from ellagic acid the most studied microbial phenolic metabolites [19, 20]. The microbial metabolites are thus likely to be responsible for the health benefits of pomegranate rather than the original compounds. However, to date no human studies have revealed whether pomegranate polyphenols could possess specific qualities to modulate the bacterial gut population and other steroid metabolites related to colonic health.

The purpose of the present study was to evaluate possible beneficial effects regarding the microbial and metabolic profile of feces from healthy adults after the addition of pomegranate juice (PJ; 200 mL/day) to the habitual diet during a period of 4 weeks. Changes in the microbial phenolic metabolites, SCFAs, and fecal steroids were evaluated. The modulation of the intestinal microbial population after the intervention was also evaluated, studying the relationship between possible changes in microbial metabolite profile and microbial composition for each individual. To our knowledge, this is the first study that evaluates the microbiota profile of healthy volunteers and the phenol metabolic profile of human feces after a PJ intervention.

2 Materials and methods

2.1 Pomegranate juice

The pomegranate fruits (*Punica granatum* L. *Mollar de Elche* cv.; growing area of Alicante, Spain) were purchased from a local market in Lleida (Catalonia, Spain). Fruits were washed and manually peeled. The PJ was prepared by blending the entire arils without the peel using a commercial blender with an incorporated filter (Molinex, France) in which pulp and intact seeds were retained. The juice was immediately divided into daily doses of 200 mL in amber glass bottles and stored at −20°C, and on the day of consumption the juice was defrosted at 4°C. The phenolic composition analysis was carried out by using Ultra-performance LC MS/MS methodology on the basis of the protocol of Mosele et al. [19]. The average phenolic content in the daily intake of 200 mL of PJ was: 878.9 mg ellagic acid and ellagitannins, 41.5 phenolic acids and its derivates, 38.0 anthocyanins, 4.21 lignans, 3.39 flavonols, 1.19 flavan-3-ols, 0.50 flavanones, and minor phenols as syringaldehyde and phloretin glucoside; Supporting Information Table 1). Two hundred milliliters of PJ contributed approximately 968 mg/day of phenolic compounds to the habitual diet of the volunteers. Values correspond to the average concentration of different aliquots ($n = 5$) collected over the 4 weeks of juice preparation.

2.2 Study design

The diet intervention study was approved by the Ethical Committee of Clinical Research of Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: CEIC-1326). Twelve healthy adults (4 men and 8 women, aged 26–40, BMI < 25 kg/m^2) were recruited to participate in the study. Inclusion criteria included healthy men and women aged between 26 and 40 years, not taking antibiotics at least 3 months

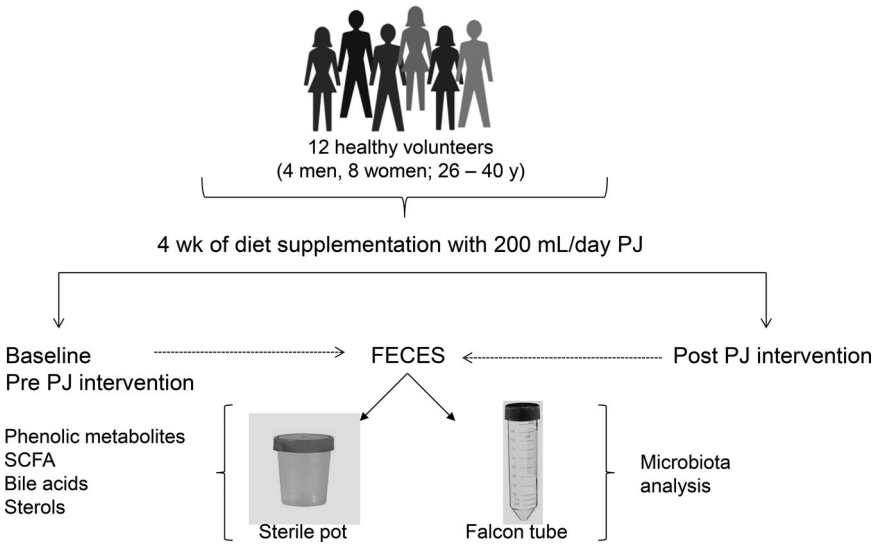

Figure 1. Graphic representation of the study design and sample collection.

before the study and reported to be in good health conditions before and during the study. After successful completion of screening, participants provided informed consent, including authorization to use their feces for analysis of microbiota and metabolites. Participants were instructed to maintain their regular diet, with the exception of probiotic consumption, in order to elucidate the gut metabolic profile and changes in microbiota population in a conventional diet complemented with the daily intake of PJ. They were also asked to complete a 3-day dietary record before and during the 4 weeks of PJ supplementation period which included 1 day of the weekend in order to consider all the dietary habits of volunteers. Mean total kilocalories of the volunteer diets and their composition in proteins, carbohydrates, and fat were estimated using Spanish food composition sheets (www.bedca.net), and the total intake of phenolic compounds was estimated using the information supplied by www.phenolexplored.eu. No significant differences were detected in energy, carbohydrate, protein, fat, and phenolic compound intake between baseline and the study period (see Supporting Information Table 2).

Before the intervention period with PJ, participants received detailed instructions to collect fecal samples and were provided with two different containers: a sterile pot and a falcon tube containing 10 mL of RNAlater® solution (Life Technologies). The volunteers were asked to transfer approximately 10 g of fresh feces from the sterile pot to the falcon tube immediately after defecation. Weekly, each volunteer received a box with seven bottles containing the daily PJ dose. During the intervention period the volunteers consumed 200 mL/day of PJ with breakfast during 4 weeks. Each volunteer collected the feces sample in fasted state before (Pre; day 0) and after (Post; day 28) the intervention period. The same day of collection, fecal samples were lyophilized and stored at −80°C until the chromatographic analysis to determinate phenolic microbial metabolites, SCFAs, and fecal steroids (BAs and sterols). The feces preserved in falcon tubes, also stored at −80°C, were used for the analysis of microbiota composition. Figure 1 represents the study design and the sample collection.

2.3 Phenolic quantification in feces

Lyophilized feces (0.1 g) were mixed in 1 mL of MeOH/HCl/H_2O (79.9:0.1:20, v/v/v) and centrifuged (8784 × *g*, 5 min, 4°C) after 15 min of shaking. The supernatant was collected and re-centrifuged under the same conditions. The resulting supernatant was filtered (0.22 μm filter pore size) and analyzed by ultra-high LC coupled to MS/MS (Supporting Information Method). All phenolic compounds present in the PJ and their potential catabolites formed by microbiota fermentation were studied. To avoid differences in the water content of feces, depending on the individual, the results are expressed in mg or ng of compound per gram of dry feces.

2.4 Determination of SCFAs in feces

Lyophilized feces (0.1 g) were mixed with 1 mL of acidified aqueous solution (1% phosphoric acid) containing 4-methyl valeric acid (Sigma-Aldrich, St. Louis, MO, USA) as internal standard (IS, final concentration 500 μM). Samples were shaken for 15 min and centrifuged (10 min, 1800 × *g*, 4°C). Before filtration (0.22 mm pore size filter), the supernatants were centrifuged (4 min, 8784 × *g*, 4°C) once more.

The analysis of acetic, propionic, butyric, isobutyric, isovaleric, and valeric acids was performed by GC (Agilent 7890A Series) using a capillary BP-21 column (30 m, 0.25 mm, 0.25 μm; SGE, Cromlab SL, Barcelona, Spain), coupled to a flame ionization detector (FID). The column temperature was programed at 90°C, rising by 15°C/min until it reached 150°C, then 5°C/min to 170°C, and then 20°C/min to 240°C, and maintained 3 min (total run time 14.5 min). Helium was the carrier gas (1 mL/min). Injection was carried out with

a split injector (1:100) at 220°C, detector temperature was 250°C, and 1 μL of the solution was injected into the GC/FID system. Identification of the SCFAs was carried out according to the retention time of standard compounds (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid; Sigma-Aldrich) and their quantification was determined with reference to the peak side of IS (4-methyl valeric acid; Supporting Information Fig. 1). All samples were analyzed in duplicate.

2.5 Analysis of fecal steroids: BAs and sterols

Silylation of sterols and BAs was carried out simultaneously. For this, 100 μL of pyridine and 100 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (both from Sigma-Aldrich) were added to a vial containing 10 mg of feces and two ISs (1000 μM of 5α-cholestane and 500 μM 5β-cholanic acid; Sigma-Aldrich) and then maintained during 30 min at 60°C. After silylation, samples were centrifuged 10 min at 8784 × *g* at room temperature. The supernatants were analyzed by GC system (Agilent 7890A) coupled to an FID. Samples were injected into a capillary column DB-1 (30 m × 0.25 mm × 0.25 i.d.) and the simultaneous quantification of sterols and BAs was performed as follows: the column temperature was set at 240–290°C (20°C/min) and maintained 2 min; 290–295°C (1°C/min) and 295–310°C (25°C/min) maintained during 5 min, total run time 16.1 min. The temperature of the injector was 280°C, the volume of injection was 1 μL and the FID temperature was 200°C. Peak identification was based on comparison of retention times with reference compounds. All quantifications were performed using calibration curves generated from different know concentrations of commercial standards. For fecal sterols quantifications, commercial standards of α-cholestane (IS), cholesterol, coprostanol, cholestanol, and cholestanone acid (Sigma-Aldrich) were used. In the case of fecal BAs commercial standards of 5β-cholanic acid (IS), cholic acid, deoxycholic acid, chenodeoxycholic acid, and lithocholic acid (Sigma-Aldrich) were used (Supporting Information Fig. 2).

2.6 Fecal microbiota composition analysis

2.6.1 DNA purification, amplicons, and sequencing

Fecal samples stored in RNAlater® were treated following Vázquez et al. [21]. Briefly, the samples were diluted with 5 mL of PBS solution. To remove fecal debris, the samples were centrifuged at 600 × *g* at 4°C for 2 min and then the supernatant was centrifuged at 25 364 × *g* for 5 min to pellet the cells. Total DNA was extracted from bacterial pellet with QIAamp® DNA Stool Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

A region of the 16S rRNA gene was amplified by PCR with the universal primers E8F (5′-AGAGTTT GATCMTGGCTCAG-3′) and 530R (5′-CCGCGGCKGC TGGCAC-3′) using the sample-specific Multiplex Identifier (MID) for pyrosequencing, and attached to the 454 Roche adaptors. The amplified region comprises hyper-variable regions V1, V2, and V3. PCR was run under the following conditions: 95° for 2 min followed by 28 cycles of 95° for 30 s, 52° for 1 min and 72° for 1 min and a final extension step at 72° for 10 min. After purification using NucleoFast® 96 PCR Clean-Up Kit (Macherey-Nagel), the PCR products were pooled and directly pyrosequenced using a Roche GS FLX sequencer and Titanium chemistry in the Centre for Public Health Research (FISABIO-Salud Pública, Valencia, Spain). All sequences were deposited in the public European Nucleotide Archive server under accession number PRJEB7859.

2.6.2 Composition, biodiversity, and interactions of fecal microbiota

16S rRNA gene reads with low-quality score (<20 out of 40 quality units assigned by the 454) and short read lengths (<170 nt) were removed. Potential chimeras were also removed from the remaining sequences using uchime in Qiime v1.8 pipe line [22]. Usearch tool in Qiime software was used to denoise data and to create the operational taxonomic unit (OTU) clusters (97%). Taxonomic information of the 16S rDNA sequences was obtained by comparison with the Ribosomal Database Project-II [23], using the pick_otus_through_otu_table.py pipe line available in QIIME v1.8.0 software. We considered only annotations that were obtained with a bootstrap value greater than 0.8, leaving the assignation at the last well-identified level and consecutive levels as unclassified (uc). To statistically assess the effect of the PJ on the bacterial composition, the Permutational Multivariate Analysis of Variance Using Distance Matrices (Adonis) was applied, as implemented in the Vegan package of the R software (Available from: http://CRAN.R-project.org/package=vegan and http://www.R-project.org/. Accessed October 14, 2013).

The association between the microbiota before PJ intervention period (Pre-treatment) and metabolite production was determined by applying a generalized linear regression model, using glmnet function in the "glmnet" R package [24]. The models were estimated by setting the genus frequencies before PJ as the predictor matrix, transformed by the Arcsin square root transformation, and the increment of the metabolites as the response vector. The Least Absolute Shrinkage and Selection Operator was used as the regression method to penalize the absolute size of the regression coefficients. The performance of the predictive model was validated by means of the *k*-mean cross-validation using cv. glmnet function in the "glmnet" R package. Those bacterial taxa that gave a good fit within the linear regression model were validated using the Spearman correlation index. Those genera that matched both criteria were selected as bacteria involved in the metabolite production.

Table 1. Changes in the phenolic compounds and their metabolites in human feces before (Pre) and after (Post) the supplementation period (4 weeks) with pomegranate juice (200 mL/day)[a)]

Compound	Pre	Post	*n* (%)
Urolithins (mg/g dry feces)			
Urolithin D (tetrahydroxy-urolithin)	n.d.	t.r.	1 (8)
Urolithin C (trihydroxy-urolithin)	0.07 ± 0.05	0.69 ± 0.31	6 (50)
Urolithin A (dihydroxy-urolithin)	7.00 ± 6.74	35.9 ± 11.3*	8 (67)
Isourolithin A (dihydroxy-urolithin)	n.d.	0.57 ± 0.57	1 (8)
Urolithin B (monohydroxyl-urolithin)	2.23 ± 2.02	9.47 ± 5.74	3 (23)
Anthocyanins (ng/g dry feces)			
Cyanidin-*O*-3,5-diglucoside	n.d.	112 ± 87.5	3 (25)
Cyanidin-*O*-3-glucoside	0.89 ± 0.89	27.5 ± 14.7	9 (70)
Cyanidin pentoside	n.d.	4.35 ± 2.98	3 (25)
Phenolic acids (mg/g dry feces)			
Phenylpropionic acid	5.80 ± 3.33	10.98 ± 3.69*	8 (67)
Protocatechuic acid	0.13 ± 0.03	0.18 ± 0.03	9 (75)
Other phenolic compounds (mg/g dry feces)			
Catechol	0.02 ± 0.01	0.05 ± 0.01**	10 (83)
Hydroxytyrosol	0.06 ± 0.01	0.13 ± 0.03*	10 (83)
Unidentified metabolites (mg/g dry feces)			
Unkown: m/z^- 333 MS^2 285, 259	1.25 ± 0.63	54.4 ± 16.8**	12 (100)
Total phenolic metabolites (mg/g dry feces)	16.6 ± 7.24	112 ± 19.1**	12 (100)

a) All values are means ($n = 12$) ± SE. Compared with corresponding Pre-value, *$p < 0.05$, **$p < 0.01$.
n.d., no detected; t.r., traces level.
n is number of volunteers who presented an increase in the concentration for each compound after pomegranate juice, and the percentage of the total 12 volunteers who presented an increase for each compound (%).

2.6.3 Functional prediction

Functional prediction was performed using the PICRUSt pipeline [25]. Usearch software was used to cluster the OTU sequences at 97% of identity, using the GreenGenes OTU database (version 13.5) as reference. OTUs were normalized by copy number using the normalize_by_copy_number.py script (version 1.0.0), and the functional predictions were estimated running the script predict_metagenomes.py, taking the KEGG database as reference. The resulting metagenomic prediction was then entered into the HUMAnN pipeline [26] in order to determine the abundance and coverage of putative microbial pathways. In the present study, we only predict the functional composition of all the OTUs whose taxonomic annotation includes *Oscillospira*, *Odoribacter*, or *Paraprevotella* genera.

2.7 General statistical analysis

The results were presented as mean values ± SE. Paired Student's *t*-test was used to analyze changes of phenolic compounds and their metabolites, SCFAs, BA, sterols, and tocopherol, in the feces before and after PJ intake. The biodiversity Shannon index was estimated with the Kruskal–Wallis rank sum test. The *p*-values in the correlation analysis were adjusted using the Benjamini–Hochberg correction.

3 Results

3.1 Effect of PJ intake on phenolic metabolite profile in human feces

Changes in some phenolic metabolite amounts were assessed in fecal samples of all volunteers before and after PJ intervention during 4 weeks (Table 1). It is most remarkable that five types of urolithins with different hydroxyl substitutions were detected in variable concentrations and high individual variability was observed in the fecal metabolism of pomegranate phenols. Urolithin A ($p < 0.05$) was the most abundant urolithin detected in larger amounts in eight out of 12 subjects, while isourolithin A (dihydroxyisourolithin) was quantified in the feces of one subject after the PJ intervention. Three volunteers produced higher amounts of urolithin B (hydroxyurolithin), especially in the isourolithin A producers. The excretion of urolithin C (trihydroxyurolithin) was detected in feces of six volunteers and urolithin D (tetrahydroxyurolithin) only in one volunteer. In three volunteers no class of urolithins was detected. Apart from urolithins, the phenolic acids 3-phenylpropionic acid ($p < 0.05$), catechol ($p < 0.01$), and hydroxytyrosol ($p < 0.05$) were also detected in significantly higher concentrations after the PJ intervention.

Native anthocyanins from PJ were detected with a large variability in concentrations (Table 1). Cyanidin 3-*O*-glucoside was present in nine out of 12 volunteers after the intake of PJ (Post) while cyanidin pentoside and cyanidin 3,5-*O*-diglucoside were detected in the feces of the same three

Table 2. Changes in the SCFAs composition in human feces before (Pre) and after (Post) the supplementation during 4 weeks with pomegranate juice (200 mL/day)[a)]

	Concentration (mg/g dry feces)		Molar ratio (% mol of total)	
SCFAs	Pre	Post	Pre	Post
Acetic acid	11.98 ± 1.78	13.53 ± 1.70	57.48 ± 1.24	57.63 ± 1.80
Propionic acid	5.05 ± 0.82	4.94 ± 0.64	19.19 ± 1.47	17.15 ± 0.90
Butyric acid	5.05 ± 1.38	5.37 ± 1.01	15.31 ± 1.31	18.08 ± 0.96
Isobutyric acid	0.63 ± 0.09	0.68 ± 0.10	2.46 ± 0.29	2.14 ± 0.38
Isovaleric acid	1.05 ± 0.14	0.93 ± 0.19	3.37 ± 0.46	2.68 ± 0.60
Valeric acid	0.73 ± 0.12	0.82 ± 0.12	2.19 ± 0.25	2.35 ± 0.42
Total	24.49 ± 4.00	26.26 ± 3.17		

a) All values are means ($n = 12$) ± SE.

volunteers. The detection of an unknown compound (m/z^- 333, MS^2 285, 259) in the feces of all volunteers is noteworthy, a significant increase of this metabolite being observed following the PJ intake ($p < 0.01$). Neither ellagic acid nor ellagitannins were recovered in their native form present in PJ in any of the feces analyzed.

3.2 Effect of PJ intake on SCFAs and steroid (BAs and sterols) composition in feces

Results of SCFAs (acetic, propionic, butyric, isobutiric, valeric, and isovaleric acids) concentrations are reported in Table 2. No significant statistical differences were observed between Pre and Post PJ intake in the concentration of total and individual SCFAs (mg/g dry feces). However, when the data were normalized to molar ratio (percentage of individual SCFAs in relation to total SCFAs) differences were observed between Pre and Post PJ intake.

Changes in fecal BA concentration are shown in Table 3. Although no significant changes were observed after the PJ consumption period, an increasing trend of primary BA was observed. Regarding the fecal sterols, cholesterol increased its concentration in feces ($p < 0.05$) and the concentration of coprostanol suffered a significant reduction ($p < 0.05$; Table 3).

Table 3. Changes in the bile acids (BA) and sterols composition in human feces before (Pre) and after (Post) the supplementation during 4 weeks with pomegranate juice (200 mL/day)[a)]

Compound	Concentration (mg/g dry feces)	
	Pre	Post
Primary BA		
Cholic acid	0.11 ± 0.07	0.19 ± 0.13
Chenodeoxycholic acid	0.09 ± 0.03	0.15 ± 0.06
Total	0.20 ± 0.08	0.33 ± 0.18
Secondary BA		
Lithocholic acid	1.54 ± 0.20	1.55 ± 0.21
Desoxycholic acid	1.99 ± 0.40	1.82 ± 0.35
Total	3.53 ± 0.57	3.37 ± 0.50
Primary + secondary BA	3.73 ± 0.61	3.70 ± 0.55
Sterols		
Cholesterol	1.88 ± 0.53	5.60 ± 1.56*
Colestanol	0.54 ± 0.12	0.51 ± 0.19
Coprostanol	20.33 ± 4.32	13.9 ± 4.12*
Total	22.7 ± 4.32	19.8 ± 3.48

a) All values are means ($n = 12$) ± SE; compared with corresponding Pre-value, *$p < 0.05$.

3.3 Effect of PJ intake on gut microbiota composition

Despite some intraindividual differences, the bacterial composition of all the fecal samples, before and after PJ intervention during 4 weeks, was rather homogeneous since the distribution of genus abundance was similar (Shannon index, p value = 0.386). *Bacteroides* (20.372 ± 9.128%) and *Faecalibacterium* (23.138 ± 12.279%) were the most abundant genera. We also found that commensal members of intestinal community, such as *Prevotella* (3.913 ± 6.76%), *Oscillospira* (3.482 ± 4.075%), *Lachnospira* (2.99 ± 2.571%), *Roseburia* (2.615 ± 1.964%), *Parabacteroides* (2.398 ± 3.345%), *Ruminococcus* (1.659 ± 2.429%), and *Coprococcus* (1.605 ± 1.354%; Fig. 2; Supporting Information Fig. 3 shows the dispersion of the samples before and after PJ). We performed a statistical analysis to explore the variation in bacterial composition introduced by the intervention, revealing that the PJ did not make significant changes in the microbiota (Adonis, $p = 0.956$). Neither PJ effect on microbiota composition was found when we analyzed independently the males (Adonis, $p = 0.842$) and the females (Adonis, $p = 0.915$).

3.4 Interactions between fecal microbiota and metabolite production

As no changes in bacterial community were detected after PJ, we asked whether the production of certain metabolites for each volunteer is related to specific bacterial genera present in initial microbiota. In order to study the relationships

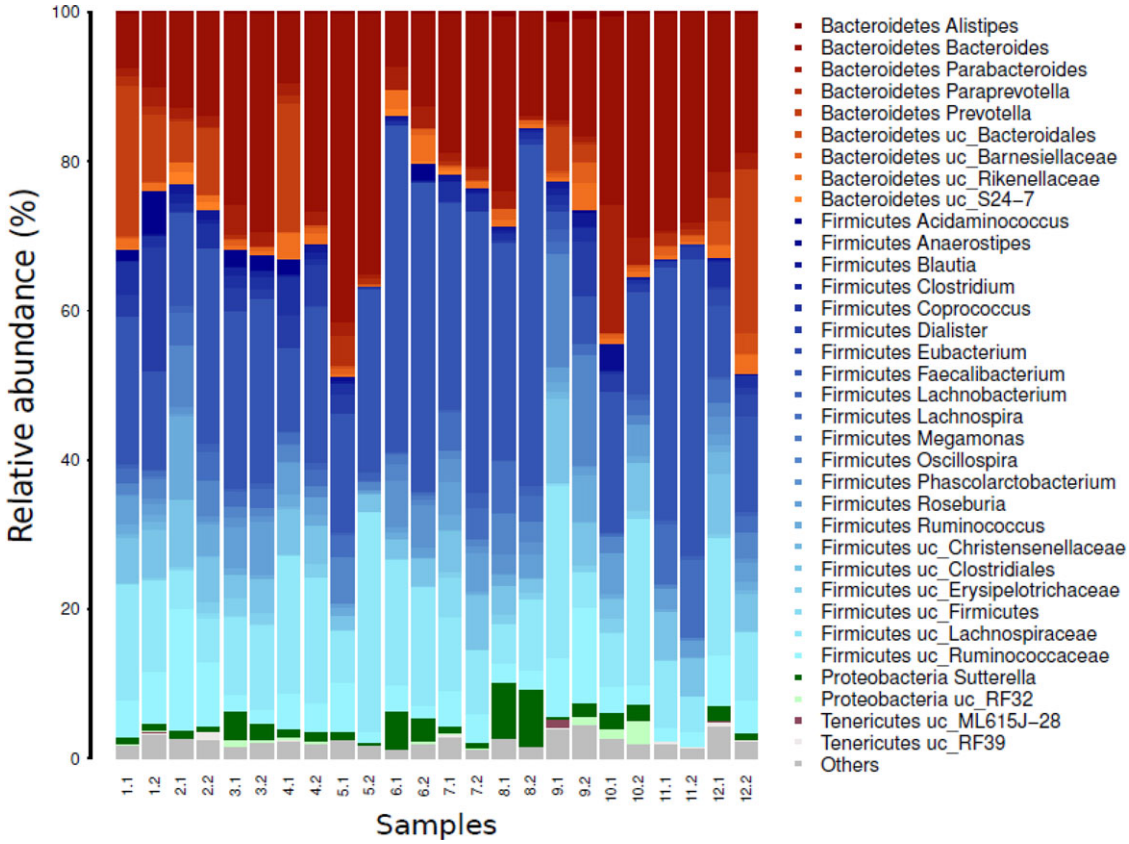

Figure 2. Fecal microbiota composition in the individuals analyzed before and after PJ. The phylum and the genus level are shown for the most abundant bacterial groups (>1%).

between metabolites produced and initial microbiota (Pre PJ), we performed a generalized linear regression model analysis. We identified three genera that showed significant correlation with two phenolic metabolites detected in feces. Catechol was positively correlated with *Oscillospira* genus and negatively correlated with *Paraprevotella* genus, while 3-phenylpropionic acid showed a positive correlation with *Odoribacter* genus (Table 4). Both phenolic metabolites presented a significant increase after the PJ supplementation (4 weeks). These three genera, *Oscillospira*, *Paraprevotella*, and *Odoribacter*, were low abundant in the initial microbiota (4.021 ± 4.245%, 0.821 ± 1.174%, and 0.123 ± 0.169%).

To determine if the metabolic pathways involved in the production of these phenolic metabolites are present in these members of fecal microbiota, we obtained a prediction of metagenome functional content from each genera using bioinformatic PICRUSt and HUMAnN tools [25, 26]. Thus, for *Oscillospira*, the functional category "Xenobiotics Biodegradation and Metabolism" contained the different pathways (benzoate degradation, naphthalene degradation, aminobenzoate degradation, polycyclic aromatic hydrocarbon degradation, dioxin degradation, and chlorocyclohexane and chlorobenzene degradation) where cathecol could be synthesized. For *Paraprevotella*, we obtained a functional prediction in eight of the 12 samples, the same metabolic pathways being represented. Table 5 shows the relative abundance and coverage of each pathway. The 3-phenylpropionic acid could be involved in three KEGG pathways:

Table 4. Significant interactions between bacterial genera and phenolic metabolites in feces

Phenolic metabolite	Genus	GLM coefficient	Spearman *rho* coefficient	*p*-Value	*q*-Value[a)]
Catechol	*Paraprevotella*	−6.809	−0.822	0.001	0.012
Catechol	*Oscillospira*	3.297	0.685	0.012	0.087
3-phenylpropionic acid	*Odoribacter*	2.179	0.643	0.0278	0.093

a) *q*-Value is the *p*-value adjusted using the Benjamini–Hochberg correction.
GLM, generalized linear regression model.

Table 5. Metabolic pathways related to the cathecol in *Oscillospira* and *Paraprevotella*

	Oscillospira		*Paraprevotella*	
Pathways	Abundance (%)	Coverage (%)	Abundance (%)	Coverage (%)
ko00361: Chlorocyclohexane and chlorobenzene degradation	0.0497 ± 0.0485	5.701755 ± 1.5193	0.0343 ± 0.0339	5.2631 ± 2.8132
ko00362: Benzoate degradation	0.4946 ± 0.1152	12.0000 ± 0.0000	0.1347 ± 0.1021	8.9999 ± 4.0158
ko00621: Dioxin degradation	0.3339 ± 0.0948	7.6923 ± 0.0000	0.0112 ± 0.0234	5.7692 ± 7.9622
ko00624: Polycyclic aromatic hydrocarbon degradation	0.2293 ± 0.0489	2.9412 ± 0.0000	0.1718 ± 0.1274	3.3088 ± 1.0398
ko00626: Naphthalene degradation	0.6160 ± 0.0604	13.6905 ± 1.3902	0.3072 ± 0.2328	16.0714 ± 3.3064
ko00627: Aminobenzoate degradation	0.2922 ± 0.1044	7.3446 ± 0.8345	0.1308 ± 0.0980	7.86128 ± 2.5255

The relative abundance is expressed as percentage based on the total pathways predicted. The coverage represents the percentage of the pathway that has been found in the prediction.

phenylalanine metabolism, degradation of aromatic compounds, and microbial metabolism in diverse environments. In the *Odoribacter* genome, we found only the phenylalanine metabolism pathway with a relative abundance and coverage of 0.3456 ± 0.0675 and 17 ± 8.00, respectively.

4 Discussion

To our knowledge, this is the first study that evaluates the interaction between the gut microbiota composition, the nature of the biotransformation products of pomegranate polyphenols, and the modulation of other fermentation products such as fecal steroids and SCFAs. Regarding the fecal microbial metabolism of pomegranate polyphenols, the main metabolites detected in feces after the intervention period with PJ were urolithins, which is in accordance with previous works where the predominant fecal metabolites after ellagitannin-rich food administration were urolithins [20]. Besides that, other phenolic metabolites such as 3-phenylpropionic acid and catechol have been described for the first time in human feces after pomegranate consumption which could be products from the anthocyanin colonic metabolism, as previously described in an in vitro colonic fermentation of a red wine extract [27] and cyanidin [28]. Hydroxytyrosol, which has normally been associated with the intake of virgin olive oil phenols [29], presented a significant increase in feces after PJ consumption in the present study. In a previous study, tyrosol appeared as a phenolic catabolite after an in vitro colonic fermentation of raspberry anthocyanins [28], so we hypothesize that hydroxytyrosol could also derive from pomegranate anthocyanins colonic metabolism. Related to the native structures of anthocyanins present in PJ, an important increase in their concentration in feces was observed after the intervention period with PJ, which indicated that these phenolic compounds could resist the digestion conditions and reach the large intestine. As demonstrated in the present study, urolithins, together with the phenolic acid metabolites and the nondegraded anthocyanins, are able to persist in the intestinal lumen and their contact with the gut epithelium and other compounds from diet could modulate some of the mechanisms involved in intestinal diseases.

The biological mechanisms proposed for these phenolic catabolites have been previously described, being the maintenance of adequate organic antioxidant status, the increase in anti-inflammatory defense and the protection against intestinal pathologies [30, 31]. Remarkable activities associated with antioxidant effects against radical oxygen species have been noticed for urolithins [32, 33], catechol [34], hydroxytyrosol [35], and cyanidin [36]. The capacity of phenolic compounds to regulate the signaling pathways, such as nuclear factor-kappa B (NF-kB) and mitogen-activated protein kinases, responsible for the regulation of proinflammatory modulators, has been the central topic in recent studies [36–39]. In the case of the fecal phenolic catabolites of pomegranate, urolithins A and C were effective in the inhibition of cytokine-induced proinflammatory markers TNF-alfa and IL-6, respectively, in THP-1 cell line-derived macrophages [38]. In addition, urolithin A was also effective at reducing the levels of PGE2, PAI-1, and IL-8 in parallel with the inhibition of colon fibroblast migration and monocyte adhesion to fibroblast [39]. Catechol also showed a reduction in the expression of adhesion molecules in a microvascular endothelial cell line [40], in addition to NF-kB factor inhibition [41]. Cytokine-stimulated human HT-29 cells treated with cyanidin 3-glucoside also reduce the production of the proinflammatory mediators as well as the amounts of activated STAT1 [42]. Several studies based on rodents induced inflammatory bowel disease (IBD) highlighted the properties of rich-ellagic acid [43] and hydroxytyrosol [44] based diets, observing a reduction in intestinal inflammation promoters and decreasing colon epithelial damage. All these data suggest that, apart from the classical urolithins derived from pomegranate, other fecal metabolites described in the present study, such as phenolic acids or intact anthocyanins, might also be potential candidates to keep down the inflammatory response, especially in IBD pathogenesis.

As inflammation is a manifestation of oxidative stress and the intensity and duration of the inflammation process were also linked to the development of cancer [45, 46], the phenolic compounds detected in feces in our study could play a role in the prevention of the proliferation of cancer cells by control of oxidative stress and inflammation episodes. In this sense, several in vitro studies have described specific anticarcinogenic effects of urolithin A [47], catechol [48], and hydroxytyrosol [49]. Molecular mechanisms, such as

inhibition of COX-2 in the case of 3-phenylpropionic acid [50] and downregulation of AP-1 and NF-kB activity by anthocyanins [36], were proposed as chemopreventive activity. We hypothesize that a broad spectrum of the latter-mentioned functions could occur, not only due to urolithins, but as a consequence of the synergistic combination of all the microbial phenolic metabolites detected after PJ intake in our study. The sustained intake of PJ could also potentiate their effects, increasing the time of their exposure to gut epithelium, with a consequent restriction of the oxidative damage and limiting the inflammatory episodes, contributing to reducing the risk of colon cancer and IBD development as well as to achieving remission of symptoms.

There is limited information regarding the ability of dietary components, including phenolic compounds, to influence the gut microbial population. To address this, we investigated the influence of pomegranate intake on the composition of human fecal microbiota. Only one study has been performed in animals suggesting that pomegranate polyphenols can be very active at the gut level, modulating the gut microbial population [51]. Contrary to this study, we showed, using high-throughput sequencing, that a 4 weeks daily ingestion of 200 mL of PJ that represented a daily intake of around 968 mg of pomegranate phenols (Supporting Information Table 1) did not significantly modify the composition of the individual gut microbiota in healthy humans. In another study, no differences in fecal microbiota profile were detected after consumption of a probiotic-enriched yogurt [52]. These results are in good agreement with our observations. This may be related to the microbial resilience reported under normal physiological conditions, which contributes to maintaining homeostasis in healthy adults [53]. Moreover, Espin et al. [54] observed the conversion of ellagic acid to urolithins in the jejunum of pigs fed with oak acorns (rich source of ellagitannins), associated with special ellagic acid converter microbial groups situated in upper segments of the gastrointestinal tract. Due to the difficulty in obtaining internal human samples, microbial analyses are normally carried out in feces, whose bacteria profile may differ from the microbial population of the intestinal mucosa and small intestine segments [55]. This could explain that no relation between the bacteria profile and urolithin production was inferred in our study.

However, we identified catechol that was positively correlated with *Oscillospora* genus and negatively correlated with *Paraprevotella*, while 3-phenylpropionic acid showed a positive correlation with *Odoribacter* genus. Since none of these genera are abundant members of the fecal microbiota, they were poorly described in human studies and scarce information is available in terms of reference genomes. However, by bioinformatics we detected several pathways involved in the production of catechol in *Oscillospira*. Thus, the positive correlation between this genus and catechol in feces indicates that *Oscillospira* could be involved in the conversion of pomegranate phenolics into catechol based on metabolic predictions and, therefore, volunteers presenting higher proportions of this specific genus could also present higher concentrations of catechol. Despite the fact that *Paraprevotella* presented the same pathway profile as *Oscillospira*, we found a negative correlation between the fecal catabolite and this genus. This result may indicate differences in enzymatic composition within the catechol pathways involved. Thus, this bacterium could transform the phenolic compounds from PJ in a different manner, yielding other products. Furthermore, *Ododribacter* genus was positively correlated with 3-phenylpropionic acid. We found that this compound is only synthesized in the phenylalanine metabolism pathway, as occurred in *Odoribacter splanchnicus*, whose genome has been recently sequenced [56]. Further studies are needed to obtain direct evidence of metabolic capabilities of these genera.

The modulation observed in the sterols profile and the slight changes in BA and SCFA might mean that pomegranate phenolic compounds are not directly involved in their formation or excretion rate but may interfere with the activity of some microbial enzyme, as has been reported after fiber fermentation [57]. This may explain the differences among molar ratios of butyrate, less conversion of secondary BAs, and less conversion of cholesterol to coprostanol. According to recent studies [57], these changes in the proportions and profile of fecal SCFAs, BAs, and sterols could contribute to the health status of the gut. Butyric acid, the main energy source of intestinal cells, supports metabolic health by preventing the development of abnormal intestinal cells as well as by the selective induction of apoptosis of cancer cells [58]. High fecal secondary BAs and coprostanol enhance the lumen toxicity, which increased the incidence of colorectal cancer, and it is therefore expected that the change in the profile of these compounds after PJ intake could potentially exert protective action against colon cancer.

In conclusion, although the inclusion of PJ in the diet did not significantly alter the gut microbiota composition, specific genera, as *Oscillospira* and *Odoribacter*, could contribute to the generation of potential health-promoting phenolic metabolites in the individuals that harbor these bacteria. Besides that, we found that the inclusion of PJ in the diet modulates the concentration in feces of fecal metabolites of interest, as SCFAs, sterols, and secondary BAs. Further research should be undertaken to unravel the bacterial mechanisms involved in metabolite production. The comprehensive identification of bacteria groups associated with certain food components, such as the case of phenolic compounds, is a pending task and must be considered essential to establish dietetic recommendation, functional food design, or provide information to develop a therapeutic target to prevent or treat intestinal diseases.

The authors' responsibilities were as follows—M.J.M. and J.I.M.: designed the research; J.I.M. and L.R.: conducted the research and had responsibility for the human intervention study; A.M. and J.I.M.: conducted the chromatographic analyses; N.J.H. and J.I.M. conducted pyrosequencing; M.J.G., J.F.V.C., N.J.H., A.M., A.L.: analyzed the gut microbiota; all authors analyzed

data and wrote the manuscript; and M.J.M.: had primary responsibility for the final content. All authors read and approved the final manuscript.

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