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Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism☆

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Abstract

Whether the beneficial effects of pomegranate are due to the ellagitannins or to their microbiota-derived urolithins is not known. Our objectives were to evaluate the effects of pomegranate intake and its main microbiota-derived metabolite urolithin-A (UROA) on colon inflammation and to assess whether UROA is the main anti-inflammatory compound. In addition, the effect of the inflammation on the phenolic metabolism was also explored. Male Fisher rats were fed with 250 mg kg^{-1} day^{-1} pomegranate extract (PE) or 15 mg kg^{-1} day^{-1} UROA for 25 days. Dextran sodium sulfate (5%) (DSS) was administered for the five last days and then rats were euthanized. DSS is a well-known model of inflammatory bowel disease. Colon tissue damage, microbiota changes, antioxidant status, prostaglandin E_2 (PGE_2), nitric oxide production, inducible nitric oxide synthase (iNOS), prostaglandin E synthase (PTGES), gene expression (microarrays and RT-PCR) and polyphenol metabolism (LC-MS-MS) were evaluated. Both PE and UROA decreased inflammation markers (iNOS, cycloxygenase-2, PTGES and PGE_2 in colonic mucosa) and modulated favorably the gut microbiota. The G_1 to S cell cycle pathway was up-regulated in both groups. UROA group showed various down-regulated pathways, including that of the inflammatory response. PE, but not UROA, decreased oxidative stress in plasma and colon mucosa. Only UROA preserved colonic architecture. The normal formation of urolithins in PE-fed rats was prevented during inflammation. Our results suggest that UROA could be the most active anti-inflammatory compound derived from pomegranate ingestion in healthy subjects, whereas in colon inflammation, the effects could be due to the nonmetabolized ellagitannin-related fraction.

Keywords: Dextran sulfate sodium; Gut microbiota; Microarray; COX-2; Bowel disease; Polyphenol

1. Introduction

Nowadays, there is an increased incidence of acute colon inflammation episodes that can be elicited by viral or bacterial infection, stress, or allergy to some peptides. Chronic inflammation bowel diseases (IBDs) like ulcerative colitis and Crohn disease have also risen in the last few years [1]. Several genes that are up-regulated in colon inflammation remain elevated in colonic cancer, and oxidative stress that accompanies chronic inflammation also contributes to the trigger of dysplasia [2].

The etiology of IBDs is not well understood, but two broad hypotheses have arisen regarding the fundamental nature of the pathogenesis of IBDs. The first contends that primary deregulation of the mucosal immune system leads to excessive immunologic responses to normal microbiota. The second one suggests that changes in the composition of gut microbiota and deranged epithelial barrier function elicit pathologic responses from the normal mucosal immune system [3].

Since ancient times, pomegranate has been regarded as a "healing food" with numerous beneficial effects on several diseases. Indeed, pomegranate has been used as an anti-helmintic and vermifuge and to cure aphtae, ulcers and diarrhea in folk medicine. The studies carried out today indicate that our ancestors were not entirely wrong and that some of properties of pomegranate were not unfounded. In vitro and in vivo studies have demonstrated antioxidant, anti-inflammatory and anticancer properties of pomegranate. Administration of 50 mg/kg of pomegranate peel extract for 28 days to rats

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with liver fibrosis decreased malondialdehyde (MDA) levels and myeloperoxidase activity as well as TNF-α and IL-1β levels [4]. Also, pomegranate juice, as a substitute for drinking water for 1 month, reduced basal levels of hepatic oxidative stress, decreasing DNA damage [5]. In a model of rheumatoid arthritis, pretreatment with 13.6 mg/kg of pomegranate extract (PE) decreased the arthritis incidence and IL-6 and IL-1β levels in arthritic joints [6]. Nevertheless, these studies did not take into account that when pomegranate compounds are ingested, only a fraction is absorbed. In fact, our group demonstrated for the first time that ellagitannins from pomegranate and other sources are metabolized to urolithins by colonic microbiota [7–10]. These metabolites are subsequently absorbed and reach a number of organs [10]. Urolithin properties have not been deeply studied. We have previously reported the anti-estrogenic/estrogenic activity of urolithins due to their structural analogy to estrogens [11] as well as their antiproliferative activity due to the modulation of gene expression including MAPK signaling pathways [12]. Other authors have reported that ellagic acid and several synthesized urolithins inhibited the growth of human prostate cancer cells in vitro [13]. In addition, an ellagitannin-rich PE has been shown to inhibit angiogenesis in prostate cancer in vitro and in vivo, suggesting that ellagitannins are the most active fraction responsible of the effect [14]. Therefore, pomegranate properties could be mediated by the in vivo produced metabolites in addition to the original phenolic compounds present in the food matrix. Human subjects can be divided into high and low urolithin producers [15]. Whether a high or low urolithin production is beneficial or indifferent remains unanswered so far.

There is increased evidence regarding the involvement of gut microbiota on the health-beneficial effects of foods. For example, Russell et al. [16] have suggested that blueberry phenolic transformations by gut microbiota determine the action of these compounds inhibiting prostanoid production.

In view of the antioxidant, anticancer and anti-inflammatory properties of pomegranate phenolics and/or its derived metabolites, we could hypothesize that PE and/or their derived metabolites could have a beneficial effect on colon inflammation. Our aim was to evaluate the effects of diets supplemented with PE and urolithin-A (UROA) (the main in vivo-derived metabolite) in a dextran sodium sulfate (DSS)-induced colon inflammation rat model (a well-known model that mimics an inflammatory bowel disease). The underlying objectives were to assess whether the effects are due to the ellagitannins or to their microbiota-derived urolithins and to check the effect of inflammation on phenolic metabolism. For this purpose, colon tissue damage, microbiota changes, reactive oxygen species (ROS), gene expression profile, polyphenol metabolism as well as a number of inflammatory markers including prostaglandin and nitric oxide production were explored.

2. Materials and methods

2.1. Animals, diets and experimental design

Experiments followed a protocol approved by the local animal ethics committee and the local government. All experiments achieved were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC). Male Fischer rats (n=32) with weights ranging from 175 to 200 g were provided by the Animal Centre of the University of Murcia (Spain). Animals were randomly assigned to four groups (n=8 rats per group). Each group was housed in four different cages (two rats per cage) in a temperature-controlled environment (22±2°C) with 55±10% relative humidity and controlled lighting (12-h light/dark cycle). All rats were fed for 25 days with rat standard chow (Panlab, Barcelona, Spain) containing 14.5% proteins, 63.9% carbohydrates and 4% fat (3.2 kcal/g). The four groups were distributed as follows: control group fed with standard feed; DSS group (DSS) received the standard diet for 25 days plus 5% DSS (dextran sulfate sodium salt, average molecular weight 36,000–50,000, MP Biomedicals, Illkirch, France) for the last 5 days of the experiment; DSS-PE and DSS-UROA groups received the standard chow supplemented with 250 mg kg^{-1} day^{-1} of PE ("Nutragranate" from Nutracitrus S.L., Elche, Spain) and 15 mg kg^{-1} day^{-1} of UROA (Kylolab S.L., Murcia, Spain), respectively, for 25 days plus 5% DSS also for the last 5 days of the assay. The PE dose assayed was equivalent to 36 mg kg^{-1} day^{-1} in humans (2.5 g PE in a 70-kg person) and the UROA dose to 2.2 mg kg^{-1} day^{-1} (154 mg UROA in a 70-kg person) according to the human equivalent dose formula: HED=animal dose in mg/kg×(animal weight in kg/human weight in kg)$^{0.33}$ [17].

Acute colitis was induced by giving 5% DSS to the DSS, DSS-PE and DSS-UROA groups for the last 5 days. DSS was administered in sterilized tap drinking water ad libitum. DSS solution was freshly prepared, and both food and water intake were measured daily. After the fifth day of colitis induction, rats were anaesthetized with a mixture (1:1 v/v; 1 ml/kg body weight) of xylazine (Xilagesic 2%, Calier Laboratories, Barcelona, Spain) and ketamine (Imalgene 1000, Merial laboratories, Barcelona, Spain) and sacrificed by decapitation.

Two more groups of male Fischer rats (n=4 per group) with the same weights than those described above were used to check the metabolism of PE and UROA in the absence of DSS, that is, metabolism in healthy rats. In this case, standard chow was supplemented with the same amounts of both PE and UROA as described above for 5 days (in the absence of DSS). Rats were anaesthetized and sacrificed as described above.

2.2. Sampling procedure

PE (10 mg) was dissolved in a solution of 1 ml water/formic acid (99:1) (PE was freely soluble). The solution was filtered through a 0.45-μm membrane filter Millex-HV13 (Millipore Corp., Bedford, USA) and an aliquot of 10 μl was analyzed by HPLC-MS-MS.

Blood samples (approximately 3 ml) were obtained immediately after decapitation and collected in EDTA-treated tubes to obtain the hematological profile.

Colons were removed and rinsed with PBS, dried on blotting and distal colon mucosa was scraped using a glass slide. Samples were either snap frozen on liquid nitrogen and stored a −80°C until analysis or kept in RNAlater solution (Ambion, Madrid, Spain). Tissue samples were excised and fixed in neutral-buffered formalin.

Samples of freeze-dried feces (0.2 g) were processed as reported previously [10]. An aliquot of 50 μl was diluted by adding 50 μl water, filtered through a 0.45-μm membrane filter Millex-HV13 (Millipore Corp.) and injected (6 μl) in the HPLC-DAD-MS-MS equipment.

2.3. Hematology

Hematological parameters were determined using an automated hematological analyzer (Abacus Junior Vet, CVM S.L., Navarra, Spain) with specific software for rat blood samples. The parameters analyzed were red blood cell (RBC) number, hemoglobin (Hb) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular Hb (MCH), mean corpuscular Hb concentration (MCHC) and white blood cell (WBC) count. The normal value range (NVR) was calculated for each parameter as the mean of control group value ±2×S.D.

2.4. Histological analyses

Tissue samples from the distal colon were fixed in 10% neutral buffered formalin, dehydrated in graded ethanol series and embedded in paraffin. For histological evaluation, 5-μm-thick tissue sections were stained with hematoxylin–eosin and observed under a Leica DMRB light microscope (Leica Ultracut, Wetzlar, Germany). The mucosal damage to evaluate the degree of colitis was scored according to Araki et al. [18]. The samples were graded 0–4 for surface epithelial loss, crypt destruction and inflammatory cell infiltration into the mucosa (maximum score=12).

2.5. Fecal microbiota analysis

Fecal samples were collected at Day 0, 10, 20 and in the day of sacrifice and homogenized in buffered peptone water (100 mg/ml) (AES Laboratoire, Combourg, France) (1:10 dilution) using filter stomacher bags (Seward Limited, London, UK) and a stomacher (IUL Instrument, Barcelona, Spain) for 90 s. The appropriate dilutions were spread onto different agar media. Lactobacilli and bifidobacteria were enumerated on Man Rogosa Sharpe (MRS) agar and MRS agar supplemented with 0.5 mg/L dicloxacilin, 3 g/L LiCl and 0.5 g/L L-cysteine hydrochloride, respectively. Enterobacteria and *Escherichia coli* were obtained by inoculating decimal dilutions in violet red bile glucose agar and chromocult coliform agar, respectively. *Clostridium* spp. were enumerated on reinforced clostridial containing 20 μg/ml of polymixine sulphadiacine. Culture plates were incubated at 37°C for 24–48 h in an anaerobic chamber (Don Whitley Scientific Limited, Shipley, UK) (CO2:H2:N2, 5:15:80). Similarly, total aerobic bacteria were enumerated by the standard plate count method on brain heart infusion agar after incubation in aerobic conditions at 37°C for 48 h. All media were obtained from Oxoid (Basingstoke, UK), whereas antibiotics and other supplements were obtained from Sigma (St. Louis, MO, USA). Microbial counts were expressed as log CFU/g. The mean and standard error per group were calculated from the log values of the CFU/g.

2.6. RT-PCR

Total RNA was isolated from rat distal colon mucosa using the Rneasy midi kit (Qiagen, Milan, Italy). For first-strand cDNA synthesis, 1 μg of RNA from each sample was reverse-transcribed using 100 U RT superscript II and 1×random hexamers (Roche, Monza, Italy). The PCR reaction was carried out on aliquots of the cDNA preparation for each gene. The PCR products were separated onto 1.5% agarose gel (w/v). The amplified products were photographed and the intensity of the bands was analyzed with Quantity-One software (Bio-rad, Segrate, Milan, Italy). For each target gene, the relative amount of mRNA was calculated in the sample using β-actin co-amplified as internal standard. The primer sequences 5′-CCT TGT TCA TCA GCT ACG CCT TC-3′ and 5′-CAT GGT GAA CAC GTT CTT GG-3′ were used for inducible nitric oxide synthase (iNOS) detection. The primers sequences 5′-ATC AGG TCA TCG GTG GAG AG-3′ and 5′-CTG CTT GTA CAG CGA TTG GA-3′ were used for cycloxygenase-2 (COX-2), and the primers described by Tsune et al. [19] were employed for β-actin analyses.

2.7. Western blot analyses

Colonic mucosa was homogenized in cold RIPA buffer with protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were centrifuged at 15,000×*g* for 20 min at 4°C, and protein concentration was measured using Bradford's reagent. To determine prostaglandin E synthase (PTGES) and COX-2, 40 μg protein/lane were loaded while GAPDH antibody (Affinity BioreagentsTM, Golden, CO) was routinely assayed for monitoring total protein load. Proteins were separated by 10–15% SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK) by electroblotting. Membranes were incubated for 2 h with the primary antibodies (Cayman Chemical, Ann Arbor, MI) and 1 h with the secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich). Proteins were detected using and ECL plus detection system (GE Healthcare) according to the manufacturer's instructions. The density of the bands was quantified by scanning densitometry using the ImageQuant TL v2005 (GE Healthcare). The Western blot assays were carried out in all the rats (n=8) for each treatment (control, DSS and DSS-PE and DSS-UROA).

2.8. Nitric oxide determination

Distal colon mucosa was homogenized in PBS pH 7.4 and centrifuged at 10,000×*g* for 20 min at 4°C. Supernatant was deproteinized by ultrafiltration using an Ultracel YM-10 membrane (10,000 NMWL) (Centricon, Millipore, Milan, Italy). Nitric oxide production was quantified using the nitrite/nitrate assay kit and according to manufacturer's instructions (Fluka, Steinheim, Germany).

2.9. Prostaglandin E_2 assay

Samples of distal colon mucosa (10 mg) were homogenized in 1 ml of 0.1 M phosphate pH 7.4 containing 1 mM EDTA and 10 μM indomethacin and centrifuged at 9000×*g* for 20 min. A dilution of sample supernatant 1:50 was assayed. Prostaglandin E_2 (PGE_2) levels were measured using an immunoenzymatic method (Cayman Chemicals, San Diego, CA, USA) according to the manufacturer's specifications.

2.10. Thiobarbituric acid-reactive substances and ferric-reducing antioxidant power determinations

Thiobarbituric acid-reactive substances (TBARS) concentration in colon tissue was quantified spectrophotometrically by the method described by Jozwik et al. [20]. 1,1,3,3-Tetramethoxypropane (Sigma, Steinheim, Germany) was used to perform a standard curve. Results were expressed as MDA equivalents per milligram of protein.

The ferric-reducing antioxidant power (FRAP) method was assayed in plasma. Briefly, FRAP reagent was freshly prepared by mixing 10 vol. of 0.3 M acetate buffer, pH 3.6, with 1 vol. of 10 mM 2,4,6,-tripyridyl-*S*-triazine in 40 mM HCl with 1 vol. of 20 mM $FeCl_3$ 6 H_2O and was warmed to 37°C. Subsequently, 100 μl of plasma was added to 900 μl of the FRAP reagent, and the absorbance at 593 nm was monitored for 45 min. A standard curve was done with $FeSO_4$ and results were expressed as μM Fe^{2+}/ml of plasma.

2.11. LC-MS-MS analyses

PE was analyzed in a 1100 series HPLC-DAD-MS-MS (Agilent Technologies, Waldbronn, Germany) according to Cerdá et al. [8]. Briefly, chromatographic separations were carried out on a reverse phase C_{18} LiChroCART column (25×0.4 cm, particle size 5 μm, Merck, Darmstadt, Germany) using water/formic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 1 ml/min. The gradient started with 7% B in A, to reach 30% B at 25 min, isocratic at 30% B for 5 min, 60% B at 36 min, 95% B in A at 37 min, isocratic at 95 for 5 min and returning to the initial conditions (7% B). UV chromatograms were recorded at 280, 360 and 510 nm.

Feces extracts were analyzed in a 1200 series HPLC-DAD system (Agilent Technologies, Waldbronn, Germany) equipped with an HTC Ultra mass detector in series (Bruker Daltonics, Bremen, Germany). The mass detector was an ion-trap mass spectrometer equipped with an electrospray ionization (ESI, capillary voltage, 4 kV; nebulizer 15 psi; dry gas 5 L/min; dry temperature, 350°C) system. Mass scan (MS) and MS/MS daughter spectra were measured from m/z 100–1200 using the Ultra scan mode (26,000 m/z per second). Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode. Chromatographic separations of feces samples were carried out on a 150×0.5 mm i.d., 5 μm, reverse phase SB C18 Zorbax column (Agilent, Waldbronn, Germany) using water/formic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 10 μl/min. The gradient started with 1% B in A to reach 60% B at 30 min, 90% B at 30 min for 5 min and returning to the initial conditions (1% B). UV chromatograms of feces samples were recorded at 280, 305 and 360 nm.

2.12. Identification and quantification of PE ellagitannins and derived metabolites

Identification of ellagic acid and punicalagin was carried out by chromatographic comparisons (UV and MS) with pure standards of both ellagic acid and punicalagin. Ellagitannins in PE (with punicalagin-like spectra) were quantified as punicalagin at 360 nm and ellagic acid derivatives as free ellagic acid at 360 nm. Urolithin-like metabolites were identified according to their UV and MS spectra as well as MS/MS fragments as previously described and quantified at 305 nm using UROA as standard [10].

2.13. Microarray assay and differential analyses

For RNA analyses, distal colon tissue that had been kept in RNAlater (Qiagen) were homogenized and total RNA was isolated using the RNeasy Midi kit (Qiagen) according to the manufacturer's instructions. RNA quantity and purity were assessed at 260 and 280 nm using a Nanodrop (ND-1000; Labtech. International). Equal quantities of extracted RNA from each individual colon sample (n=8) were pooled to create control and DSS groups. Five micrograms of each sample were amplified and labeled according with Luceri et al. [21]. Gene expression was analyzed using whole genome microarrays (Rat Oligo Microarray kit, Agilent, Waldbronn, Germany) and slides were scanned using GenePix 4000A scanner (Axon Instruments Inc., Foster City, CA, USA). Data files were generated by the Agilent Feature Extraction 9.5 software (Agilent, Waldbronn, Germany) and data were normalized in log-space using locally weighted polynomial regression (Loess) using R software (http://www.R-project.org) and the Limma library of Bioconductor (http://www.bioconductor.org). Multiple probes corresponding to a single gene were collapsed using the median, and genes with log ratios not present at least on three arrays were removed. The differential expression of genes was evaluated comparing the expression of the DSS-PE and DSS-UROA groups with the DSS group using normalized nonredundant log ratios. The comparison was performed using a t-moderated test with the Benjamini–Hochberg correction of the false discovery rate (FDR) for the multiple tests [22]. The genes with a FDR-adjusted P value of less than .01 were considered differentially expressed.

2.14. Functional analyses

Data were subjected to functional cluster analysis using the visualization tool GenMAPP (Gene Map Annotator Pathway Profiler) version 2.1. This is a program for viewing and analyzing microarray data on microarray pathway profiles (MAPPs) representing biological pathways or any other functional grouping of genes [23]. Local rat MAPPS: Rn_Contributed_20060824 and as gene database: Rn-Std_20070817.gdb were used for Gen-MAPP analysis. All the gene expression data are dynamically linked to the MAPPs with a tool called MAPPFinder. This program calculated the percentage of genes found differentially expressed by the t-moderated test and the number of genes found differentially expressed on each MAPP. The Z score, a standardized difference score, is calculated using the expected value and the standard deviation of the number of genes meeting the criterion on a MAPP and attempts to take into account that a larger fraction of changed genes is less likely to occur in a large MAPP than is a small one. A positive Z score indicates that there are more genes meeting the criterion in a MAPP that would be expected by random chance. The criteria selected to consider MAPP pathways were Z score >2 and adjusted P<.05.

2.15. Statistical analysis

All analyses, except microarray analysis, were carried out using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov test showed agreement of the empirical distribution on the data with normality assumption. Therefore, all data are expressed as the mean±S.D. of independent measurements. Statistical significance was determined by Student's t test, using P<.05 as the level of significance. In the case of the histological evaluation, nonparametric tests were performed because the assumption of normality can be questioned and the sample size was small for the central limit theorem to be invoked. The results were in accordance with the results of the parametric tests. Inflammatory infiltrate scores were compared between treatment groups within animals by Wilcoxon's signed-rank test.

3. Results

3.1. Hematology

The DSS-treated rats showed a significant decrease ($P<.05$) in the hematological parameters RBC count, Hb and HCT compared to control rats (Table 1). These reductions were significantly attenuated in the DSS-PE group ($P<.05$). The effect in the DSS-UROA group was stronger than in the DSS-PE group as both Hb and HCT were not reduced upon DSS treatment and even a slight increase was observed for RBC count (Table 1). No effect was observed in the rest of the hematological parameters analyzed.

3.2. Histological analyses

Microscopically, colon samples from control group (Fig. 1A) showed the normal histology of the rat colon. In the DSS group (Fig. 1B), colonic sections showed typical inflammatory changes in colonic architecture such as crypt and surface epithelial loss as well as infiltration of inflammatory cells (mononuclear cells, neutrophils and eosinophils). In addition, a complete destruction of the epithelial architecture was observed in some areas. The DSS-UROA group showed an attenuation in the severity of colon injury, a higher integrity of mucosal architecture and the epithelial loss ($P<.0005$) and total histological scores ($P<.005$) were significantly reduced compared to the DSS group (Fig. 1C; Table 2). Although the loss of epithelium and the infiltration of inflammatory cells were lower in the DSS-PE group compared to the DSS group, the differences were not statistically significant (Fig. 1D; Table 2).

3.3. Fecal microbiota

The DSS-PE and DSS-UROA rats showed an increase of bifidobacteria and lactobacilli in feces after 10 days of starting the standard diet supplemented with 250 and 15 mg kg^{-1} day^{-1} of PE and UROA, respectively (Fig. 2A, B). In contrast, the control rat group did not exhibit changes either in the bifidobacteria or in lactobacilli levels (Fig. 2A, B). The increase of bifidobacteria and lactobacilli counts in feces of the DSS-PE and DSS-UROA groups became significant ($P<.001$) after 10 days of pomegranate and urolithin intake, and these differences were maintained in the course of 20 days of consumption. *Clostridium* counts were also increased during PE and UROA ingestion with significant differences ($P<.001$) in relation to the control rat group (Fig. 2C).

After DSS administration for 5 days, the increment observed for bifidobacteria and lactobacilli in DSS-pomegranate rats was significantly reduced while the level of these bacterial groups in feces of UROA rats was maintained (Fig. 2A, B). In feces of the control group, bifidobacteria were also reduced as a consequence of DSS ingestion (Fig. 2A). DSS administration also produced a decrease in *Clostridium* levels in feces of the DSS-PE group achieving levels similar to the UROA group (Fig. 2C). Although lactobacilli level in feces of the control group was not significantly reduced 5 days after DSS administration, a significant growth induction of *E. coli* ($P<.001$), enterobacteria ($P<.01$) and total aerobic bacteria ($P<.05$) was observed (Fig. 3). In contrast, the increase of *E. coli*, enterobacteria and total aerobic bacteria upon DSS administration was significantly lower in the groups fed with the PE- and UROA-supplemented diets (Fig. 3).

3.4. FRAP and TBARS assays

Antioxidant status of control, DSS, DSS-PE and DSS-UROA rats was assessed measuring the capacity of their plasma to reduce Fe^{3+} to Fe^{2+} (FRAP assay). Also, lipid peroxidation levels in colon tissue were evaluated by TBARS measurement. Plasma of the rats treated with 5% DSS in drinking water for 5 days showed a 2.3-fold lower antioxidant status (Table 3). This reduction was significantly ($P<.001$) lower in the DSS-PE group (1.4-fold decrease) but not in the DSS-UROA rats. TBARS levels significantly increased in the DSS rats (1.8-fold) compared with those found in the control rats (Table 3). However, when the DSS-PE rats were treated with DSS, the TBARS increase was lower ($P<.001$) and lipid peroxidation increased by 1.3-fold. In contrast, peroxidation levels in DSS-UROA rats were not significantly different from DSS rat levels.

3.5. COX-2 and iNOS mRNA expression and NO production

COX-2 expression at mRNA level was significantly ($P<.001$) up-regulated 3.5-fold by the DSS treatment (Fig. 4A). Dietary administration of PE and UROA partially prevented this up-regulation, especially in the case of UROA (Fig. 4A).

iNOS expression was significantly ($P<.001$) up-regulated 2-fold (Fig. 4B) and NO levels 3.3-fold upon DSS treatment (Table 4) ($P<.001$). Dietary supplementation with PE and UROA prevented the up-regulation of iNOS expression, keeping its levels similar to those in control group (Fig. 4B). According to iNOS expression, NO levels were significantly lower in rats fed with PE and with UROA with respect to the DSS-treated rats (Table 4). There were no statistically significant differences between PE and UROA dietary interventions.

3.6. COX-2 and PTGES protein levels and PGE2 production

PGE_2 levels increased 11-fold ($P<.001$) upon DSS administration (Table 4). Dietary supplementation with PE and UROA significantly ($P<.01$) counteracted PGE_2 production where the DSS-UROA group showed only a 1.5-fold increase over the control in comparison to the DSS-PE group, which showed a 3.3-fold increase (Table 4).

Both COX-2 (Fig. 5A) and PTGES (Fig. 5B) protein levels were increased significantly ($P<.0001$) by the DSS treatment. According to our results for PGE_2, both PE and UROA dietary supplementations

Table 1
Hematological parameters

Parameter	Control	DSS	DSS-PE	DSS-UROA	NVR
WBC (1 ml)	7.81±2.02	5.40±3.70	8.01±3.64	7.76±2.11	6.1–7.9
RBC count (L)	8.2±0.52	6.50±1.64[a]	7.60±1.00[a]	9.33±1.56[b]	6.7–8.2
Hb (g/dl)	15.65±1.35	12.23±2.81[a]	13.7±2.04[a]	15.89±1.83	12.7–15.9
HCT (%)	47.13±4.30	36.98±7.08[a]	40.34±4.83[a]	47.23±4.95[b]	37.9–46
MCV (μm^3)	54.5±1.97	56.37±4.31	54.12±0.99	54.37±1.77	54.5–58.0
MCH (pg)	17.83±0.44	18.53±0.83	18.00±0.51	17.71±0.32	17.8–20.5
MCHC (g/dL)	33.25±1.17	33.06±1.59	33.87±1.30	33.35±1.17	32.1–36.0
PLT (1 ml)	356.33±67.41	340.12±69.92	462.87±102.26	457±87.09	300.9–466.8

Results are expressed as mean±S.D. and correspond to the end of the experiment (Day 26). NVR was calculated as the mean of reference values ±2×S.D. PLT, platelets.
[a] Significant differences ($P<.05$) over control group.
[b] Significant difference ($P<.05$) over DSS group.

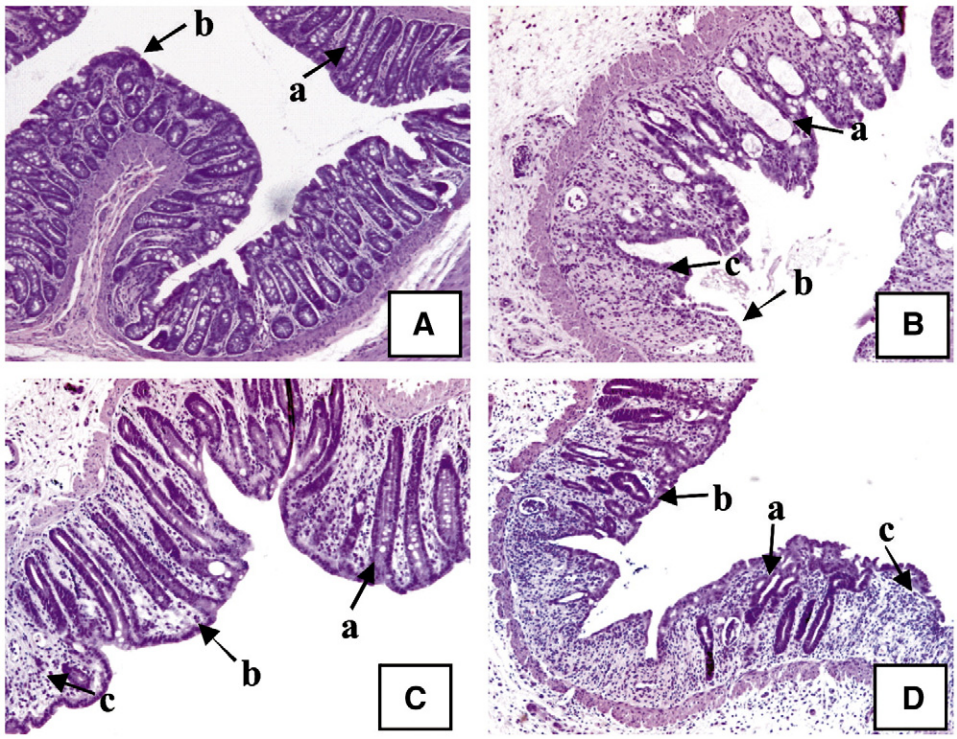

Fig. 1. Histological sections of rat colonic samples stained with hematoxylin and eosin. Scores are reported in Table 2. (A) Control group showing normal histology of rat colon. (B) Mucosal injury produced after DSS administration with loss of crypts and epithelial integrity as well as severe inflammatory cell infiltration. (C) DSS-UROA samples. Morphological alteration associated with DSS treatment is reduced upon UROA administration (15 mg kg^{-1} day^{-1}) keeping colonic architecture and reducing epithelium loss. (D) DSS-PE (250 mg kg^{-1} day^{-1}). Slight reduction of inflammatory cells and epithelium loss were observed. Original magnification ×100. (a) Crypts, (b) epithelium, (c) cell infiltration.

significantly ($P<.01$) prevented to a similar extent the up-regulation of COX-2 (Fig. 5A), whereas the prevention was more efficient for PTGES in the case of the DSS-PE group (Fig. 5B).

3.7. Changes in gene expression

Gene expression profile of colonic mucosa of rats treated with DSS was compared with those of DSS-PE and DSS-UROA groups. From a total of 27,056 genes, the DSS-PE group showed a differential expression (at least 2-fold and $P<.01$) in the 7.6% (2058 genes), the majority of them being up-regulated (84%). In the case of DSS-UROA rats, the 25.8% (6,996 genes) showed differential expression (at least 2-fold and $P<.01$), being up-regulated the 57%. The 32% of the genes (667 genes) modified by PE supplementation were also modified by UROA. Among some important genes changed upon PE supplementation; the up-regulation of the tumor suppressors retinoblastoma 1 (Rb1) and the protein p53 (up-regulation of 3-fold in both cases) is remarkable. In the case of the DSS-URO group, the above tumor suppressors were also up-regulated in the same extent as well as the down-regulation (2-fold) of the CD40 molecule (belonging to the TNF receptor family) and the pro-inflammatory interleukins IL-1β (3-fold) and IL-4 (2-fold).

Functional analysis showed an up-regulation of the G_1 to S cell cycle reactome pathway in both experimental groups, and the DSS-UROA group also up-regulated the cell cycle KEGG pathway and down-regulated the GPCRDB_other pathway (G protein-coupled receptor), the striated muscle contraction and the inflammatory response pathways (Table 5). DSS-PE did not show any significantly down-regulated pathway.

Table 2
Histological scores for evaluating colonic tissue damage

Parameter	Control	DSS	DSS-PE	DSS-UROA
Loss of epithelium	0	3.38±0.52	2.63±1.19	1.75±0.71 *
Crypt damage	0	3.13±0.64	3.13±0.83	2.63±1.19
Infiltration of inflammatory cells	0	3.38±0.52	2.88±.0.64	3.00±0.53
Total	0	9.88±1.13	8.63±1.69	7.38±1.69 **

Each value represents the mean±S.D. ($n=8$). Significant differences over DSS group. Typical distal colonic architecture present in control rats was referenced as 0 value.
* $P<.0005$.
** $P<.005$.

3.8. Polyphenolic content of PE and occurrence of PE-derived metabolites and UROA in feces from healthy and DSS-treated rats

The PE assayed contained 35% punicalagins (three isomers with m/z^- 1083), 13% punicalin ($m/z-$ 781), 4.5% ellagic acid glucoside ($m/z-$ 463) and 8.9% free ellagic acid (m/z^- 301). In addition, other ellagitannins with punicalagin-like UV spectra and MS-MS fragment of 301 were detected. Therefore, the DSS-PE group ingested at least 25 mg kg^{-1} day^{-1} ellagic acid-related molecules.

The phenolic profile of feces extracts from healthy rats (no DSS supplementation) showed phenolics present in the standard rat chow (Fig. 6A; Peaks 1 and 2, vicenin-2 and ferulic acid derivative, respectively) as well as the pomegranate ellagic acid-derived metabolites urolithin C, A and B (Fig. 6A; Peaks 3, 4 and 5, respectively). UROA was quantified, detecting a mean value of 190±70 μg/g. Neither ellagitannins nor free ellagic acid were detected. However, the phenolic profile was different in the PE-DSS group (Fig. 6B) showing the presence of ellagic acid (Peak 6 that co-eluted with the ferulic derivative), much lower presence of UROA (8±3 μg/g) and the absence of urolithins C and B (Fig. 6). In addition, the MS-MS analysis revealed the presence of punicalagin (not detected in UV).

In contrast to the different metabolism observed in PE groups, no significant differences were observed in the accumulation of UROA in

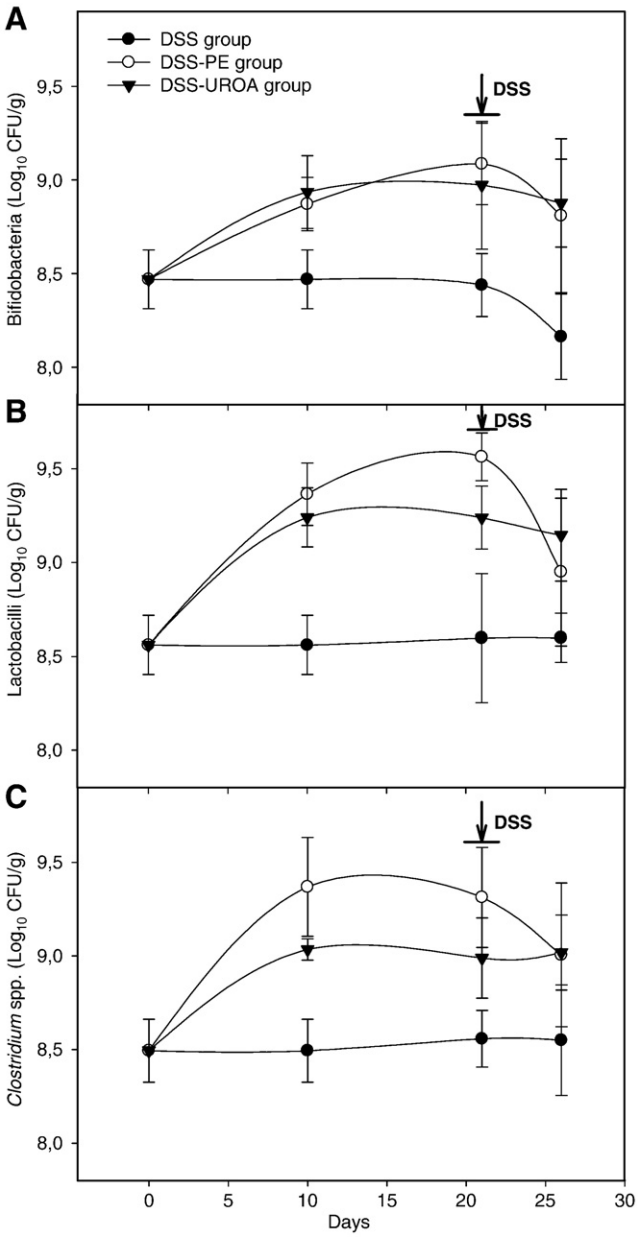

Fig. 2. Counts of (A) *Bifidobacterium*, (B) *Lactobacillus* and (C) *Clostridium* spp. in rat feces upon consumption of 250 and 15 mg kg^{-1} day^{-1} of pomegranate and UROA, respectively, and before and after DSS treatment. Arrows designate the beginning of DSS treatment, which was maintained for 5 days. Results are expressed as the mean±S.D.; n=8.

the feces of healthy rats (135±30 μg/g) (Fig. 6C) and after DSS treatment (120±40 μg/g) (Fig. 6D).

4. Discussion

Inflammatory bowel diseases are characterized by high levels of ROS that are produced by neutrophils and macrophages recruited in the inflamed tissue, as well as a decreased antioxidant capacity of plasma. Moreover, cytokines like TNF-α and IL-1β can also induce ROS production [24]. According to a previous study carried out with the DSS model [25], an increase in lipid peroxidation levels in colon mucosa and a decrease in antioxidant capacity of plasma were observed in our study. From the two dietary interventions assayed in

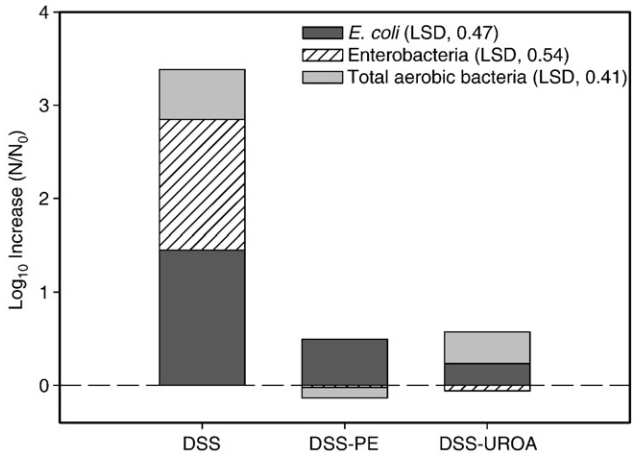

Fig. 3. Increase of *E. coli*, enterobacteria and total aerobic counts in rat feces after DSS treatment. Increased values ($\log_{10} N/N_0$) represent microbial counts in rat feces 5 days after DSS treatment (N) in relation to microbial counts in rat feces just before DSS treatment (N_0).

the present study, only the PE administration counteracted the depletion of the antioxidant status probably because UROA has low antioxidant activity [8].

DSS model of colitis is characterized by high levels of nitric oxide (NO) produced by the induction of iNOS expression. While small amounts of NO exert anti-inflammatory action in endothelial cells, during colon inflammation the isoform enzyme iNOS produces large amounts of NO that contributes to the inflammation status [26]. Our results showed that both PE and UROA supplementation were able to abrogate the NO production by avoiding iNOS induction caused by DSS treatment. These results agree with those from Tugcu et al. [27] that demonstrated a decrease in NO levels and iNOS expression in rats with nephrolithiasis fed with pomegranate juice. Our results also agree with the studies carried out in human chondrocytes stimulated with IL-1β and in mouse macrophages stimulated with LPS showing that NO production was inhibited by plasma containing non-identified pomegranate metabolites and ellagic acid and pretreatment with PE, respectively [6,28].

PGE_2 levels in an inflammation state are determined by two inducible enzymes, COX-2 and PTGES. Our results showed that both PE and UROA supplementations led to a decrease in PGE_2 levels in colon mucosa by down-regulating the over-expressed COX-2 and PTGES levels. Another study has also demonstrated a down-regulation of COX-2 protein by pomegranate compounds in vitro [29], but this is the first time that an up-regulation of PTGES by DSS treatment and subsequent down-regulation by pomegranate and the derived metabolite UROA have been described in vivo. This is important since the mechanisms responsible for the in vivo anti-inflammatory properties of pomegranate have not been described so far.

Table 3
Lipid peroxidation levels in colonic mucosa (TBARS) and plasma antioxidant status (FRAP) in control and DSS-treated groups (DSS, DSS-PE and DSS-UROA)

	FRAP	TBARS
Control	189.9±49.2	0.82±0.18
DSS	80.9±11.9[a]	1.45±0.17[a]
DSS-PE	135.4±37.7[a,b]	1.11±0.09[a,b]
DSS-UROA	60.8±30.6[a]	1.27±0.20[a]

Results are expressed as mean±S.D. (n=8). FRAP values are expressed as μM Fe^{2+}/ml plasma. TBARS values are expressed as nM MDA/mg protein.
[a] Significant differences over control group (P<.05).
[b] Significant differences over DSS group (P<.001).

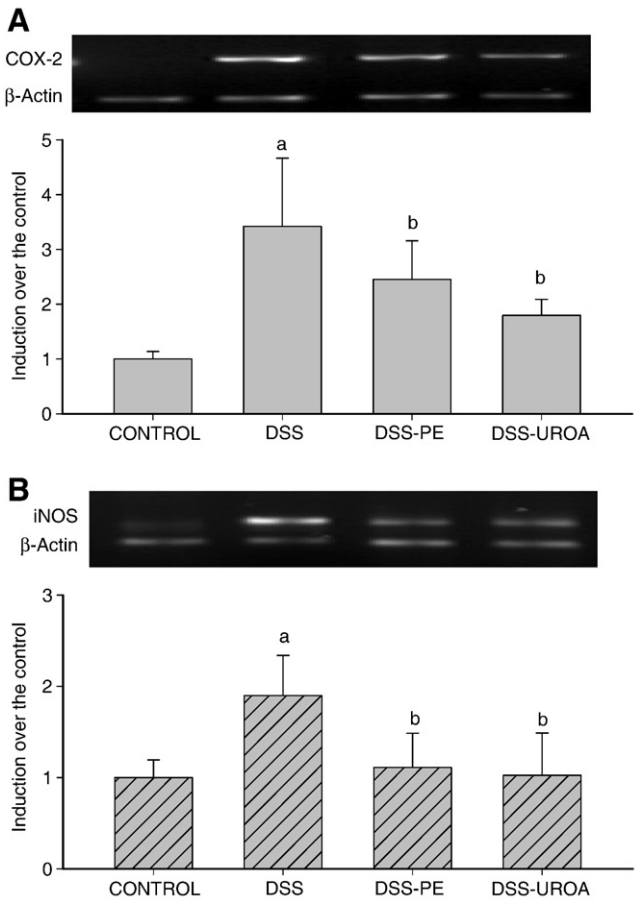

Fig. 4. RT-PCR analyses of (A) COX-2 and (B) iNOS expression in colonic mucosa. Densitometric data were calculated following normalization to β-actin. Results are expressed as the mean±S.D.; n=8. (a) Significant difference (P<.0001) over the control group, (b) significant difference (P<.03) over the DSS group.

IBD is characterized by an abnormal mucosal immune response, but microbial factors and epithelial cell abnormalities can facilitate this response [3]. Indeed, the treatment of the acute DSS-induced colitis model with antibiotics, prebiotics and probiotics has demonstrated to reduce the symptoms of disease and preventing inflammation [30]. In the present study, when PE and UROA were administered for 20 days previous to induction of colitis, an increase in lactobacilli and bifidobacteria was observed. To our knowledge, this is the first time that a modulation of gut microbiota by pomegranate and any urolithin has been reported. In the case of UROA, the effect could be the result of the inhibition of some specific gut microbiota species while other microbial groups such as lactobacilli and bifidobacteria were less severely affected. In the case of PE, the same explanation can be given, although a typical prebiotic effect cannot be discarded as the PE contained other compounds such as fibers and sugars apart from the phenolic content (information provided by Nutracitrus S.L.).

Table 4
PGE_2 and NO levels in distal colon mucosa

	PGE_2	NO
Control	261±138	3.02±1.72
DSS	2871±1349[a]	10.13±2.04[a]
DSS-PE	874±584[ab]	4.49±2.44[b]
DSS-UROA	416±244[b]	3.88±1.23[b]

Data are expressed as mean±S.D.; n=8. Results for PGE_2 are expressed as pg/mg protein. Results for nitric oxide are expressed as μM/mg protein.
[a] Significant difference (P<.001) over the control group.
[b] Significant difference (P<.01) over the DSS group.

Table 5
Pathways modified by the dietary administration of PE and UROA in colon mucosa of DSS-treated rats

MAPP name	Changed	Up/down	Measured	On MAPP	Z score	Adjusted P
DSS-UROA						
GPCRDB Other	17	3/14	36	62	4.13	.004
Striated muscle contraction	12	5/7	25	39	3.53	.046
Inflammatory response pathway	15	3/12	18	29	2.68	.047
G_1 to S cell cycle reactome	28	25/3	47	69	4.72	.000
Cell cycle KEGG	29	25/4	53	80	4.22	.001
DSS-PE						
G_1 to S cell cycle reactome	7	5/2	33	69	6	.038

The analysis was performed using the MAPPFinder program.

The effects of PE and UROA on the intestinal environment by modulating the intestinal bacterial population could contribute to the protective effect of the phenolics against the DSS-induced colitis. There is growing evidence about the involvement of the intestinal microbiota in IBD. For instance, in humans, there is an inverse relationship between the presence of *Faecalibacterium prausnitzii* in colon microbiota and the incidence of IBD [31]. In fact, several studies have demonstrated a protective effect of lactic acid bacteria probiotics in induced colitis affecting several parameters such as pro-inflammatory cytokines and oxidative damage [32], and the growth stimulation of *Lactobacillus* and *Bacteroides* by aminoacid supplementation promoted epithelial repair in a DSS-colitis model [33]. Other predominant group in the human gut is the *Clostridium coccoides*

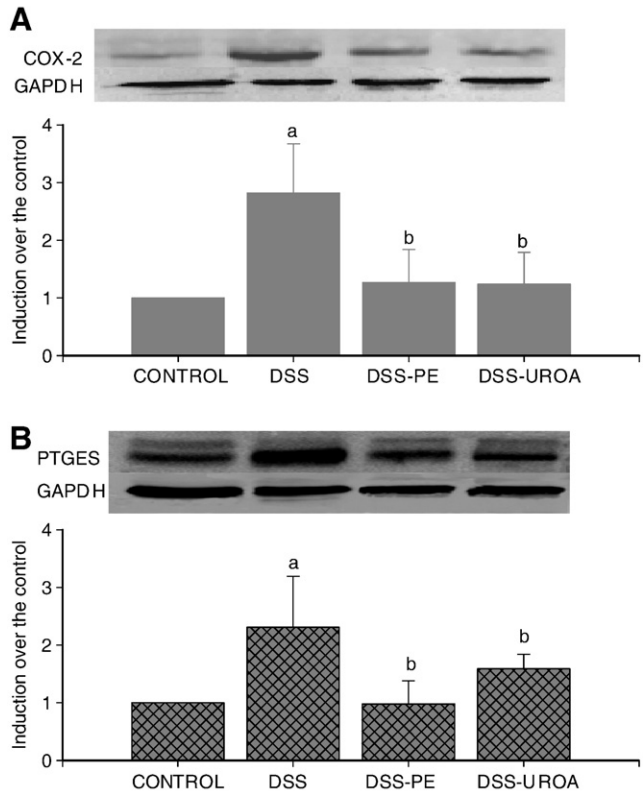

Fig. 5. Western blot analysis of (A) COX-2 and (B) PTGES. Densitometric data were calculated following normalization to GAPDH. Results are expressed as the mean±S.D.; n=8. (a) Significant difference (P<.0001) over the control group, (b) significant difference (P<.03) over the DSS group.

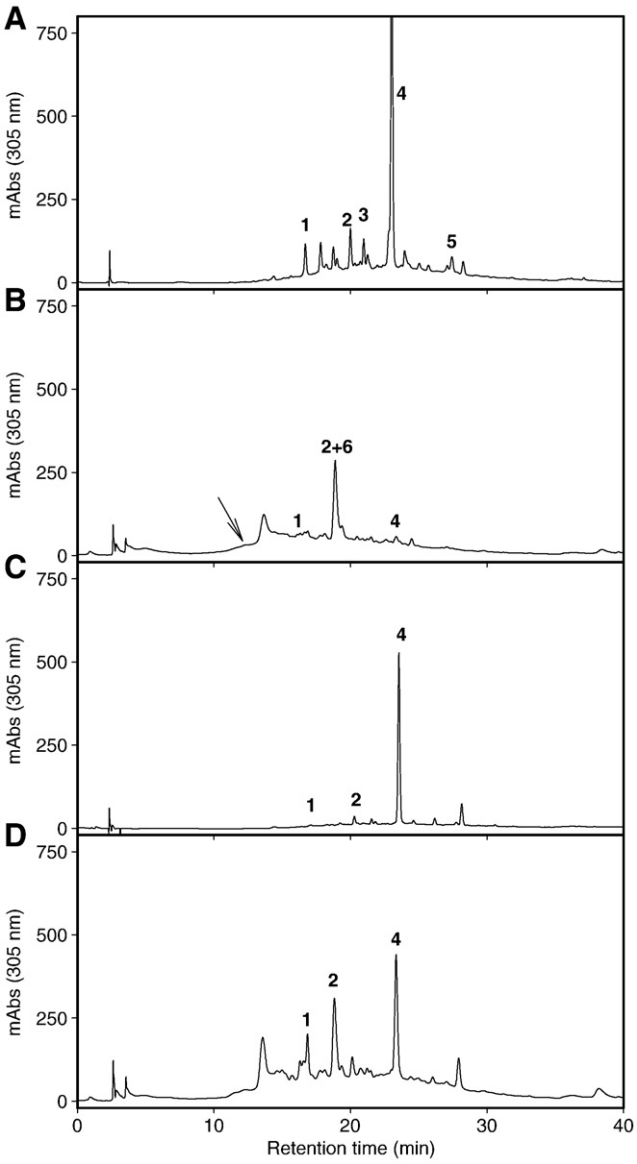

Fig. 6. HPLC chromatograms of rat feces extracts. Administration of PE in (A) healthy and (B) DSS-treated rats. Administration of UROA in (C) healthy and (D) DSS-treated rats. 1, vicenin-2; 2, ferulic acid derivative; 3, urolithin-C; 4, UROA; 5, urolithin-B; 6, ellagic acid. The arrow designates accumulation of the ion 1083 (punicalagin) after extracted ion chromatogram analysis.

group. Most of the butyrate-producing bacteria from colon belong to this group and can thereby contribute to important processes to colonic health [34]. In fact, it has also been confirmed that butyrate has anti-inflammatory actions through its suppression of the inflammatory cytokine cascade [35]. Therefore, butyrate may be an important energy source and immunomodulator in the colon of patients with ulcerative colitis. Furthermore, oral administration of butyrate-producing bacteria such as some *Clostridium* spp. alleviates DSS-induced colitis as previously reported [35].

When colitis was induced, *E. coli* and enterobacteria increased. It has been previously described that gut inflammation changes microbiota composition, disrupts colonization resistance and enhances pathogen growth. In fact, some pathogens such as pathogenic enterobacteria species can benefit from inflammatory defenses [3]. The increase in both *E. coli* and enterobacteria levels after DSS treatment was lower in rats fed with PE and UROA than those in control rats. This could be the result of an indirect effect of phenolic-supplemented diet which increased *Bifidobacterium*, *Lactobacillus* and *Clostridium* counts preventing the colonization and invasion of tissues by enterobacteria including *E. coli*. Moreover, a previous study has shown that *Bifidobacterium* and *Lactobacillus* are also effective in modulating the pro-inflammatory response in intestinal epithelial cells challenged by pathogenic enterobacteria [36].

Both PE and UROA seemed to exert similar effects by down-regulating either PGE_2 levels or the expression of enzymes involved in PGE_2 production or iNOS induction or NO levels. However, these effects seemed not to be enough to protect the colonic architecture in the case of PE as shown in the histological studies.

Previous studies have reported that ellagitannins from pomegranate are metabolized by the colonic microbiota to yield urolithins in rats, pigs and humans [7–10,15]. However, the study by HPLC-MS of the feces revealed that the pomegranate metabolism of rats with colitis was quite different from those without colon inflammation. The low metabolism exerted by the altered microbiota of DSS-PE rats allowed ellagic acid and even punicalagin from the PE to reach the colon and exert a possible antioxidant action. This finding is consistent with Ogawa et al. [25] who demonstrated that microspheres of ellagic acid reached the colon and exerted an antioxidant effect, lowering TBARS levels. However, the ingestion of non-encapsulated ellagic acid did not cause any effect probably because ellagic acid did not reach the colon. It is important to note the different metabolism of compounds in inflamed colons compared to normal colons since this effect has never been described and the majority of studies assume that metabolism of compounds or foods does not vary with the inflammation state. In this context, under an inflammation status, phenolic composition as found in the PE [37] or the main in vivo hydrolysis product, ellagic acid [38], could exert some effects on the colon environment.

In the present study, the effects observed for PE were not mainly due to the accumulation of UROA in the colon, whereas the effect observed in DSS-UROA group was clearly attributed to this metabolite. Overall, UROA supplementation was more effective than PE for ameliorating the inflammation; however, a clear effect was also observed on inflammatory markers, antioxidant status, microbiota and gene expression in the DSS-PE group. In this case, the effect could be due to either the remaining ellagic acid or the ellagitannin fraction that can reach the colon together with the small amount of UROA produced by the microbiota. In addition, the involvement of other non-phenolic compounds in the anti-inflammatory action of PE cannot be discarded. Based on these results, different hypotheses can arise. (i) UROA is the main active anti-inflammatory compound related to pomegranate consumption by healthy subjects (with normal metabolism); however, in the case of inflamed colon such as in IBD, the ellagitannin and ellagic acid fraction together with some minor amount of UROA could act as a synergic anti-inflammatory cocktail. (ii) UROA (formed in the healthy rat colon before DSS treatment during PE administration) is the only anti-inflammatory compound that provided protection in the induced colitis. This effect was prolonged and lasted during the colitis process. (iii) The increase of lactobacilli, bifidobacteria and clostridia before DSS treatment in both PE and UROA groups could have an important effect on the effects observed. (iv) A combination of all the above.

More research is needed to elucidate which is the most likely explanation. However, independently of the main compound responsible for the action, our results indicate that pomegranate composition could provide prevention against colon inflammation before and during the disease process.

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