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Effect of a Low Dose of Dietary Resveratrol on Colon Microbiota, Inflammation and Tissue Damage in a DSS-Induced Colitis Rat Model

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The naturally occurring polyphenol resveratrol has been acknowledged with health-beneficial properties. Most of the studies dealing with its in vivo effects assay huge doses, not representative from a dietary point of view. Our aim was to ascertain whether resveratrol can exert anti-inflammatory activity in vivo at an attainable dietary dose. Rats were fed with 1 mg of resveratrol/kg/day (a human equivalent dose) for 25 days, and in the last 5 days, 5% dextran sulfate sodium (DSS) was administered to induce colitis. Effects on colon tissue damage, gut microbiota, reactive oxygen species, inflammatory markers and nitric oxide production as well as gene expression profile with microarrays were evaluated. Resveratrol increased lactobacilli and bifidobacteria as well as diminished the increase of enterobacteria upon DSS treatment. Resveratrol significantly protected the colonic mucosa architecture, reduced body weight loss, diminished the induced anemia and reduced systemic inflammation markers, colonic mucosa prostaglandin E_2, cycloxygenase-2, prostaglandin E synthase and nitric oxide levels. In addition, the expression of 2,655 genes in distal colon mucosa related to important pathways was varied. These results reinforce the concept of resveratrol as a dietary beneficial compound in intestinal inflammation at doses possibly attainable with resveratrol-enriched nutraceuticals.

KEYWORDS: Dextran sulfate sodium; gut microbiota; microarray; polyphenol; colon; COX-2; prostaglandin

INTRODUCTION

Inflammatory bowel diseases (IBD), whose major forms are Crohn disease and ulcerative colitis (UC), affect millions of individuals in the Western world. The pathogenesis of IBD is not clearly understood, but the influence of genetic, immunological and environmental factors is known. The loss of balance among host susceptibility, enteric microbiota and mucosal immunity results in an exacerbated immune system response with an imbalance of pro-inflammatory cytokines, adhesion molecules and reactive oxygen metabolites, which react with normal gut microbiota and provoke tissue injury. Genetic factors that alter epithelial barrier and increase intestinal permeability or deregulate innate or adaptative immune system response to gut microbiota or pathogens might trigger IBD (*1*). For experimental studies, the administration of chemicals such as dextran sulfate sodium (DSS) in drinking water can induce a syndrome similar to IBD with diarrhea, rectal bleeding, weight loss, multiple erosions, abscesses and changes in intestinal microbiota (*2*).

Among environmental and dietary factors that influence the development of colon inflammatory diseases, probiotics have demonstrated clinical efficacy in DSS-induced colitis (*3*). Recently, dietary polyphenols have been proposed as protective agents in different models of colon inflammation. For example, curcumin, a polyphenol of *Curcuma longa*, reduces colonic levels of nitrites and induces downregulation of cyclooxygenase-2 (COX-2) and inducible nitric oxide (iNOS) expression in chronic colitis (*4*).

Resveratrol, (3,5,4′-trihydroxy-*trans*-stilbene), which naturally occurs in grapes and grape-derived foodstuffs such as red wine, has been reported to exert many different health-promoting effects including antioxidant, anti-inflammatory, antitumor, antiplatelet aggregation, cardioprotective and aging-delay effects

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(*5*). The anti-inflammatory effect of resveratrol has been reported in several diseases, including arthritis, in which 2.3 mg/kg injected resveratrol inhibited synovial inflammation (*6*), and pancreatitis, in which resveratrol at 10 mg/kg reduced inflammatory infiltration and edema (*7*).

However, despite the huge number of studies regarding the health-beneficial effects of resveratrol, its effectiveness from a dietary point of view is not clear. Many studies report the effects either by ip administration or assaying quite unrealistic doses upon oral administration that cannot be reached in a common diet. The main dietary source of resveratrol is red wine with content ranging from 0.2 to 14 mg/L (*8*), and thus the presence of resveratrol in the human diet is scarce. Resveratrol is a phytoalexin, i.e. a stress-inducible metabolite synthesized in the plant to face unfavorable environmental conditions (*9*). This explains the high variability in resveratrol content found. The estimated dose of daily resveratrol intake in the Spanish population is 0.9 mg/day (*10*) although the dietary intake can be increased through resveratrol-enriched nutraceuticals (*11*).

Taking into account the lack of reports dealing with the anti-inflammatory effects of resveratrol at an achievable nutraceutical dose, the aim of the present study was to ascertain whether a human equivalent dose of 10 mg resveratrol/day (in a 70 kg person) is capable of ameliorating acute colon inflammation in a rat model (1 mg/kg/day). For this purpose, colon tissue damage, colonic microbiota, reactive oxygen species, cyclooxygenase-2 (COX-2) and prostaglandin E synthase protein levels (PTGES), prostaglandin E_2 (PGE_2) and nitric oxide (NO) production as well as gene expression profile using microarrays were evaluated.

MATERIALS AND METHODS

Animals and Diets. Male Fischer F344 rats weighting 175−200 g were provided by the Animal Centre of the University of Murcia (Spain). Animals were randomly assigned to three groups and housed in groups of four rats per cage in a temperature-controlled environment (22 ± 2 °C) with 55 ± 10% relative humidity and controlled lighting (12 h light/dark cycle). They 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 three groups were distributed as follows: the control group, which was fed with standard feed; the DSS group, which 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) during the last five days of the experiment (from day 20 to day 25); and the DSS-Res group, which received the standard chow supplemented with 1 mg/kg/day of resveratrol (>99% purity) (Sigma-Aldrich, St. Louis, MO, USA) for 25 days plus 5% DSS also during the last five days of the assay. Resveratrol was homogenously mixed with grinded standard chow and further repelleted. The resveratrol-enriched diet was protected from light. Resveratrol content in the diet was routinely checked by HPLC along the study.

The resveratrol dose assayed is equivalent to 0.143 mg/kg/day in humans (10 mg resveratrol in a 70 kg person) according to the human equivalent dose formula (HED), which takes into account the body surface area instead of the direct animal weight−human weight extrapolation: HED = animal dose in mg/kg × (animal weight in kg/human weight in kg)$^{0.33}$ (*12*).

Experimental Design. Experiments followed a protocol approved by the local animal Ethics Committee and the Local Government. All experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC). Rats were fed for 20 days with control and resveratrol-supplemented diets before the DSS treatment. After this period, acute colitis was induced by giving 5% DSS to both DSS and DSS-Res groups in drinking tap sterilized water ad libitum for five days. DSS solution was freshly prepared, and both food and water intake were measured daily. After the fifth day of colitis induction, rats were anesthetized 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.

Sampling Procedure. Blood samples (approximately 3 mL) were obtained immediately after decapitation and collected in EDTA-treated tubes to obtain the hematological profile. For the determination of serobiochemical parameters, the collected blood was immediately separated in plasma by centrifugation at 3000*g* for 10 min at 4 °C in a Sigma 1-13 microcentrifuge (Braun Biotech. International, Melsungen, Germany). The plasma was immediately frozen at −80 °C for further analyses.

Colons were removed and rinsed with PBS and blotted dry, and the length was measured. Distal colon mucosa was scraped using a glass slide, and samples were either snap frozen on liquid nitrogen and stored a −80 °C until analysis or kept in RNAlater solution (Ambion, Madrid, Spain). A tissue sample was excised and fixed in neutral-buffered formalin.

Samples of freeze-dried feces (0.5 g) were processed as reported elsewhere (*13*). An aliquot of 50 μL was diluted with 50 μL of water, filtered through a Millex-HV13 0.45 μm membrane filter (Millipore Corp., Bedford, MA) and injected (6 μL) in the HPLC−DAD−MS−MS equipment.

Hematology and Clinical Chemistry. Hematological parameters were determined using an Abacus Junior Vet automated hematological analyzer (CVM S.L., Navarra, Spain), with specific software for rat blood samples. The parameters analyzed were red blood cell number (RBC); hemoglobin concentration (Hb); hematocrit (HCT); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); and white blood cell count (WBC).

Serum concentrations of haptoglobin (Hp) were quantified by a spectrophotometric method using a phase Range Haptoglobin assay (Tridelta Development, Ireland). Albumin was determined by the bromocresol green method using a commercial kit (Spinreact, Spain). All these assays were performed in a Cobas Mira Plus analyzer (ABX Diagnostic, Montpellier, France) following the manufacturer's instructions. Fibrinogen was measured according to the method of Clauss as previously described (*14*).

The normal values range (NVR) was calculated for each parameter as the mean of control group value ± 2SD.

Histological Analyses. Tissue samples from the distal colon were fixed in 10% neutral buffered formalin, dehydrated in graded ethanol series and embedded in paraffin. The samples were cut into 5 μm thick sections on a Leica Ultracut rotary microtome. For histological evaluation, tissue sections were stained with hematoxylin−eosin and observed under a light microscope. The mucosal damage scoring system of Araki et al. (*2*) was used to evaluate the degree of colitis. The samples were graded 0−4 for surface epithelial loss, crypt destruction and inflammatory cell infiltration into the mucosa (maximum score = 12).

Fecal Microbiota Analysis. Fresh fecal samples were collected at day 0, and 10, 20 and in the day of sacrifice after starting the supplementation with resveratrol. Fecal samples were homogenized in buffered peptone water (100 mg/mL) (AES Laboratoire, Combourg, France) (1:10 dilution) using filter stomacher bags (Seward Limited, London, U.K.) and a stomacher (IUL Instrument, Barcelona, Spain) for 90 s. 10-fold serial dilutions were made in the same medium, and aliquots of 0.1 mL of the appropriate dilution were spread onto different agar media. *Lactobacillus* and *Bifidobacterium* 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 were obtained by inoculating decimal dilutions in violet red bile glucose agar. *Escherichia coli* were enumerated using chromocult coliform agar. *Clostridium* spp. were enumerated on reinforced clostridial containing 20 μg/mL of polymixine sulfadiacine. Culture plates were incubated at 37 °C for 24−48 h in an anaerobic chamber(CO_2:H_2:N_2, 5:15:80). Similarly, total aerobic bacteria were enumerated by the standard plate count method on brain heart infusion (BHI) agar after incubation in aerobic conditions at 37 °C for

48 h. All media were obtained from Oxoid (Basingstoke, U.K.) whereas antibiotics and other supplements were obtained from Sigma. 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.

Nitric Oxide Determination. Distal colon mucosa was homogenized in phosphate buffered saline pH 7.4 and centrifuged at 10000*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 indirectly as nitrite plus nitrate concentration using the nitrite/nitrate assay kit (Sigma-Aldrich). Nitrate in the supernatant was reduced to nitrite by incubation with nitrate reductase and NADPH at room temperature for 1 h. Nitrite concentration in the reduced samples was measured by the Griess reaction according to the manufacturer's protocol. Nitrite concentrations were calculated by comparison with standard solutions of sodium nitrate prepared in saline solution after reduction according to manufacturer's instructions.

Prostaglandin Assay. PGE_2 levels were measured in distal colon mucosa homogenates using an EIA immunoenzymatic method (Cayman Chemicals, San Diego, CA) according to the manufacturer's specifications. Samples of distal colon mucosa (10 mg) were homogenized in 1 mL of 0.1 M sodium 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 used.

Western Blot Analyses. Colonic mucosa was homogenized in cold RIPA buffer with protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were centrifuged at 15000*g* for 20 min at 4 °C, and protein concentration was measured usingtBradford's reagent. To determine COX-2 and prostaglandin E synthase-1 (PTGES) 40 μg of protein/lane was loaded. GAPDH antibody (Affinity Bioreagents, 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, U.K.) 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). Unless otherwise stated, proteins were detected using and ECL plus detection system (GE Healthcare) according to the manufacturer's instructions. For quantification, the density of the bands was detected with 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-Res).

TBARS and FRAP Determinations. TBARS (thiobarbituric acid-reactive substances) concentration in colon tissue was quantified spectrophotometrically by the method described by Jozwik et al. (*15*). A standard curve was done with 1,1,3,3-tetramethoxypropane (Sigma); results were expressed as MDA equivalents per mg of protein.

The ferric-reducing antioxidant power (FRAP) was measured in plasma according to Benzie and Strain (*16*) with some modifications. FRAP reagent was freshly prepared by mixing 10 vol of 300 mM 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 were 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 mM Fe^{2+}/mL of plasma.

LC−MS−MS Analyses. The 1200 series HPLC−DAD system (Agilent Technologies, Waldbronn, Germany) was equipped with an HTC Ultra mass detector in series (Bruker Daltonics, Bremen, Germany). The mass detector was an ion-trap mass spectrometer equipped with either an electrospray ionization (ESI, capillary voltage, 4 kV; dry temperature, 300 °C) or an atmospheric pressure chemical ionization chamber (APCI, crown voltage 4 kA, dry temperature 250 °C, vaporizer temperature 350 °C) system. Mass scan (MS) and MS/MS daughter spectra were measured from m/z 100 to 600 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 (ESI) and positive (APCI) ionization modes. Chromatographic separations of samples were carried out on a 150 × 0.5 mm i.d., 5 μm, reverse phase SB C18 Zorbax column (Agilent) 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 samples were recorded at 280 and 320 nm. UV and MS spectra as well as MS/MS fragments were used to identify both resveratrol and dihydroresveratrol. The commercially available resveratrol standard was used for comparison.

Microarray Assay and Differential Analyses. Total RNA from distal colon mucosa was extracted using the RNeasy Midi kit (Qiagen) and quantified spectrophotometrically. Control and DSS pooled groups were created by mixing equal quantities of RNA from each extracted individual colon RNA sample ($n = 8$). Sample labeling and processing were performed according to Luceri et al. (*17*). A 44K Rat Oligo Microarray kit (Agilent) was used for microarray analysis. Hybridization was carried out for 18 h with rotation at 63 °C. After washing, slides were scanned using a GenePix 4000A scanner (Axon Instruments Inc., Foster City, CA). Minimun information about a microarray experiment (MIAME) compliant data from the DSS group and the DSS-Res group have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE14544. For each array the quality control was performed in order to delete bad spots from the analysis with the Agilent Feature Extraction 9.5 software (Agilent). The log-ratio of the background adjusted intensity on the red channel vs green channel was normalized by the smoothing function LOESS to rescale the log-ratios to zero, using R software and the Limma library of Bioconductor. The normalized log-ratios of multiple probes corresponding to a single gene were collapsed using the median to obtain one normalized log-ratio for single gene. Genes with log-ratios not present at least on three arrays were removed during the quality control step.

To evaluate the differential expression of genes of both DSS and DSS-Res groups, the expression of the DSS-Res group was compared with the expression of the reference, using normalized nonredundant log-ratios. The genes which corresponded to a log-ratio value not significantly different from zero were considered not differentially expressed. The comparisons were performed using a t-moderated test (*18*) with the Benjamini-Hochberg correction of the FDR (false discovery rate) for the multiple tests (*19*). The genes with a FDR-adjusted P-value of less than 0.01 were considered differentially expressed.

Functional Analyses. To identify biological processes affected by resveratrol treatment, the visualization tool GenMAPP (Gene Map Annotator and Pathway Profiler) version 2.1 was used. This is a generally accessible program for viewing and analyzing gene array data on microarray pathway profiles (MAPPs) representing biological pathways or any other functional grouping of genes (*20*). For Gen-MAPP analysis we used the local rat MAPPS, Rn_Contributed_20060824, and as gene database, Rn-Std_20070817.gdb. The functional analysis was performed using the program and dynamically linked it to the MAPPs with a tool called MAPPFinder. This calculates the percentage of genes meeting a user-defined criterion, in our case the percentage of genes present on the maps, founded differentially expressed by the t-moderated analysis. 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, taking into account the MAPP sizes. The criteria selected to having in consideration MAPP pathways were Z-score > 2 and PermuteP < 0.01.

Statistical analysis. All analyses, except microarray analysis, were carried out using SPSS14.0 software (SPSS Inc., Chicago, IL). The Kolmogorov−Smirnov test showed that the data had a normal distribution. Data are expressed as the mean ± SD of independent measurements. Statistical significance was determined by Student's t test, using $P < 0.05$ as the level of significance.

RESULTS

Food and Water Intake and Body Weight Gain. Food and water intake as well as body weight were monitored daily. The mean food intake was 18 ± 0 g for control and DSS-Res groups whereas the food intake in the DSS group was significantly

Table 1. Hematological and Serobiochemical Inflammatory Parameters in Rat Groups[a]

parameter	control	DSS	DSS-Res	NVR
WBC (1 mL)	6.09 ± 3.03	6.53 ± 4.74	7.11 ± 4.12	6.1–7.9
RBC (1 L)	8.2 ± 0.52	6.50 ± 1.64*	8.27 ± 0.74**	6.7–8.2
Hb (g/dL)	15.65 ± 1.35	12.23 ± 2.81*	14.31 ± 2.17	12.7–15.9
HCT (%)	47.13 ± 4.30	36.98 ± 7.08*	43.07 ± 5.64	37.9–46
MCV (μm^3)	53.88 ± 1.81	56.38 ± 2.31	54.00 ± 1.15	54.5–58
MCH (pg)	17.90 ± 0.53	18.56 ± 0.83	17.89 ± 0.17	17.8–20.5
MCHC (g/dL)	33.25 ± 1.11	33.06 ± 1.60	33.10 ± 0.88	32.1–36
PLT (1 mL)	332.57 ± 20.8	340.50 ± 16.92	438.14 ± 15.49	300.9–466.8
haptoglobin (g/L)	1.19 ± 0.18	4.69 ± 1.68*	3.51 ± 1.85*	0.83–1.55
fibrinogen (g/L)	4.06 ± 1.89	16.86 ± 4.78*	11.7 ± 4.94*	2.17–7.84
albumin (g/dL)	3.06 ± 0.19	2.6 ± 0.13*	2.8 ± 0.17**	2.68–3.44

[a] Results are expressed as mean ± SD. Normal values range (NVR) was calculated as the mean of reference values ± 2SD. Results shown correspond to the end of the experiment (day 26). RBC, red blood cell number; Hb, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; and WBC, white blood cell count. *Significant differences ($P < 0.05$) over the control group. **Significant difference ($P < 0.05$) over the DSS-group.

lower, 12.7 ± 4.5 g ($P < 0.01$). There were no significant differences in water intake among the three groups (data not shown). The body weight gain in the last five days of the assay was 6.1 ± 2.5 g for control group. The weight gain decreased significantly ($P < 0.05$) to −5.25 ± 9.7 g for the DSS group and was significantly counteracted by resveratrol treatment ($P < 0.05$) in the DSS-Res group (2 ± 3.9 g).

Hematological and Serobiochemical Parameters. Rats in the DSS-treated group exhibited a significant ($P < 0.05$) anemia (lower RBC levels, Hb and HCT) in comparison with the control group. Supplementation of the diet with 1 mg/kg/day of resveratrol attenuated these symptoms, but significant differences ($P < 0.05$) were only found for RBC values in the DSS group (**Table 1**). The normal values range (NVR) is included in **Table 1** as values are influenced by reagents, methods, analyzers or even blood extraction systems. For this reason, the establishment of reference values by each laboratory is highly recommended.

The DSS-treated group showed a significant increase in haptoglobin and a significant decrease in albumin compared with the control group. The group supplemented with resveratrol had both lower increase in haptoglobin (Hp) and decrease in albumin values.

Colon Length and Histological Evaluation. The control group showed a colon length of 17.1 ± 1.4 cm that was shortened to 12.7 ± 1.6 cm in the DSS group ($P < 0.01$) upon consumption of DSS for 5 days in drinking water. Administration of resveratrol significantly prevented colon shortening (14.9 ± 0.9 cm) ($P < 0.004$) in the DSS-Res group. Microscopically, colon samples from the control group (**Figure 1A**) showed the normal histology of the rat colon. In the DSS group (**Figure 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). Complete destruction of the epithelial architecture was observed in some areas. Treatment with resveratrol (**Figure 1C**) reduced the morphological signs of cell damage protecting the mucosal architecture. In some areas, the epithelium remained intact. Damage to the colonic mucosa assessed by total histological score was significantly higher ($P < 0.005$) in the DSS group compared with the DSS-resveratrol group (**Table 2**).

COX-2 and PTGES Protein Levels. DSS treatment increased 2.3-fold COX-2 level ($P < 0.01$) in colon mucosa over the control whereas this increase was significantly prevented

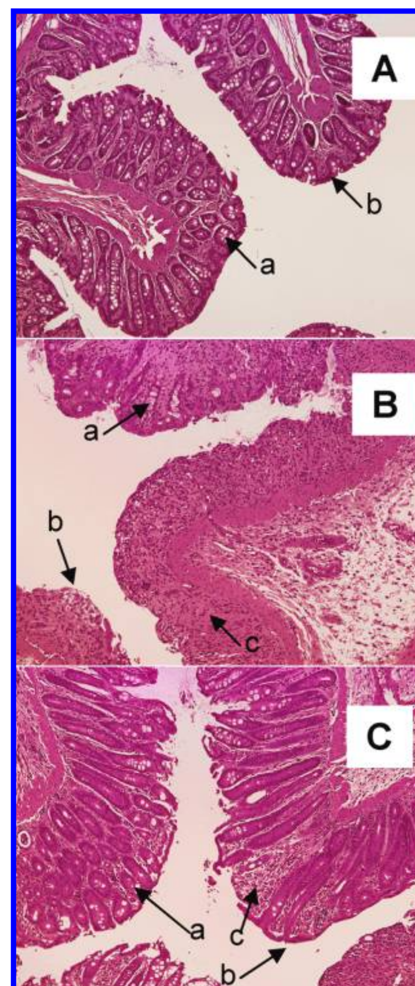

Figure 1. Histology of rat colonic samples stained with hematoxylin and eosin. (**A**) Control group showing normal histology of rat colon. (**B**) Mucosal injury produced after dextran sulfate sodium (DSS) administration with loss of crypts and epithelial integrity as well as severe inflammatory cell infiltration. (**C**) Reduction of morphological alteration associated with DSS treatment upon resveratrol administration (1 mg/kg/day) and thus protecting the colonic structure. Original magnification ×100. (a) Crypts, (b) epithelium, (c) cell infiltration.

Table 2. Histological Scores for Evaluating Colonic Tissue Damage[a]

parameter evaluated	control	DSS	DSS-Res
epithelium loss	0	3.38 ± 0.52	2.00 ± 0.93**
crypt damage	0	3.13 ± 0.64	3.00 ± 0.53
infiltration of inflammatory cells	0	3.38 ± 0.52	2.88 ± 0.35*
total	0	9.89 ± 1.13	7.88 ± 1.39**

[a] Each value represents the mean ± SD ($n = 8$). *$P < 0.05$. **$P < 0.005$.

($P < 0.01$) in the presence of resveratrol (**Figure 2A**). In the case of PTGES, its level increased 2-fold ($P < 0.01$) over the control upon DSS treatment whereas its level decreased 2-fold over the control (4-fold over DSS-treatment) in the presence of resveratrol ($P < 0.01$) (**Figure 2B**).

Nitric Oxide and Prostaglandin Levels. A significant increase in NO production ($P < 0.01$) was observed after DSS administration. Whereas NO levels were increased from 3.02 ± 1.72 μM/mg protein in control colon mucosa to 10.13 ± 2.04 in DSS-treated rats, NO levels were significantly lowered in the DSS-Res group (2.82 ± 1.20 μM/mg protein) ($P < 0.004$) (**Figure 3**). PGE_2 levels increased 11-fold ($P < 0.01$) in colon

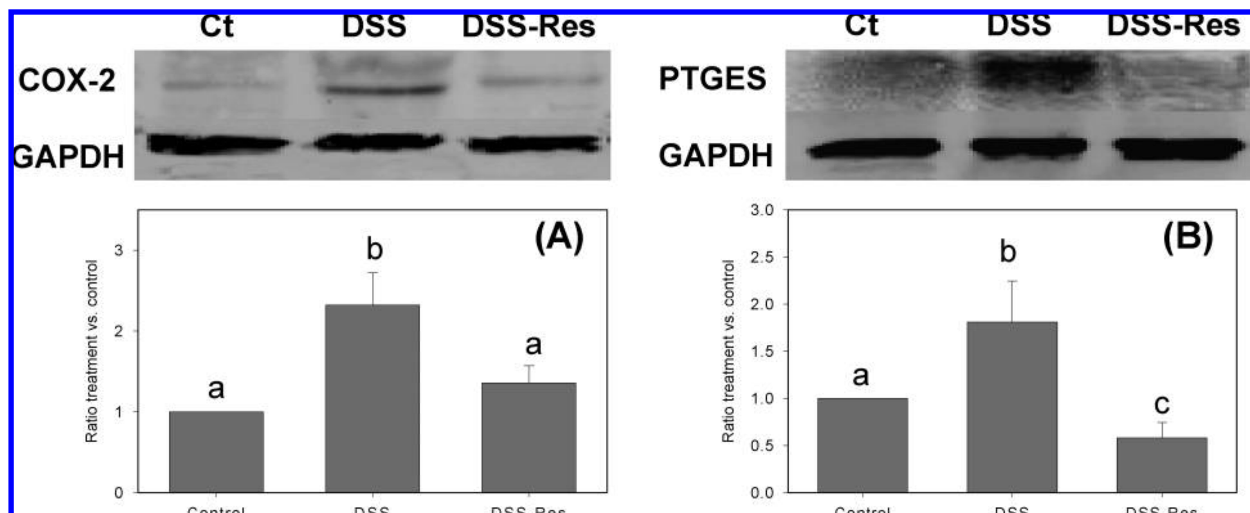

Figure 2. Western blot analysis in colon mucosa: (**A**) cyclooxygenase-2 (COX-2); (**B**) prostaglandin E synthase (PTGES). The corresponding densitometry analyses are shown below each Western blot. Results are shown as the mean ± SD ($n = 8$). Bars with the same letter are not significantly different at $P < 0.01$.

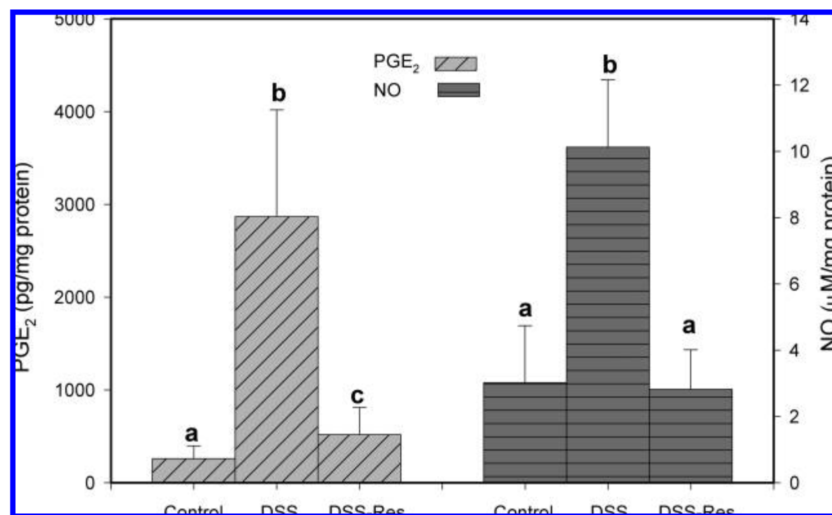

Figure 3. Prostaglandin E_2 (PGE_2) and nitric oxide (NO) levels in distal colonic mucosa. Each bar is the mean ± SD ($n = 8$). Bars with the same letter are not significantly different at $P < 0.01$.

mucosa after DSS treatment. The dietary supplementation of resveratrol attenuated 5.5-fold ($P < 0.01$) the increase of PGE_2 levels (**Figure 3**).

TBARS in Colon Tissue and FRAP in Plasma Samples. TBARS were significantly raised ($P < 0.01$) from 0.82 ± 0.18 (nM TBARS/mg protein ± SD) in control colon mucosa to 1.45 ± 0.17 after DSS-treatment. Resveratrol treatment had no effect on the accumulation of TBARs. In addition, the capacity of plasma to reduce Fe^{3+} to Fe^{2+} (FRAP assay) in DSS-treated rats significantly ($P < 0.05$) decreased (80.9 ± 11.9 μM Fe^{2+}/mL of plasma) in comparison with control rats (189.9 ± 49.2 μM Fe^{2+}/mL of plasma). However, resveratrol treatment (189.9 ± 49.2 μM Fe^{2+}/mL plasma) was not able to ameliorate the decrease of plasma antioxidant status upon DSS-treatment.

Resveratrol Content in Feces. Neither resveratrol nor its colonic metabolite dihydroresveratrol was found in feces using LC−MS−MS (ESI or APCI). Neither resveratrol-like UV spectra nor the selectively extracted ions (m/z^- 227 with ESI and m/z^+ 229 with APCI for resveratrol and m/z^- 229 with ESI and m/z^+ 231 for dihydroresveratrol) were detected.

Fecal Microbiota. The DSS-Res group showed an increase of *Lactobacillus* and *Bifidobacterium* in feces after 10 days of starting the standard diet supplemented with 1 mg/kg/day of resveratrol while the control group did not exhibit changes in the lactic acid bacteria levels (**Figure 4**). The increase of *Lactobacillus* and *Bifidobacterium* counts in feces of the DSS-Res group became significant ($P < 0.001$) after 20 days of resveratrol intake. After DSS administration, the increases observed for *Lactobacillus* and *Bifidobacterium* in DSS-Res group were reduced to the levels found at day 10 (**Figure 4**). Although *Lactobacillus* and *Bifidobacterium* levels in feces of the control group were not significantly reduced after the end of DSS administration, a significant growth induction of *E. coli* ($P < 0.001$) and enterobacteria ($P < 0.01$) was observed (**Figure 5**). In contrast, the increase of *E. coli* and enterobacteria, upon DSS administration, was significantly lower in the group fed with the resveratrol-supplemented diet (**Figure 5**).

Microarray Analyses. From all analyzed genes, 6.03% of genes (2,655) in distal colon mucosa were differentially regulated by resveratrol treatment ($P < 0.01$). The upregulated genes (at least 2-fold) were the 61.4% (1,630) and the down-regulated genes (at least 0.5- fold) represented the 38.6% (1,025) of total differentially expressed genes. **Tables 3** and **4** show upregulated and dowregulated genes by resveratrol, respectively, which have been previously reported to be related in TNBS-induced inflammation model, UC or Crohn disease.

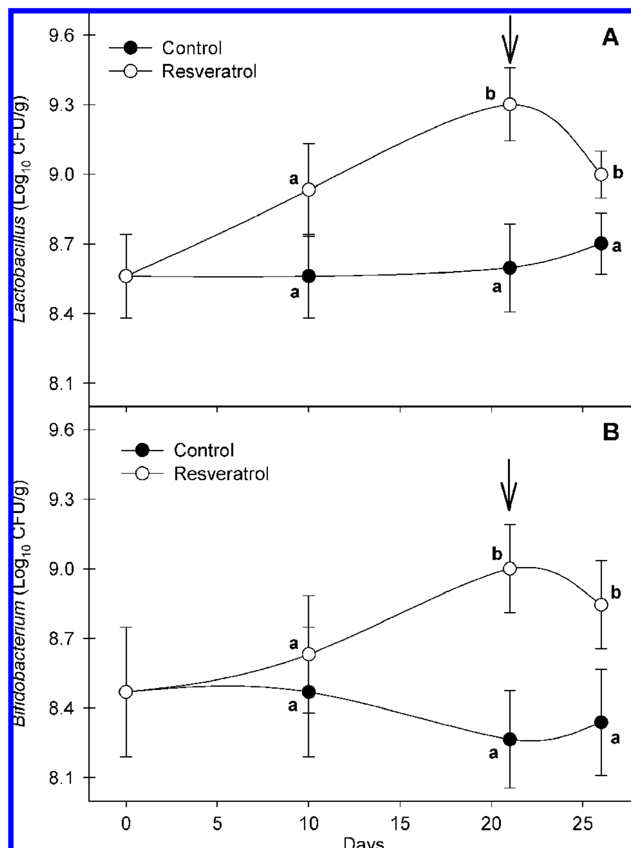

Figure 4. Counts of *Lactobacillus* (**A**) and *Bifidobacterium* (**B**) in rat feces upon consumption of 1 mg/kg/day of resveratrol before and after DSS treatment. Arrows designate the beginning of DSS treatment which was maintained for 5 days. The vertical bars correspond to SD means within control and resveratrol groups followed by different letters are significantly different ($P < 0.001$).

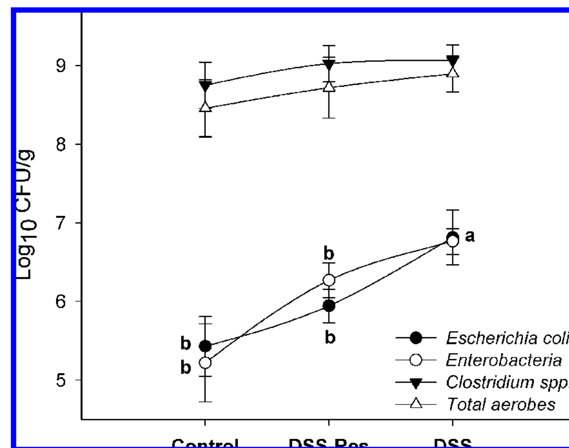

Figure 5. Increase of *E. coli*, enterobacteria, *Clostridium* spp. and total aerobic counts in rat feces after dextran sodium sulfate (DSS) treatment. Means within control and resveratrol groups followed by different letters are significantly different at $P < 0.05$. No significant differences were found for *Clostridium* spp. and total aerobes.

Functional analysis of differentially expressed genes showed alteration of genes implicated on interleukin 6 (IL-6) pathway NetPath 18, HSP70 and apoptosis and mitochondrial fatty acid oxidation, Wnt signaling and T-cell-receptor net path 11 (**Table 5**).

DISCUSSION

DSS is a physical agent that when orally administered disrupts the epithelial barrier causing acute clinical symptoms (diarrhea and bloody stools), epithelial crypt loss and subsequently inflammation (*2*). The increased colonic permeability leads to an increased interaction between microbiota and immune system, one of the important factors on initiation of IBD. In fact, previous studies demonstrate that interleukin-10 gene-deficient mice do not develop a Crohn's-like colitis when the mice are raised under germ-free conditions (*21*).

In our study, rats treated with DSS in drinking water showed anemia marked by lower levels of RBC, HCT and Hb. This normocytic (with normal MCV) and normochromic (with normal MCHC and MCH) anemia is usually associated with inflammation. In addition, DSS-treated rats showed a decrease in body weight gain and food intake. The clinical symptoms correlated with histological changes. The architecture of the colon mucosa was completely altered with inflammatory cell infiltration. These histological lesions were severe enough to compromise the normal colon function, such as absorption, and explain the loss in the body weight and anemia due to the loss of blood in stool.

Dietary administration of 1 mg/kg/day resveratrol for 20 days before and during the five days of DSS administration attenuated significantly the clinical symptoms and reduced the histological degradation of colon wall structure. In some areas, the epithelium remained intact. These results are in agreement with former studies using different models of induced acute and chronic colitis that have shown the protective effects of resveratrol against tissue damage (*22, 23*). However, it should be stressed that the resveratrol dose assayed in those studies was 10-fold higher than that assayed in our study.

Rats treated with DSS showed a typical acute phase response with increases in the positive acute phase proteins of haptoglobin and fibrinogen (although the increase in fibrinogen was not significant) and decreases in the negative acute phase albumin. The presence of 1 mg of resveratrol/kg/day in the diet attenuated this response. Acute phase response with change in concentration of acute phase proteins is associated with inflammatory processes, and particularly in the rat, serum haptoglobin is considered a more sensitive and useful marker of acute inflammation than fibrinogen (*14*).

Nitric oxide is a free radical produced by three enzyme isoforms. While it is well-known that inducible nitric oxide synthase (iNOS) is involved in intestinal inflammation such as ulcerative colitis and contributes to DSS-induced inflammation in mice, the role of both endogenous and neuronal nitric oxide synthases (eNOS and nNOS, respectively) on colon inflammation is controversial and poorly understood (*24*). In the present study, NO production decreased to levels similar to those of control rats. This effect was probably due to the downregulation of both eNOS and more marked iNOs expression by resveratrol as the gene expression analysis revealed that nNOS expression remained unchanged (results not shown). Recently, an upregulation of eNOS expression has been described in different models of colon inflammation while nNOS expression was not altered (*24*). Downregulation of iNOS expression by resveratrol has previously been reported in arteries of rats subjected to cigarette smoke-induced vascular oxidative stress and inflammation (*25*) and in peritoneal macrophages of rats with pancreatitis (*7*). However, in some cases, the decrease of NO levels is not correlated with a downregulation of iNOS, the lower levels of NO being attributed to the scavenging properties of resveratrol (*26*). An effect of resveratrol as free radical scavenger does not seem to be the main mode of action for resveratrol in

Table 3. Upregulated Genes Upon Resveratrol Supplementation[a]

gene name	description	fold change	Padj	TNBS	UC	Crohn	refs
Smad4	MAD homologue 4	3.36	0.000	×			*41*
Tob2	transducer of ERBB2	2.84	0.000	×			*41*
Rsbn1	rosbin, round spermatid basic protein 1	2.82	0.001		×		*31*
Mep1a	meprin 1 alpha	2.76	0.001		×	×	*42*
Slc16a1	solute carrier family 16 member 1	2.62	0.000		×		*31*
Hsd17b2	hydroxysteroid (17-beta) dehydrogenase 2	2.50	0.007		×		*42*
Ptprr	protein tyrosine phosphatase, receptor type	2.38	0.004		×		*31*
Mapk9	mitogen-activated protein kinase 9	2.38	0.000	×		×	*41, 43*
Cobl	cordon-bleu	2.34	0.000		×		*31*
Top2a	topoisomerase (DNA) 2 alpha	2.30	0.003	×			*41*
Akap9	A kinase (PRKA) anchor protein (yotiao) 9	2.25	0.001		×		*31*
Epor	erythropoietin receptor	2.23	0.003	×			*41*
Lamb3	laminin, beta 3	2.15	0.000	×			*41*
Nat1	*N*-acetyltransferase 1	2.14	0.000	×	×		*41, 42*
Eif3s6ip	eukaryotic translation initiation factor 3, subunit 6 interacting protein	2.11	0.000	×			*41*
Nedd4l	neural precursor cell expressed, developmentally downregulated 4-like	2.10	0.000		×		*31*
Gstm1	glutathione *S*-transferase	2.09	0.000	×			*41*
Lbp	lipopolysaccharide binding protein	2.08	0.000	×			*41*
Ca4	carbonic anhydrase 4	2.05	0.001		×	×	*42*
Mgst3	microsomal glutathione *S*-transferase 3	2.05	0.001	×			*41*
Enpp4	ectonucleotide pyrophosphatase/phosphodiesterase 4	2.03	0.001		×		*31*
Ccnt2	cyclin T2	2.01	0.002		×		*31*

[a] References are related to the studies in which these genes have been downregulated in TNBS-induced inflammation model, UC or Crohn disease. In the present study, resveratrol upregulated the expression of these genes and thus contributed to the anti-inflammatory effect observed. Padj, adjusted *t* test *P* values; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

Table 4. Downregulated Genes Upon Resveratrol Supplementation[a]

gene name	description	fold change	Padj	TNBS	UC	Crohn	refs
S100a8	S100 calcium binding protein A8	0.08	0.000	×	×	×	*41, 43*
Col1a1	procollagen, type 1, alpha 1	0.14	0.000	×	×	×	*41, 43, 44*
Thbs1	thrombospondin 1	0.15	0.000	×			*35, 41*
Cxcl2	chemokine (C-X-C motif) ligand 2	0.19	0.000		×	×	*31*
Col3a1	procollagen, type III, alpha 1	0.19	0.000	×			*41, 42*
Col1a2	procollagen, type I, alpha 2	0.23	0.000	×	×	×	*41, 42, 44*
S100a9	S100 calcium binding protein A9	0.23	0.000	×	×	×	*31, 41, 42*
Fn1	fibronectin 1	0.25	0.000	×	×	×	*41−43*
Pap	pancreatitis-associated protein	0.26	0.004		×	×	*42*
Igfbp5	insulin-like growth factor binding protein 5	0.29	0.000		×		*31*
Tf	transferrin	0.31	0.001	×			*41*
Il1b	interleukin-1β	0.32	0.000		×		*42*
Apoe	apolipoprotein E	0.33	0.000	×			*41*
Ifitm3	interferon induced transmembrane protein 3	0.36	0.000			×	*31*
Ptgs2	prostaglandin-endoperoxide synthase 2	0.37	0.000	×			*41*
Lyz	lysozyme	0.42	0.001		×		*42*
Hspb1	heat shock 27 kDa protein 1	0.44	0.001	×			*41*
Mmp9	matrix metallopeptidase 9	0.44	0.000	×	×	×	*41−43*
Il1rn	interleukin 1 receptor antagonist	0.44	0.000		×		*42*
Dusp1	dual specificity phosphatase 1	0.44	0.000	×			*41*
Nos2	nitric oxide synthase 2	0.45	0.000		×	×	*31, 42, 43*
Sparc	secreted acidic cysteine rich glycoprotein	0.45	0.002	×			*41, 42*
Ero1l	ERO1-like (*S. cerevisiae*)	0.46	0.000	×			*41*
Col6a3	similar to alpha 3 type VI collagen isoform 1 precursor	0.46	0.000		×		*42*
PF-4	platelet factor 4 precursor	0.48	0.001	×			*41*
S100a1	S100 calcium binding protein A1	0.48	0.001		×		*42*
Igf1	rat insulin-like growth factor I	0.49	0.001	×			*41*

[a] References are related to the studies in which these genes have been upregulated in TNBS-induced inflammation model, UC or Crohn disease. In the present study, resveratrol downregulated the expression of these genes and thus contributed to the anti-inflammatory effect observed.

our assay since TBARS and FRAP levels were not affected by resveratrol, in agreement with other results previously reported (*27*).

The role of PGE_2 production in colon inflammation is controversial. Whereas inhibition of PGE_2 production is generally considered beneficial in inflammation, some reports have shown that exogenously applied PGE_2 reduces mucosal inflammatory cytokines and epithelial erosion after DSS (*28*). Previous studies with the TNBS-induced colitis model have reported that resveratrol at 10 mg/kg/day had no influence on the increase of PGE_2 but diminished PGD_2 levels in acute conditions and counteracted the downregulation of PGE_2 produced by the TNBS instillation in chronic conditions (*22, 23*). Our results show that the intake of 1 mg/kg/day of resveratrol abrogated the increase in PGE_2 production in colon mucosa. Although the downregulation of COX-1 expression by resveratrol has previously been reported (*29*), the mechanism involved seems to be related to COX-2 inhibition in accordance with Martin et al. (*22, 23*) as 2.3-fold decrease of COX-2 levels (**Figure 2A**) and no effects on COX-1 expression (results not shown) were found in our study. In addition, our study reports the decrease of the levels of the

Table 5. Main Pathways Affected by Resveratrol

MAPP name	changed	up/down	measured	on MAPP	Z-score	PermuteP
IL-6 NetPath 18	62	37/25	86	97	3.83	0.000
HSP70 and apoptosis	14	12/2	16	18	2.86	0.004
mitochondrial fatty acid betaoxidation	15	14/1	18	29	2.68	0.008
Wnt signaling	22	10/12	29	44	2.60	0.020
T-cell-receptor Net Path 11	60	29/31	95	122	2.24	0.020

enzyme prostaglandin E synthase in colon for the first time by resveratrol (**Figure 2B**).

The microarray data showed the downregulation of the expression of secreted phospholipases $sPLA_2g3$ and PLA_2g2d (results not shown). $sPLA_2g3$ is a recently described phospholipase preferentially distributed in microvascular endothelial cells in inflamed joints, ischemic hearts and various types of cancer that increase prostaglandin production in response to interleukin-1β. PLA_2g2d seems to be involved in nasal mucosa inflammation although its role is still unclear (*30*). In this context, the effect of resveratrol on these enzymes deserves further investigation as these enzymes could be critical to explain the effect of resveratrol on prostaglandin production.

A number of genes have been reported to be previously altered in IBD or other experimental inflammation models (*31*). Our data indicated that resveratrol supplementation counteracted either the up- or downregulation observed during colon inflammation (**Tables 3** and **4**). In the functional analysis of microarray data, resveratrol modified the IL-6 pathway in accordance with previous reports using diverse models of inflammation after treatment with resveratrol (*32*). The HSP70 and apoptosis pathway was also changed by resveratrol treatment. DSS-induced colitis increase DNA mutagenic lesions in colon mucosa (*33*). Apoptosis of damaged cells is a mechanism of defense to prevent mutations accumulation in cells. Induction of apoptosis cell death by resveratrol has been reported in many in vitro models (*34*), however, data about resveratrol's effect on apoptosis in vivo are scarce. Our data agrees with Martín et al. (*22*), who reported an increased apoptosis caused by resveratrol in rats with TNBS-induced colitis. Another pathway modified by resveratrol in our microarrays is the Wnt signaling pathway. Previous studies with mice mutated in the Wnt pathway showed a strong inhibitory effect of resveratrol (12 mg/kg/day) on the formation of intestinal tumors (*35*). In addition, in different colon cell lines, resveratrol, at lower concentrations than those required for apoptosis induction, inhibited Wnt signal. However, its mechanism of action is still unknown and probably involves multiple effects (*36*).

In recent years, an important role in IBD has been attributed to resident microbiota. Indeed, the treatment of the acute DSS-induced colitis model with antibiotics, prebiotics and probiotics has been demonstrated to reduce the symptoms of disease and prevent inflammation (*3*). In the present study, when resveratrol was administered for 20 days prior to induction of colitis, an increase in *Lactobacillus* and *Bifidobacterium* was observed. To our best knowledge, this is the first time that specific changes in colonic microbiota by resveratrol have been reported, although an increase in *Lactobacillus* has also been described in rats treated with dimethylhydrazine and fed with 50 mg/kg of red wine polyphenols extract for fifteen weeks (*37*). Although this deserves further research, as the resveratrol dose assayed was very low (HED of 10 mg/kg/day), and we employed cultural methods that may distort the actual balance of microbiota species due to selective advantages of some species during cultivation in vitro, the specific changes in microbial composition do not seem to be due to its fermentative properties (as in the case of typical prebiotics such as inulins) but to the inhibition of some specific gut microbiota species, thus allowing the increase of lactic acid bacteria and bifidobacteria. The increase of lactic acid bacteria in the DSS-Res group could contribute to the protective effect of resveratrol against DSS-induced colitis. In fact, several studies have demonstrated a protective effect of *Lactobacillus* and *Bifidobacterium* in DSS-induced colitis, affecting several parameters like proinflammatory cytokines and oxidative damage (*38*). When colitis was induced, *E. coli*, enterobacteria increased. This also occurs under some pathological circumstances, such as acute diarrheal illnesses and antibiotic treatment, where the individual microbiota is depleted. In fact, the reduction of the intestinal microbiota generally has negative effects on the host's well-being and can be associated with a higher susceptibility and development of enteropathogenic bacteria (*39*). The increase in both *E. coli* and enterobacteria proliferation after DSS treatment was lower in rats treated with resveratrol than those in control rats. This could be the result of an indirect effect of resveratrol-supplemented diet which increased *Bifidobacterium* and *Lactobacillus* 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 proinflammatory response in intestinal epithelial cells challenged by pathogenic enterobacteria (*39*). The changes in microbial composition observed for resveratrol are even more important taking into account the low (dietary) dose assayed.

Neither resveratrol nor its colonic metabolite dihydroresveratrol was detected in feces. Resveratrol is efficiently absorbed and extensively conjugated to be excreted (*40*), which hampers its accumulation in the colon. However, a local (colonic) effect was observed as anti-inflammatory as well as changing specifically microbial composition. This effect was produced by a resveratrol concentration below our detection limit (6 nM) in the LC−MS−MS analyses. In addition, the systemic anti-inflammatory effect (serobiochemical inflammatory markers) (**Table 1**) also suggested that either resveratrol exerts systemic effects at very low doses or its circulating metabolites are also active.

In summary, it should be stressed that most studies dealing with the health-beneficial effects of resveratrol usually assay huge doses, not representative from a dietary or nutraceutical point of view. The present study describes the protective effect of dietary resveratrol (1 mg/kg/day for 25 days) in a rat model of DSS-induced colitis. This dose is equivalent to 10 mg of resveratrol/day in a 70 kg person which could be attainable in the diet or with nutraceuticals. Whether the anti-inflammatory effect observed is due exclusively to the direct resveratrol action or it is also mediated by the gut microbiota deserves further investigation. The microarray analysis revealed that 2,655 genes (6.03% of the total analyzed) in distal colon mucosa were significantly regulated. The functional analysis showed alteration of genes involved on IL-6 pathway NetPath 18, HSP70 and apoptosis and mitochondrial fatty acid oxidation, Wnt signaling and T-cell-receptor net path 11. Therefore, taking all together, our results reinforce the important role of resveratrol as a multitargeted anti-inflammatory compound and suggest that this molecule is not only a promising drug to potentially treat diseases at pharmacologic doses but also a polyphenol whose presence in the diet at a low dose can exert protective effects derived from anti-inflammatory activity and specific changes in microbial composition.

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