ANTI-INFLAMMATORY BOWEL EFFECT OF INDUSTRIAL ORANGE BY-PRODUCTS IN
DSS-TREATED MICE

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ABSTRACT

This work addresses the role of different by-products derived from industrial extraction of orange juice on a possible anti-inflammatory effect in mice with colitis induced by dextran sulfate sodium (DSS). The fresh orange residue (FOR), the dry orange residue (DOR), orange liqueur (OL) and animal feed (AF), as well as commercial citrus pectin (CP) were supplied to C57BL/6J mice for 15 days before starting the DSS treatment. Macroscopic parameters such as Disease Activity Index (DAI) and colonic weight/length ratio revealed an anti-inflammatory effect following intake of FOR, AF or CP. Moreover, q-PCR of RNA from colonic tissue indicated measurable changes in the expression of TNF-α, IL-1β, iNOS, and intercellular adhesion molecules ICAM I, as well as in intestinal barrier proteins as MUC-3, Occludin, and ZO-1. Pectin, phenolic compounds and/or Maillard reaction products formed at initial steps were identified as relevant components exerting the ascribed beneficial effects. Our findings could open up the further application of a variety of orange by-products as food supplements in the potential amelioration of inflammatory bowel diseases.

Keywords: orange residues, Amadori compounds, citrus pectin, inflammatory bowel disease.
1. Introduction

Inflammatory bowel disease (IBD) is commonly divided into ulcerative colitis (UC) and Crohn’s disease (CD).¹ UC is usually confined to the colon, while CD usually affects any part of the gastrointestinal tract. A small segment of patients with IBD is classified as undetermined IBD, showing symptoms of UC and CD.² The IBD prevalence is between 37.5-248.6 per 100,000 in North America and 4.9-505 per 100,000 in Europe.³

Among various potential causes implicated in the IBD pathology, genetic susceptibility coupled with environmental risk factors attributed to lifestyle changes such as dietary habits, smoking, stress and lack of exercise, as well as other changes associated with medications, surgery or those leading to alteration of the bacterial flora of the gut are the most frequently described.⁴,⁵

Current strategies for the treatment of IBD involve first induction of remission, followed by maintaining remission. Patients are usually treated with corticosteroids, immunomodulators, and anti-TNFα agents; although immunosuppressive therapies and anti-TNFα agents are associated with a higher risk of infections⁶ and they eventually require surgical intervention, indicating that current therapeutic options are insufficient.⁷ Moreover, the high cost of biological therapies contributes to the increasing financial burden of health care. The disadvantages of pharmacological therapies on IBD emphasize the need for non-pharmacological options.⁸ Exclusion diets are generally not recommended and there is little evidence to support any particular food when nutritional regimens are recommended.⁹ In this sense, low dietary fiber intake has been associated with the incidence of IBD,¹⁰ since the prebiotic activity of fiber can act on IBD by stimulating the selective growth of the intestinal lactobacilli and bifidobacteria, which produce short-chain fatty acids (SCFA). SCFA could improve
mucosal barrier functions and modulate the immune system. However, there is scarce evidence regarding prebiotics use in this type of pathologies.

Fiber can be found in a plethora of products and derivatives of vegetal origin. Industrial processing of citrus generates huge amounts of wastes (24.3 million of tons per year worldwide, and 1.3 million of tons in Spain) that, without further treatment, cause environmental problems, since their fermentation implies high chemical and biological oxygen demand. In most of the cases, these by-products are processed to obtain animal feed, and depending on the composition, and the thermal treatment applied, Maillard reaction products (MRPs) may be formed. Some of these MRPs may have anti-oxidant, anti-mutagenic, carcinogenic and anti-bacterial activities, but their anti-inflammatory effect is not yet well studied.

In past decades, dozens of animal models have been developed as indispensable tools for investigating the pathogenesis of IBD and evaluating therapeutic options. These approaches are mainly based on spontaneous colitis models, inducible colitis models, genetically modified models, and adoptive transfer models. Chemically induced murine models of intestinal inflammation are one of the most commonly used models because they are simple to induce and the onset, duration, and severity of inflammation are immediate and controllable. The dextran sulfate sodium (DSS)-induced mouse colitis model is characterized by bloody faeces, diarrhea, weight loss and tissue inflammation, as well as an increase of proinflammatory cytokines (IL-1β, TNF-α and IL-6) release in the intestine, which can impair permeability of intestine and mucosal barrier function and correlate with the severity of intestine inflammation. These changes are similar to those found in humans by using molecular techniques to demonstrate changes in the composition of the mucosa-associated and fecal microbiota,
in patients with Crohn’s disease. In addition, DSS-induced colitis model has some advantages when compared to other animal models of colitis. For example, the severity of the disease can be produced easily by changing the concentration of administration of DSS, and the dysplasia that resembles the clinical course of human UC occurs frequently in the chronic phase of DSS-induced colitis.

In this context, a recent study allowed the determination of phenolic compounds, pectin and the Amadori compound N-fructosyl-lysine (furosine) in by-products from the industrial extraction of orange juice, and, in order to explore their potential functionality, the aim of this work was to investigate the anti-inflammatory effect of a variety of orange by-products consumption in a DSS model in mice, which is the most commonly approach used to assess the in vivo therapeutic activity, since it exhibits certain characteristics similar to those present in human IBD.

2. Material and methods

2.1. Analytical standards

Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). DSS (36-50 kDa) was purchased from MP Biomedicals (Santa Ana, CA, USA). RNA later® was obtained from Sigma Aldrich (St. Louis, MO, USA), and Tri-Reagent® was acquired from Thermo Fisher Scientific (Invitrogen, USA). The oligo (dT) primers (Promega, Southampton, UK) and KAPA SYBRsFAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) were used to perform the qPCR analyses.

2.2. Orange by-products

Citrus pectin (CP) was provided by CEAMSA (O’Porriño, Spain). By-products from the orange juice extraction industry: fresh orange residue (FOR), the dry orange residue
(DOR), orange liqueur (OL) and animal feed (AF) were provided by the company García-Carrión (Daimiel, Spain). After the industrial extraction of orange juice, the FOR was pressed with calcium oxide to facilitate the obtainment of the OL. Then, OL was concentrated from 10 to 50°Brix by heating at 80°C, and the pressed orange residues were dried from 10 to 30% dry matter at 70°C, obtaining the DOR. In the final stage, OL and DOR were mixed, dried at 100°C and grounded to get the AF in the form of pellets. These samples were lyophilized, grounded and characterized as follows: dry matter, protein, fat, and fiber content were determined according to the AOAC methods. Total and reducing carbohydrates were measured using phenol-sulfuric acid and 3,5-dinitrosalicylic acid methods, respectively. Total phenolic content (TPC) was determined following the Folin-Ciocalteu method described by Soria et al. Antioxidant capacity was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. 2-Furoylmethyl-Amino Acids (2-FM-AA) were analyzed by ion-pair RP-HPLC-UV and monosaccharide composition was determined through the hydrolysis with 2 M trifluoroacetic acid and subsequent formation of trimethylsilyl-oximes derivatives by GC-FID (Supplementary Tables S1 and S2).

2.3. Animals and diets

This study was carried out in accordance to the Guide for the Care and Use of Laboratory Animals as promulgated by the National Institutes of Health. The experimental protocol was approved by the Commission of Ethics in Animal Experimentation (Protocol CEEA 2010-286) of the University of Granada (Spain). C57BL/6 male mice of 7-9 weeks old were obtained from Janvier (St Berthevin Cedex, France). They were housed in Makrolon cages, maintained in an air-conditioned...
atmosphere with a 12-h light–dark cycle, and provided with free access to tap water and
standard rodent diet (Panlab A04 diet, Panlab S.A., Barcelona, Spain).

2.4. Experimental design
Mice (23±2 g) were maintained in specific pathogen-free conditions in the facilities
of Licinio de la Fuente Center and were randomly assigned to seven groups (n=10):
healthy, DSS control, FOR, DOR, OL, AF and CP. Solid by-products samples were added
to standard food at 10%. CP and OL were diluted in water at concentrations of 2.5 and
1.25 % (w/v), respectively, and supplied at the rate of 100 μL/mouse per day. Induction
of colitis was performed 15 days after the beginning of the experiment by adding 2.7%
(w/v) DSS in the drinking water for seven days (Table 1).

2.5. Macroscopic indicators
Weight variation, daily food intake, Disease Activity Index (DAI) and the ratio
between colon weight and length were considered as macroscopic indicators. Mice were
monitored recording the animal and food weight, the presence of gross blood in the
faeces and the stool consistency. These parameters were each assigned a score
according to the criteria proposed by Cooper et al.31 and used to calculate the DAI (Table
2). Once the animals were sacrificed, the colon (from the ileocaecal junction to the anal
verge) was quickly excised and carefully washed with a cold saline solution, weighed and
its length was measured.

2.6. Biochemical markers
The expression of pro-inflammatory cytokines as tumour necrosis factor (TNF)-α,
interleukin (IL)-6, (IL)-1β and inducible nitric oxide synthase (iNOS), as well as barrier
intestinal proteins as intercellular adhesion molecule (ICAM)-1, Mucin (MUC)-3,
Ocludin and Zonula occludens-1 (ZO)-1, were evaluated.
The colon tissue was longitudinally divided into different fragments and stored at -80°C in RNAlater®. Total RNA from colonic samples was isolated using Tri-Reagent® following the manufacturer’s protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 2 µg of RNA was reverse transcribed using oligo (dT) primers (Promega). Real-time quantitative PCR, was carried out on optical grade 48-well plates in an Eco™ Real-Time PCR System (Illumina, CA, USA) with 20 ng of cDNA, the KAPA SYBR®FAST qPCR Master Mix (Kapa Biosystems), and specific primers at their annealing temperature (Table 3). In order to normalize mRNA, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was measured. The mRNA relative quantitation was calculated using the ΔΔCt method.

2.7. Statistical analysis

Biochemical analyses were performed at least in duplicate. Indicators of each group were expressed as mean ± standard deviation. Means were compared using the Tukey test (significance limit was set at P < 0.05). Statistical analysis was carried out using SPSS software 22.0.

3. Results

3.1. Physicochemical characterization of test materials

The main physicochemical characteristics determined in the orange by-products and citrus pectin are summarized in Table S1. Overall, all studied orange by-products had a similar composition in protein or fat content, whereas substantial differences were found in carbohydrate, fiber or total phenolic content. By comparing the general chemical composition among the fresh orange residue (FOR), dry orange residue (DOR),
orange liqueur (OL), animal feed (AF) and citrus pectin (CP), FOR presented the greatest total phenolic content (211.9 mg GAE/100 g) and the highest antioxidant capacity (23.3 mM of Trolox /100 g), whereas CP showed the greatest content of soluble dietary fiber (SDF) (96.58 g/100g) as expected, followed by FOR (23.47 g/100g) and animal feed (AF) (12.46 g/100g). CP also showed the highest content in furosine, an indirect marker of the Amadori compound N-fructosyl-lysine,\textsuperscript{32} (827.4 mg/100 g of protein), followed by AF (455.1 mg/100 g of protein).

All samples had galacturonic acid (GalA), xylose (Xyl), arabinose (Ara), rhamnose (Rha), galactose (Gal) and mannose (Man) (Table S2), which are core monomers of the main domains of pectin (homogalacturonan, rhamnogalacturonan-I, and rhamnogalacturonan-II).\textsuperscript{33}

3.2. Assessment of macroscopic indicators of Inflammatory bowel disease in DSS-treated mice

Among symptoms of colitis induced by the oral administration of DSS are haematochezia, body weight loss, shortening of the intestine, mucosal ulcers and infiltration of neutrophils.

Except for the healthy control group, the tested groups experimented a loss of weight (Figure 1A) which was higher in DSS control group, probably due to the inflammation located in the intestine, as well as to a systemic status of illness occasioned by DSS as a general toxic.\textsuperscript{17} Remarkably, the weight reduction was significantly less in those animals that consumed CP, FOR and AF groups.

Reduction of food intake is also a symptom of illness (Figure 1B) possibly due to the discomfort caused by the intestinal inflammation.\textsuperscript{34} During the DSS period the intake
decreased significantly in the DSS control group, while animals treated with CP and FOR showed food consumption levels similar to that observed in the healthy group, and significantly different to DSS group (P< 0.05). Similarly, the AF group showed a higher intake compared to DSS group, but non-significant differences were found.

The DAI, registered during the 7 days of DSS supply, allowed to infer that FOR, CP and AF groups vs. DSS control colitic group presented the lowest DAI (p<0.05) (Figure 1C). Weight/length ratio of the colon was significantly less in those groups that consumed FOR, AF and CP vs. the DSS group (Figure 1D), indicating a lower severity of inflammation and minor colonic cells infiltration (p< 0.05).

Overall, these macroscopic parameters showed a consistent anti-inflammatory effect for CP, FOR and AF, with a significant reduction of symptoms and improvement of the animals’ general status. As mentioned in subsection 3.1, the most important compositional differences found in the tested orange by-products were in fiber, total phenolic, and Amadori compounds (measured as furosine) content.

3.3. **Assessment of biochemical markers of Inflammatory bowel disease in DSS-treated mice**

Cytokine profile of DSS acute colitis is consistent with acute inflammatory response characterized by a macrophage-derived cytokine profile, strong chemotactic pattern and a polarized Th1 panel with high participation of TNF-α and IL-1β among others, similar to human IBD disease. This acute situation is also accompanied by a high expression of iNOS, and an increase of adhesion molecule ICAM I that facilitates the leukocyte endothelial transmigration, contributing to the tissue damage and the exacerbation of the gut inflammation.
The expression of inflammatory cytokine panel (TNF-α, IL-1β, IL-6) was consistent and significantly reduced in the groups fed with FOR, AF and CP vs DSS control group (Figures 2A-2C); and additionally, a reduction in the expression of ICAM I was observed in those groups that consumed the orange by-products, in comparison with DSS control group (Figure 2D) (p<0.05). The expression of ICAM I was statistically similar between the healthy group and the CP and FOR groups, followed by AF group.

MUC 3, occludin and ZO-1 expression was significantly greater in FOR, AF and CP treated groups as compared with the DSS control group (p<0.05) (Figure 2E-2H). Increased expression of these proteins indicated a potential protective effect for those products which can not only reduce the symptoms and seriousness in acute conditions but also have a preventative effect against future IBD crisis.36

Nitric oxide synthase (iNOS) is an enzyme dominantly expressed during inflammatory reactions. The synthesis of high amounts of nitric oxide (NO) by iNOS has been demonstrated in pathophysiological processes, such as acute or chronic inflammation and tumorigenesis; however, the role of iNOS activity in these diseases is still not well understood.37 Presence of nitric oxide (NO) is responsible for the generation of substantial reactive oxygen species (ROS) as peroxynitrites or anion superoxide that may affect the microbial agent and produce apoptosis of host cells.38 Results indicated that all tested orange by-products produced a significant reduction in iNOS expression as compared to the DSS group (Figure 2H). There was no significant difference between the AF, CP and the healthy group; and the groups that consumed DOR and OL showed a higher expression of iNOS, slightly lower than that observed in the DSS control group, which could be related with their low GalA content (Table S2).
4. Discussion

By correlating the biochemical effects of orange by-products and citrus pectin ingestion to their physicochemical characteristics (Table S1), the beneficial effect observed following FOR consumption might be mainly due to its phenolic content and, consequently, antioxidant capacity. FOR had the highest content in total polyphenols, followed by DOR, OL, AF, and CP, respectively (Table S1). The reduction of phenols observed among the tested orange by-product samples seems to be mainly due to the heat treatment applied by the industry for the obtainment of animal feed from orange residues, since it is well known that phenolic compounds are vulnerable to heat. Thus, the application of pressing and subsequent drying of FOR at 70°C to obtain DOR generated a reduction of ~30% of initial phenolic content (Table S1). The process of evaporation and concentration of OL at 80°C resulted in approximately a two-fold reduction in the phenolic content as compared to the initial sample (FOR). Finally, in the case of AF, despite being a product obtained by mixing the DOR and OL (145.5 and 115.6 mg GAE/100gDM), its phenolic content was reduced to 90.2 mg GAE/100gDM, which represents 42.6% of the phenolic content of the initial sample (211.9 mg GAE/100gDM), which may be due to the previous concentration step carried out at 100°C. In consequence, the effect of phenolic compounds of FOR could be attributed to the fact that DSS acts mainly by breaching the intestinal barrier function, thereby exposing subepithelial immune cells to commensal bacteria, focusing on ROS and reactive nitrogen species (RNS) as the etiologic factors for IBD, and, thereby, phenols act as antioxidants counteracting the effect of ROS. Recent studies have suggested that the administration of antioxidants from different sources, with the additional anti-inflammatory action may be beneficial in IBD’s treatment. The beneficial effect
observed after FOR consumption is in accordance to that described by Chen et al.\textsuperscript{48} who detected hesperidin, hesperetin, nobiletin and tangeretin in orange peel and reported a significant cytoprotective effect against oxidative stress in HepG2 cells. Consequently, FOR could be potentially considered as a useful and inexpensive source of compounds to prevent or minimize the effect of intestinal inflammation.

Prebiotic fiber (e.g. pectin, oligofructose, inulin), on the other hand, helps to recover and/or maintain the normal state of the intestinal microbiota, which in turn produces short-chain fatty acids (SCFA) that decrease the synthesis or expression of inflammatory cytokines, and maintain the balance of the T regulatory cells (Treg) and T helper cells (Th)\textsuperscript{17}, counteracting and/or avoiding inflammation.\textsuperscript{49,50} Hartog et al.\textsuperscript{51} observed that oral intake of a multi-fibre mix (MF) counteracts IBD-like intestinal inflammation and weight loss in DSS treated mice, likely due to the fact that MF may induce a decrease in inflammatory cytokines levels and an increase in Treg cells in the mesenteric lymph nodes. These authors concluded that the optimization of enteral nutritional concepts dealing with the tested fibre mix could lead to the potential modulation of the gut microbiota composition and SCFA production to, subsequently, improve the inflammatory state and/or even induce remission.

In line with our results, Popov et al. (2014)\textsuperscript{52} demonstrated the \textit{in vitro} anti-inflammatory and antioxidant activities of pectic polysaccharides from fresh plums. Lastly, the dissimilar behavior observed for other assayed products as DOR and OL, which did not show any positive effect, especially when analyzing macroscopic parameters, might be explained by their low SDF and GalA content (Tables S1 and S2). Furthermore, Xiao et al.\textsuperscript{53} studied the preventive effects of cranberry (\textit{Vaccinium macrocarpon}) products on experimental colitis induced by DSS in mice and indicated
that dried cranberries were more effective in preventing colitis than cranberry extract. The same phenolic content and different blueberry fiber content in those products suggested that the fiber content could also contribute to the preventive effects on the development of colitis by its prebiotic action. A similar beneficial effect derived from the consumption of FOR or CP was observed by Cazarin et al.\textsuperscript{10} when supplying \textit{Passiflora edulis} peel flour before colitis induction by DSS in drinking water to female C57BL/6J mice. These authors reported that \textit{P. edulis} peel flour exerted an intestinal anti-inflammatory effect and attenuated the colonic damage due to its content of dietary fiber and polyphenols.

Patients with UC have presented deficiencies of antioxidant nutrients at the time of diagnosis,\textsuperscript{54,55} and patients with CD have shown a reduction of antioxidants in plasma and a decrease of total intestinal antioxidant capacity,\textsuperscript{56} suggesting an increase in ROS. Moura et al.\textsuperscript{57} indicated that this scenario could be balanced with the consumption of bioavailable functional foods, isolated nutrients, pro- and prebiotics, natural active compounds from vegetal sources, among other substances, all of them reported as effective antioxidants in IBD.

On the other hand, the anti-inflammatory effect caused by the AF ingestion could be associated to the \textit{N}-fructosyl-lysine content, formed as MRP during the concentration process and industrial drying at high temperature (100°C) and determined through the quantification of furosine. Some MRPs have been associated with different beneficial properties, such as antioxidant activities\textsuperscript{58} and anti-inflammatory effects.\textsuperscript{59} Our results are in good agreement to those reported by Hong et al.\textsuperscript{60} who indicated that glucose-lysine MRPs ameliorated DSS-induced colitis as determined by a decrease in DAI, colon weight/length ratio, nitric oxide levels in serum, recovery of body weight loss, and serum...
lysozyme levels, as well as suppression of mRNA level of the inflammatory cytokines in colon tissues, highlighting the potential of these MRPs in preventing or treating IBDs. Oh et al. (2017)\textsuperscript{61} have recently reported that MRPs derived from lysine and galactose decreased the production of TNF-α in macrophages and the expression of mRNA of interleukin IL-8 and IL-1b in Caco-2 cells in the model of DSS-induced colitis.

Concerning the effects derived from the intake of AF vs. CP, the variation of biochemical markers suggested a better anti-inflammatory response after the ingestion of CP, which could be due to its higher content of soluble dietary fibre (SDF) (96.58 vs. 12.46 g/100g DM) and N-fructosyl-lysine (827.4 vs. 455.1 mg of furosine/100g of protein) as compared to AF (Table S1).

To summarize, polyphenol and/or fiber are the key components previously reported to exert intestinal anti-inflammatory activity based on therapies with natural products. In this sense, the anti-inflammatory effects related to the consumption of FOR or CP found in the current work seem to confirm previous findings regarding the functional activity of polyphenols and fiber on IBD. Additionally, further attention should be also paid to the potentially preventive role of certain Amadori compounds against IBD.

5. Conclusions

Samples rich in pectin, Amadori compounds and phenolic compounds, such as citrus pectin, animal feed and fresh orange residue, gave rise to a lower expression of pro-inflammatory cytokines, intercellular adhesion molecules ICAM I, iNOS enzyme, and a higher expression of protective chemokines MUC 3, occludin and ZO-1. These indicators underlined the potential role of pectin and Maillard reaction compounds in ameliorating some IBD symptoms. These promising results warrant the performance of further
studies using superior doses of the selected orange by-products, as well as to broaden
the assessment of biochemical indicators in order to increase certainty about the effect
derived from the consumption of these singular by-products.

Conflict of interest
The authors declare no conflict of interest.

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**LEGENDS OF FIGURES**

**Figure 1.** Macroscopic indicators. (A) Weight gain. (B) Food intake. (C) Disease Activity Index (DAI). (D) Colonic weight/length ratio. FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. CP: citrus pectin.

**Figure 2.** Effects of orange by-products intake on the expression of pro-inflammatory cytokines and chemokines in colonic tissue of the DSS mice colitis. FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. CP: citrus pectin. Data are expressed as mean ± S. Different letters on the bars, indicate statistical differences (P < 0.05) amongst groups.
Table 1. Experimental conditions applied to the study of the consumption effect of orange by-products.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal weight (kg)</th>
<th>Daily dose of treatment</th>
<th>Average food intake (g/mice*day)</th>
<th>SDF content of treatment (g/kg animal*day)</th>
<th>Treatment supply (Days)</th>
<th>DSS (2.7%) supply (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>0.025</td>
<td>0.0</td>
<td>14.6</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DSS Control</td>
<td>0.025</td>
<td>0.0</td>
<td>10.5</td>
<td>0.0</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>FOR</td>
<td>0.025</td>
<td>10% in standard food</td>
<td>10.6</td>
<td>10.0</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>DOR</td>
<td>0.025</td>
<td>10% in standard food</td>
<td>10.9</td>
<td>2.1</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>OL</td>
<td>0.025</td>
<td>50 mg/kg of animal</td>
<td>11.2</td>
<td>0.9</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>AF</td>
<td>0.025</td>
<td>10% in the standard food</td>
<td>11.1</td>
<td>5.5</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>CP</td>
<td>0.025</td>
<td>10 mg/kg of animal</td>
<td>11.9</td>
<td>9.9</td>
<td>14</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 2. Clinical parameters considered to determine the Disease Activity Index (DAI).

<table>
<thead>
<tr>
<th>Bleeding</th>
<th>Stool consistency</th>
<th>Weight loss (WL) (%)</th>
<th>Value assigned according to WL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0: normal</td>
<td>0: normal</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1: presence of blood</td>
<td>1: moderate soft stools</td>
<td>1-4</td>
<td>1</td>
</tr>
<tr>
<td>2: moderate bleeding</td>
<td>2: soft stools</td>
<td>5-9</td>
<td>2</td>
</tr>
<tr>
<td>3: moderately high bleeding</td>
<td>3: soft stools and diarrhoea</td>
<td>10-19</td>
<td>3</td>
</tr>
<tr>
<td>4: abundant bleeding</td>
<td>4: diarrhoea</td>
<td>&gt;20</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 3. Primer sequences used in real-time qPCR assays in colonic tissue.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5′–3’</th>
<th>Annealing T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>FW: CCATCACCATCTTCCAGGAG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>RV: CCTGCTTCACCACCTTTTTG</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>FW: TGATGAGAATGACCTTTCT</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>RV: CTTCTTCAAAGATGGAAGAAA</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>FW: TAGTCCTTCCTACCCCCAATTCC</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>RV: TTGGTCCTTACGCACTCTTCC</td>
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<tr>
<td>TNF-α</td>
<td>FW: AACTAGTGGTGCCAGCCGAT</td>
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<td>RV: CTTCACAGAGCAATGACTCC</td>
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<td>ICAM-1</td>
<td>FW: AGGAGGTGAATGTATAAGTTATG</td>
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<td>RV: GGATGTGGAGGAGCAGAG</td>
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<td>iNOS</td>
<td>F: GCCAGAATGAGAAGCTGAG</td>
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<td>R: GAAGGCGTAGCTGAACAGA</td>
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<td>RV: CGGCTCTATCTCTAGCTCT</td>
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<td>ZO-1</td>
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<td>RV: TGGAGATGAGGCTCTGCT</td>
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<td>Occludin</td>
<td>FW: ACGGACCTGACCACTATGA</td>
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<tr>
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<td>RV: TCAGCAGCCATGTACTC</td>
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Figure 1. Pacheco et al.

A) Weight gain (g) over 21 days for different groups:
- Healthy
- DSS Control
- FOR
- DOR
- OL
- AF
- CP

B) Food intake (g) over 21 days for different groups:
- Healthy
- DSS Control
- FOR
- DOR
- OL
- AF
- CP
Figure 1. Pacheco et al.

C) Disease Activity Index (DAI)

D) Colonic weight/length ratio

Group

Healthy DSS Control FOR DOR OL AF CP
Figure 2. Pacheco et al.

A) TNF-α (Fold increase)

B) IL-1β (Fold increase)

C) IL-6 (Fold increase)
Figure 2. Pacheco et al.
Figure 2. Pacheco et al.

G) ZO-1 (Fold increase)

H) INOS (Fold increase)