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

M. Teresa Pacheco[a], Teresa Vezza[b], Patricia Diez-Echave[b], Pilar Utrilla[b], Mar Villamiel[a] and F. Javier Moreno*[a]

[a]Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM). Campus de la Universidad Autónoma de Madrid, Nicolás Cabrera 9 28049-Madrid, Spain.

[b]Departamento de Farmacología. Centro de Investigaciones Biomédicas en Red – Enfermedades Hepáticas y Digestivas (CIBER-EHD), Centro de Investigación Biomédica, Universidad de Granada, Granada, Spain.

*Corresponding author: javier.moreno@csic.es

Tel (+34) 91 0017948

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 infections[6] 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.[11] However, there is scarce evidence regarding prebiotics use in this type of pathologies.[12]

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)[13] that, without further treatment, cause environmental problems, since their fermentation implies high chemical and biological oxygen demand.[14,15] 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,[16] 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.[17] These approaches are mainly based on spontaneous colitis models, inducible colitis models, genetically modified models, and adoptive transfer models.[18-20] 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[21], as well as an increase of proinflammatory cytokines (IL-1β, TNF-α and IL-6) release in the intestine[22], which can impair permeability of intestine and mucosal barrier function and correlate with the severity of intestine inflammation.[23] 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.[24] 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.[25,26]

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,[27] 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.[28]

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[27]. 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.[29]. Antioxidant capacity was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method[30]. 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).[27]

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

126 atmosphere with a 12-h light–dark cycle, and provided with free access to tap water and
127 standard rodent diet (Panlab A04 diet, Panlab S.A., Barcelona, Spain).

128 **2.4. Experimental design**

129 Mice (23±2 g) were maintained in specific pathogen-free conditions in the facilities
130 of Licinio de la Fuente Center and were randomly assigned to seven groups (*n*=10):
131 healthy, DSS control, FOR, DOR, OL, AF and CP. Solid by-products samples were added
132 to standard food at 10%. CP and OL were diluted in water at concentrations of 2.5 and
133 1.25 % (w/v), respectively, and supplied at the rate of 100 µL/mouse per day. Induction
134 of colitis was performed 15 days after the beginning of the experiment by adding 2.7%
135 (w/v) DSS in the drinking water for seven days (Table 1).

136 **2.5. Macroscopic indicators**

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

145 **2.6. Biochemical markers**

146 The expression of pro-inflammatory cytokines as tumour necrosis factor (TNF)-α,
147 interleukin (IL)-6, (IL)-1β and inducible nitric oxide synthase (iNOS), as well as barrier
148 intestinal proteins as intercellular adhesion molecule (ICAM)-1, Mucin (MUC)-3,
149 Occludin and Zonula occludens-1 (ZO)-1, were evaluated.

150 The colon tissue was longitudinally divided into different fragments and stored at -
151 80°C in RNAlater®. Total RNA from colonic samples was isolated using Tri-Reagent®
152 following the manufacturer's protocol. All RNA samples were quantified with the
153 Thermo Scientific NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc.,
154 Waltham, MA, USA) and 2 µg of RNA was reverse transcribed using oligo (dT) primers
155 (Promega). Real-time quantitative PCR, was carried out on optical grade 48-well plates
156 in an Eco™ Real-Time PCR System (Illumina, CA, USA) with 20 ng of cDNA, the KAPA
157 SYBR®FAST qPCR Master Mix (Kapa Biosystems), and specific primers at their annealing
158 temperature (Table 3).[10] In order to normalize mRNA, the expression of the
159 housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was
160 measured. The mRNA relative quantitation was calculated using the ΔΔCt method.

161 **2.7. Statistical analysis**

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

166

167 **3. Results**

168 *3.1. Physicochemical characterization of test materials*

169 The main physicochemical characteristics determined in the orange by-products and
170 citrus pectin are summarized in Table S1. Overall, all studied orange by-products had a
171 similar composition in protein or fat content, whereas substantial differences were
172 found in carbohydrate, fiber or total phenolic content. By comparing the general
173 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,[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).[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.[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.[34] During the DSS period the intake

198 decreased significantly in the DSS control group, while animals treated with CP and FOR
199 showed food consumption levels similar to that observed in the healthy group, and
200 significantly different to DSS group ($P< 0.05$). Similarly, the AF group showed a higher
201 intake compared to DSS group, but non-significant differences were found.

202 The DAI, registered during the 7 days of DSS supply, allowed to infer that FOR,
203 CP and AF groups vs. DSS control colitic group presented the lowest DAI ($p<0.05$) (Figure
204 1C). Weight/length ratio of the colon was significantly less in those groups that
205 consumed FOR, AF and CP vs. the DSS group (Figure 1D), indicating a lower severity of
206 inflammation and minor colonic cells infiltration ($p< 0.05$).

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

212

213 *3.3. Assessment of biochemical markers of Inflammatory bowel disease in*
214 *DSS-treated mice*

215 Cytokine profile of DSS acute colitis is consistent with acute inflammatory response
216 characterized by a macrophage-derived cytokine profile, strong chemotactic pattern
217 and a polarized Th1 panel with high participation of TNF-α and IL-1β among others,
218 similar to human IBD disease. This acute situation is also accompanied by a high
219 expression of iNOS, and an increase of adhesion molecule ICAM I that facilitates the
220 leukocyte endothelial transmigration, contributing to the tissue damage and the
221 exacerbation of the gut inflammation.[35]

222 The expression of inflammatory cytokine panel (TNF-α, IL-1β, IL-6) was consistent
223 and significantly reduced in the groups fed with FOR, AF and CP vs DSS control group
224 (Figures 2A-2C); and additionally, a reduction in the expression of ICAM I was observed
225 in those groups that consumed the orange by-products, in comparison with DSS control
226 group (Figure 2D) ($p<0.05$). The expression of ICAM I was statistically similar between
227 the healthy group and the CP and FOR groups, followed by AF group.
228 MUC 3, occludin and ZO-1 expression was significantly greater in FOR, AF and CP
229 treated groups as compared with the DSS control group ($p< 0.05$) (Figure 2E-2H).
230 Increased expression of these proteins indicated a potential protective effect for those
231 products which can not only reduce the symptoms and seriousness in acute conditions
232 but also have a preventative effect against future IBD crisis.[36]
233 Nitric oxide synthase (iNOS) is an enzyme dominantly expressed during
234 inflammatory reactions. The synthesis of high amounts of nitric oxide (NO) by iNOS has
235 been demonstrated in pathophysiological processes, such as acute or chronic
236 inflammation and tumorigenesis; however, the role of iNOS activity in these diseases is
237 still not well understood.[37] Presence of nitric oxide (NO) is responsible for the generation
238 of substantial reactive oxygen species (ROS) as peroxynitrites or anion superoxide that
239 may affect the microbial agent and produce apoptosis of host cells.[38] Results indicated
240 that all tested orange by-products produced a significant reduction in iNOS expression
241 as compared to the DSS group (Figure 2H). There was no significant difference between
242 the AF, CP and the healthy group; and the groups that consumed DOR and OL showed a
243 higher expression of iNOS, slightly lower than that observed in the DSS control group,
244 which could be related with their low GalA content (Table S2).
245

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,[39] since it is well known that phenolic compounds are vulnerable to heat.[40] 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 sub-epithelial immune cells to commensal bacteria,[41,42] 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[43-45]. 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.[46,47] The beneficial effect

observed after FOR consumption is in accordance to that described by Chen et al.[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 citoquines, and maintain the balance of the T regulatory cells (Treg) and T helper cells (Th) 17, counteracting and/or avoiding inflammation.[49,50] Hartog et al.[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)[52] demonstrated the *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.[53] studied the preventive effects of cranberry (*Vaccinium macrocarpon*) products on experimental colitis induced by DSS in mice and indicated

294 that dried cranberries were more effective in preventing colitis than cranberry extract.
295 The same phenolic content and different blueberry fiber content in those products
296 suggested that the fiber content could also contribute to the preventive effects on the
297 development of colitis by its prebiotic action. A similar beneficial effect derived from the
298 consumption of FOR or CP was observed by Cazarin et al.[10] when supplying *Passiflora*
299 *edulis* peel flour before colitis induction by DSS in drinking water to female C57BL/6J
300 mice. These authors reported that *P. edulis* peel flour exerted an intestinal anti-
301 inflammatory effect and attenuated the colonic damage due to its content of dietary
302 fiber and polyphenols.

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

310 On the other hand, the anti-inflammatory effect caused by the AF ingestion could
311 be associated to the *N*-fructosyl-lysine content, formed as MRP during the concentration
312 process and industrial drying at high temperature (100°C) and determined through the
313 quantification of furosine. Some MRPs have been associated with different beneficial
314 properties, such as antioxidant activities[58] and anti-inflammatory effects.[59] Our results
315 are in good agreement to those reported by Hong et al.[60] who indicated that glucose-
316 lysine MRPs ameliorated DSS-induced colitis as determined by a decrease in DAI, colon
317 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)[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

342 studies using superior doses of the selected orange by-products, as well as to broaden
343 the assessment of biochemical indicators in order to increase certainty about the effect
344 derived from the consumption of these singular by-products.
345
346 **Conflict of interest**
347 The authors declare no conflict of interest.
348
349 **Acknowledgements**
350 The authors are grateful to García Carrión SA for providing samples and funding projects
351 AGL2014-53445-R (MINECO) and ALIBIRD-CM S-2013/ABI-272 (CAM). M.T.P. thanks the
352 National Secretary of Superior Education, Science, Technology and Innovation
353 SENESCYT–Ecuador.
354

References

1. A. Szilagyi and X. Xue, Comparison of geographic distributions of Irritable Bowel Syndrome with Inflammatory Bowel Disease fail to support common evolutionary roots: Irritable Bowel Syndrome and Inflammatory Bowel Diseases are not related by evolution, *Med. Hypotheses,* 2018, **110**, 31–37.

2. C. J. Moran, Very early onset inflammatory bowel disease, *Semin. Pediatr. Surg.,* 2017, **26**, 356–359.

3. A. N. Ananthakrishnan, Epidemiology and risk factors for IBD. *Nature Reviews Gastroenterol. Hepatol.,* 2015, **12**, 205–217.

4. B. R. R. De Mattos, M. P. G. Garcia, J. B. Nogueira, L. N. Paiatto, C. G. Albuquerque, C. L. Souza, L. G. R. Fernandes, W. M. da S. C. Tamashiro and P. U. Simioni, Inflammatory bowel disease: An overview of immune mechanisms and biological treatments. *Mediators Inflamm,* 2015, Article ID 493012.

5. D. Lee, L. Albenberg, Ch. Compher, R. Baldassano, D. Piccoli, J. D. Lewis and G. D. Wu, Diet in the Pathogenesis and Treatment of Inflammatory Bowel Diseases, *Gastroenterology,* 2016, **148**, 1087–1106.

6. P. Frei, L. Biedermann, O. H. Nielsen and G. Rogler, Use of thiopurines in inflammatory bowel disease, *World J. Gastroenterol.*, 2013, **19**, 1040-1048.

7. P. Andersson and J. D. Soderholm, Surgery in ulcerative colitis: indication and timing, *Digest. Dis.*, 2009, **27**, 335-340.

8. L. A. A. P. Derikx, L. A. Dieleman and F. Hoentjen, Probiotics and prebiotics in ulcerative colitis, *Best Pract. Res. Clin. Gastroenterol.,* 2016, **30**, 55–71.

9. A. Forbes, J. Escher, X. Hébuterne, S. Kłęk, Z. Krznaric, S. Schneider, R. Shamir, K. Stardelova, N. Wierdsma, A. E. Wiskin and S. C. Bischoff, ESPEN guideline: Clinical nutrition in inflammatory bowel disease, *Clin. Nutr.*, 2017, **36**, 321–347.

10. C. B. B. Cazarin, A. Rodriguez-Nogales, F. Algieri, M. P. Utrilla, M. E. Rodríguez-Cabezas, J. Garrido-Mesa, E. Guerra-Hernández, P. A. de C. Braga, F. G. Reyes Reyes, M. R. Jr. Maróstica and J. Gálvez, Intestinal anti-inflammatory effects of *Passiflora edulis* peel in the dextran sodium sulphate model of mouse colitis, *J. Funct. Foods,* 2016, **26**, 565–576.

11. J. Gálvez, M. Comalada and J. Xaus, in *Bioactive Foods in Promoting Health*, ed. R. R. Watson and V. R. Preedy (Eds.), Academic Press, Cambridge, MA, First edition, 2010, Chapter 35, pp. 589–610.

12. L. Lichtenstein, I. Avni-Biron and O. Ben-Bassat, Probiotics and prebiotics in Crohn's disease therapies, *Best Pract. Res. Clin. Gastroenterol.,* 2016, **30**, 81–88.

13. Food and Agriculture Organization of the United Nations - Intergovernmental Group on Citrus Fruits - A Subsidiary Body of the FAO Committee on Commodity Problems (CCP), Citrus Fruits Statistics 2015, Rome, 2016, 1–53. Available from: http://www.fao.org/3/a-i5558e.pdf (accessed February 2018).

14. D. Mamma and P. Christakopoulos, Biotransformation of citrus by-products into value added products, *Waste Biomass Valori.,* 2014, **5**, 529–549.

15. C. S. K. Lin, L. A. Pfaltzgraff, L. Herrero-Davila, E. B. Mubofu, S. Abderrahim, J. H. Clark, A. A. Koutinas, N. Kopsahelis, K. Stamatelatou, F. Dickson, S. Thankappan, Z. Mohamed, R. Brocklesby and R. Luque, Food waste as a valuable resource for the production of chemicals, materials and fuels. Current situation and global perspective, *Energy Environ. Sci.,* 2013, **6**, 426-464.

16. I. G. Hwang, H. Y. Kim, K. S. Woo, J. Lee and H. S. Jeong, Biological activities of Maillard reaction products (MRPs) in a sugar-amino acid model system, *Food Chem.,* 2011, **126**, 221-227.

17. M. Perše and A. Cerar, Dextran sodium sulphate colitis mouse model: Traps and tricks. *J. Biomed. Biotechnol.,* 2012, Article ID 718617.

18. M. Neurath, I. Fuss and W. Strober, TNBS-colitis, *Int. Rev. Immunol.,* 2000, **19**, 51–62.

19. S. Wirtz and M. F. Neurath, Mouse models of inflammatory bowel disease, *Adv. Drug Deliv. Rev.*, 2007, **59**, 1073–1083.

20. S. Wirtz, C. Neufert, B. Weigmann and M. F. Neurath, Chemically induced mouse models of intestinal inflammation, *Nat. Protoc.*, 2007, **2**, 541–546.

21. I. Okayasu, S. Hatakeyama, M. Yamada, T. Ohkusa, Y. Inagaki and R. Nakaya, A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice, *Gastroenterology*, 1990, **98**, 694–702.

22. D. Q. Shih and S.R. Targan, Immunopathogenesis of inflammatory bowel disease, *World J. Gastroenterol.,* 2008, **14**, 390-400.

23. G. Zhu, H. Wang, T. Wang and F. Shi, Ginsenoside Rg1 attenuates the inflammatory response in DSS-induced mice colitis, *Int. Immunopharmacol.*, 2017, **50**, 1–5.

24. R. B. Sartor, Microbial influences in inflammatory bowel diseases, *Gastroenterology*, 2008, **134**, 577-594.

25. M. De Robertis, E. Massi, M. L. Poeta, S. Carotti, S. Morini, L. Cecchetelli, E. Signori and V. M. Fazio, The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies, *J. Carcinog.,* 2011, **10**, 9.

26. M. Kanneganti, M. Mino-Kenudson and E. Mizoguchi, Animal models of colitis-

associated carcinogenesis, *J. Biomed. Biotechnol.,* 2011, Article ID 342637.

27. M. T. Pacheco, F. J. Moreno and M. Villamiel, Chemical and physicochemical characterization of orange by-products derived from industry, *J. Sci. Food Agr.,* 2018, in press.

28. Q. Sun, L. Luan, M. Arif, J. Li, Q. J. Dong, Y. Gao, Z. Chi and C. G. Liu, Redox-sensitive nanoparticles based on 4-aminothiophenol-carboxymethyl inulin conjugate for budesonide delivery in inflammatory bowel diseases, *Carbohydr. Polym.*, 2018, **189**, 352–359.

29. A. C. Soria, M. Corzo-Martínez, A. Montilla, E. Riera, J. Gamboa-Santos and M. Villamiel, Chemical and physicochemical quality parameters in carrots dehydrated by power ultrasound, *J. Agric. Food Chem.,* 2010, **58**, 7715–7722.

30. W. Brand-Williams, M. E. Cuvelier and C. Berset, Use of a free radical method to evaluate antioxidant activity, *LWT - Food Sci. Technol.,* 1995, **28**, 25–30.

31. H. S. Cooper, S. N. Murthy, R. S. Shah and D. J. Sedergran, Clinicopathologic study of dextran sulfate sodium experimental murine colitis, *Lab. Investig.,* 1993, **69**, 238–249.

32. H. F. Erbersdobler and V. Somoza, Forty years of furosine - Forty years of using Maillard reaction products as indicators of the nutritional quality of foods, *Mol. Nutr. Food Res.,* 2007, **51**, 423–430.

33. J. Holck, A. T. Hotchkiss, Jr., A. S. Meyer, J. D. Mikkelsen and R. A. Rastall, in *Food Oligosaccharides: Production, Analysis and Bioactivity*, ed. F. J. Moreno and M. L. Sanz, John Wiley & Sons, Inc., Hoboken-New Jersey, First Edition, 2014, Chapter 5, pp. 76–87.

34. Y. R. Yu and J. R. Rodriguez, Clinical presentation of Crohn's, ulcerative colitis, and

449 indeterminate colitis: Symptoms, extraintestinal manifestations, and disease
450 phenotypes, *Semin. Pediatr. Surg.,* 2017, **26**, 349–355.
451 35. M. Abdelbaqi, J. H. Chidlow, K. M. Matthews, K. P. Pavlick, S. C. Barlow and A. J.
452 Linscott, Regulation of dextran sodium sulfate induced colitis by leukocyte beta 2
453 integrins, *Lab. Investig.,* 2006, **86**, 380–390.
454 36. Y. Nie, Q. Lin and F. Luo, Effects of non-starch polysaccharides on inflammatory
455 bowel disease, *Int. J. Mol. Sci.,* 2017, **18**, 1372.
456 37. E. Gochman, J. Mahajna, P. Shenzer, A. Dahan, A. Blatt, R. Elyakim and A. Z. Reznick,
457 The expression of iNOS and nitrotyrosine in colitis and colon cancer in humans, *Acta*
458 *Histochem.,* 2012, **114**, 827–835.
459 38. I. Soufli, R. Toumi, H. Rafa and Touil-Boukoffa, C, Overview of cytokines and nitric
460 oxide involvement in immuno-pathogenesis of inflammatory bowel diseases, *World*
461 *J. Gastrointest. Pharmacol. Ther.,* 2016, **7**, 353–360.
462 39. M. Marić M, A. N. Grassino, Z. Zhu, F. J. Barba, M. Brnčić and S. Rimac Brnčić, An
463 overview of the traditional and innovative approaches for pectin extraction from
464 plant food wastes and by-products: Ultrasound-, microwaves-, and enzyme-assisted
465 extraction, *Trends Food Sci. Technol.*, 2018, **76,** 28–37.
466 40. C. Brownmiller, L. Howard and R. Prior, Processing and storage effects on monomeric
467 anthocyanins, percent polymeric color, and antioxidant capacity of processed
468 blueberry products. *J. Food Sci.,* 2008, **73**, H72–H79.
469 41. L. A. Dieleman, B. U. Ridwan, G. S. Tennyson, K. W. Beagley, R. P. Bucy and C. O.
470 Elson, Dextran sulfate sodium-induced colitis occurs in severe combined
471 immunodeficient mice, *Gastroenterology,* 1994, **107**, 1643–1652.

42. L. G. Axelsson, E. Landstrom, T. J. Goldschmidt, A. Gronberg and A. C. Bylund-Fellenius, Dextran sulfate sodium (DSS) induced experimental colitis in immunodeficient mice: effects in CD4(+)-cell depleted, athymic and NK-cell depleted SCID mice, *Inflamm. Res.,* 1996, **45**, 181–191.

43. J. Fang, T. Seki, T. Tsukamoto, H. Qin, H. Yin, L. Liao, H. Nakamura and H. Maeda, Protection from inflammatory bowel disease and colitis-associated carcinogenesis with 4-vinyl-2,6-dimethoxyphenol (canolol) involves suppression of oxidative stress and inflammatory cytokines, *Carcinogenesis,* 2013, **34**, 2833–2841.

44. A. Rezaie, R. D. Parker and M. Abdollahi, Oxidative stress and pathogenesis of inflammatory bowel disease: An epiphenomenon or the cause?, *Dig. Dis. Sci.,* 2007, **52**, 2015–2021.

45. A. Roessner, D. Kuester, P. Malfertheiner and R. Schneider-Stock, Oxidative stress in ulcerative colitis-associated carcinogenesis, *Pathol. Res. Pract.,* 2008, **204**, 511–524.

46. S. C. Ng, Y. T. Lam, K. K. F. Tsoi, F. K. L. Chan, J. J. Y. Sung and J. C. Y. Wu, Systematic review: The efficacy of herbal therapy in inflammatory bowel disease, *Aliment. Pharmacol. Ther.,* 2013, **38**, 854–863.

47. R. Rahimi, M. R. Shams-Ardekani and M. Abdollahi, A review of the efficacy of traditional Iranian medicine for inflammatory bowel disease, *World J. Gastroenterol.,* 2010, **16**, 4504–4514.

48. Z. T. Chen, H. L. Chu, C. C. Chyau, C. C. Chu and P. Der. Duh, Protective effects of sweet orange (*Citrus sinensis*) peel and their bioactive compounds on oxidative stress, *Food Chem.,* 2012, **135**, 2119–2127.

49. K. M. Maslowski, A. T. Vieira, A. Ng, J. Kranich, F. Sierro, D. Yu, H. C. Schilter, M. S. Rolph, F. Mackay, D. Artis, R. J. Xavier, M. M. Teixeira and C. R. Mackay, Regulation

496 of inflammatory responses by gut microbiota and chemoattractant receptor GPR43,
497 *Nature*, 2009, **461**, 1282-1286.
498 50. I. I. Ivanov, R. de L. Frutos, N. Manel, K. Yoshinaga, D. B. Rifkin, R. B. Sartor, B. B.
499 Finlay and D. R. Littman, Specific microbiota direct the differentiation of IL-17-
500 producing T-helper cells in the mucosa of the small intestine, *Cell Host Microbe,*
501 2008, **4**, 337-349.
502 51. A. Hartog, F. N. Belle, J. Bastiaans, P. De Graaff, J. Garssen, L. F. Harthoorn and A. P.
503 Vos, A potential role for regulatory T-cells in the amelioration of DSS induced colitis
504 by dietary non-digestible polysaccharides, *J. Nutr. Biochem.,* 2015, **26**, 227-233.
505 52. S. V. Popov, R. G. Ovodova, V. V. Golovchenko, D. S. Khramova, P. A. Markov, V. V.
506 Smirnov, A. S. Shashkov and Y. S. Ovodov, Pectic polysaccharides of the fresh plum
507 *Prunus domestica* L. isolated with a simulated gastric fluid and their anti-
508 inflammatory and antioxidant activities, *Food Chem.,* 2014, **143**, 106–113.
509 53. X. Xiao, J. Kim, Q. Sun, D. Kim, C. S. Park, T. S. Lu and Y. Park, Preventive effects of
510 cranberry products on experimental colitis induced by dextran sulphate sodium in
511 mice, *Food Chem.,* 2015, **167**, 438–446.
512 54. R. Razack and D. L. Seidner, Nutrition in inflammatory bowel disease, *Curr Opin*
513 *Gastroenterol.,* 2007, **23**, 400–405.
514 55. D. L. Seidner, B. A. Lashner, A. Brzezinski, P. L. C. Banks, J. Goldblum, C. Fiocchi, J.
515 Katz, G. R. Lichtenstein, P. A. Anton, L. Y. Kam, K. A. Garleb and S. J. Demichele, An
516 oral supplement enriched with fish oil, soluble fiber, and antioxidants for
517 corticosteroid sparing in ulcerative colitis: a randomized, controlled trial, *Clin.*
518 *Gastroenterol. Hepatol.,* 2005, **3**, 358–369.
519 56. D. Genser, M. H. Kang, H. Vogelsang and I. Elmadfa, Status of lipidsoluble

520 antioxidants and TRAP in patients with Crohn's disease and healthy controls, *Eur. J.*
521 *Clin. Nutr.*, 1999, **53**, 675–679.
522 57. F. A. Moura, K. Q. de Andrade, J. C. F. dos Santos, O. R. P. Araújo and M. O. F. Goulart,
523 Antioxidant therapy for treatment of inflammatory bowel disease: Does it work?,
524 *Redox Biol.,* 2015, **6**, 617–639.
525 58. A. P. Echavarria, J. Pagan and A. Ibarz, Optimization of Maillard reaction products
526 isolated from sugar-amino acid model system and their antioxidant activity,
527 *Afinidad,* 2013, **70**, 86–92.
528 59. D. D. Kitts, X. M. Chen and H. Jing, Demonstration of antioxidant and anti-
529 inflammatory bioactivities from sugar-amino acid Maillard reaction products, *J.*
530 *Agric. Food Chem.,* 2012, **60**, 6718–6727.
531 60. C.-O. Hong, C. H. Rhee, M. C. Pyo and K.-W. Lee, Anti-inflammatory effect of glucose-
532 lysine Maillard reaction products on intestinal inflammation model *in vivo*, *Int.*
533 *Immunopharmacol.,* 2017, **52**, 324–332.
534 61. J.-G. Oh, S.-H. Chun, D. H. Kim, J. H. Kim, H. S. Shin, Y. S. Cho, Y. K. Kim, H. Choi and
535 K.-W. Lee, Anti-inflammatory effect of sugar-amino acid Maillard reaction products
536 on intestinal inflammation model *in vitro* and *in vivo*, *Carbohydr. Res.,* 2017, **449**,
537 47–58.
538

539 ***LEGENDS OF FIGURES***

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

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

Table 1. Experimental conditions applied to the study of the consumption effect of orange by-products.

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

SDF: soluble dietary fiber. DSS: Dextran sulfate sodium. FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. CP: citrus pectin.

548

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

Bleeding	Stool consistency	Weight loss (WL) (%)	Value assigned according to WL
0: normal	0: normal	0	0
1: presence of blood	1: moderate soft stools	1-4	1
2: moderate bleeding	2: soft stools	5-9	2
3: moderately high bleeding	3: soft stools and diarrhoea	10-19	3
4: abundant bleeding	4: diarrhoea	>20	4

Table 3. Primer sequences used in real-time qPCR assays in colonic tissue.

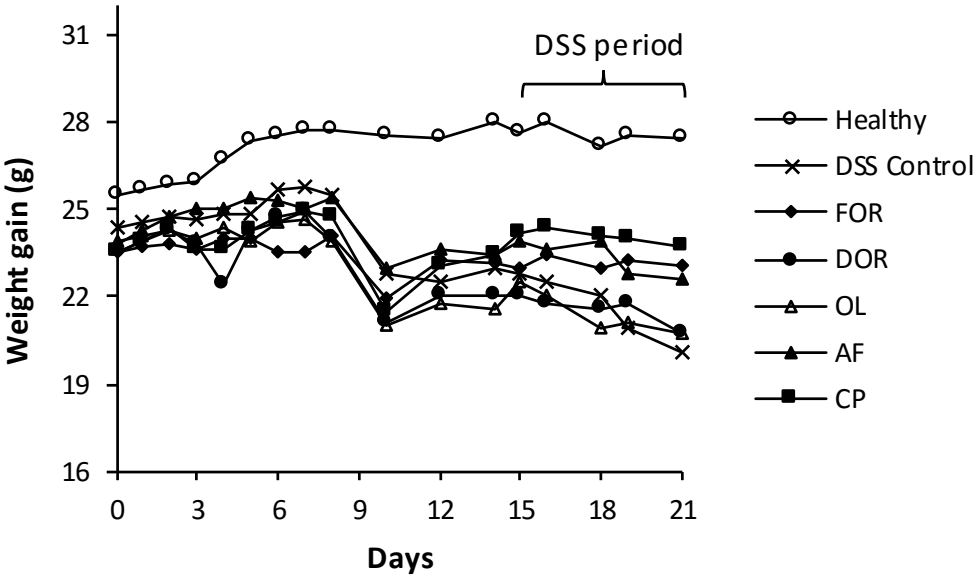
Gene	Sequence 5'–3'	Annealing T (°C)
GAPDH	FW: CCATCACCATCTTCCAGGAG	60
	RV: CCTGCTTCACCACCTTCTTG	
IL-1β	FW: TGATGAGAATGACCTCTTCT	55
	RV: CTTCTTCAAAGATGAAGGAAA	
IL-6	FW: TAGTCCTTCCTACCCCAATTTCC	60
	RV: TTGGTCCTTAGCCACTCCTTC	
TNF-α	FW: AACTAGTGGTGCCAGCCGAT	56
	RV: CTTCACAGAGCAATGACTCC	
ICAM-1	FW: AGGAGGTGAATGTATAAGTTATG	60
	RV: GGATGTGGAGGAGCAGAG	
iNOS	F: GGCAGAATGAGAAGCTGAGG	55
	R: GAAGGCGTAGCTGAACAAGG	
MUC-3	FW: CGTGGTCAACTGCGAGAATGG	62
	RV: CGGCTCTATCTCTACGCTCT	
ZO-1	FW: GGGGCCTACACTGATCAAGA	56
	RV: TGGAGATGAGGCTTCTGCT	
Occludin	FW: ACGGACCCTGACCACTATGA	56
	RV: TCAGCAGCAGCCATGTACTC	

549

550

Figure 1. *Pacheco et al.*

A)



B)

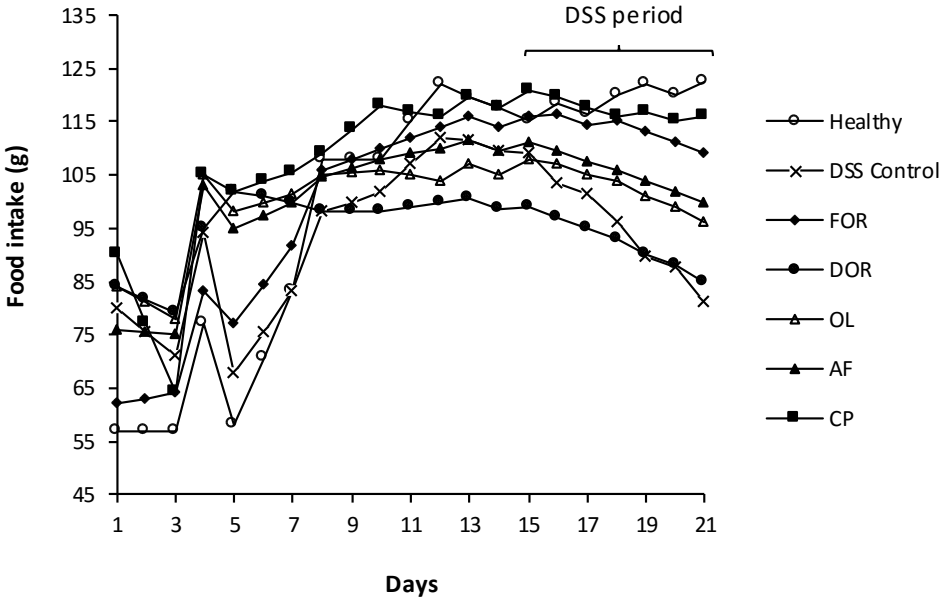
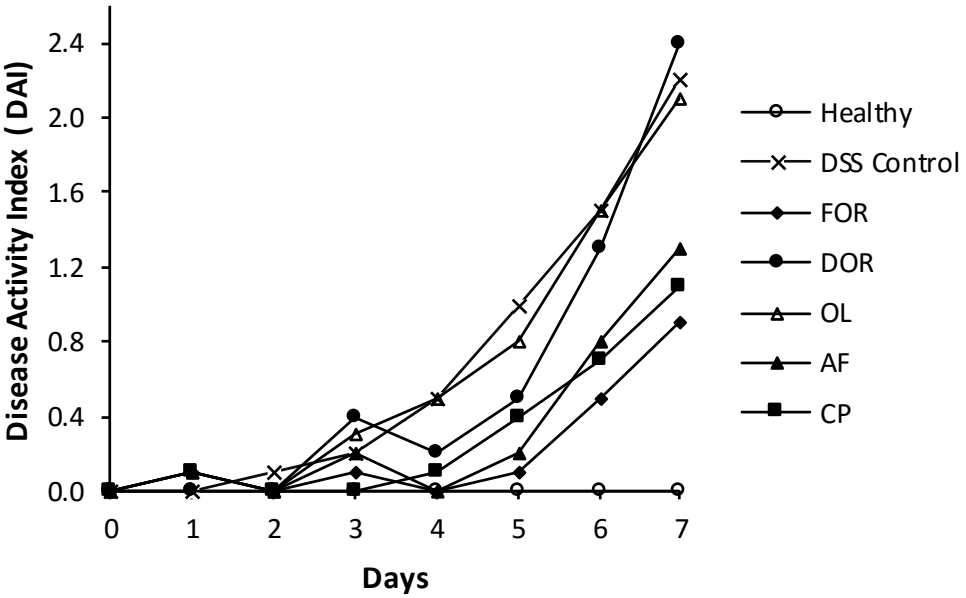


Figure 1. *Pacheco et al.*

C)



D)

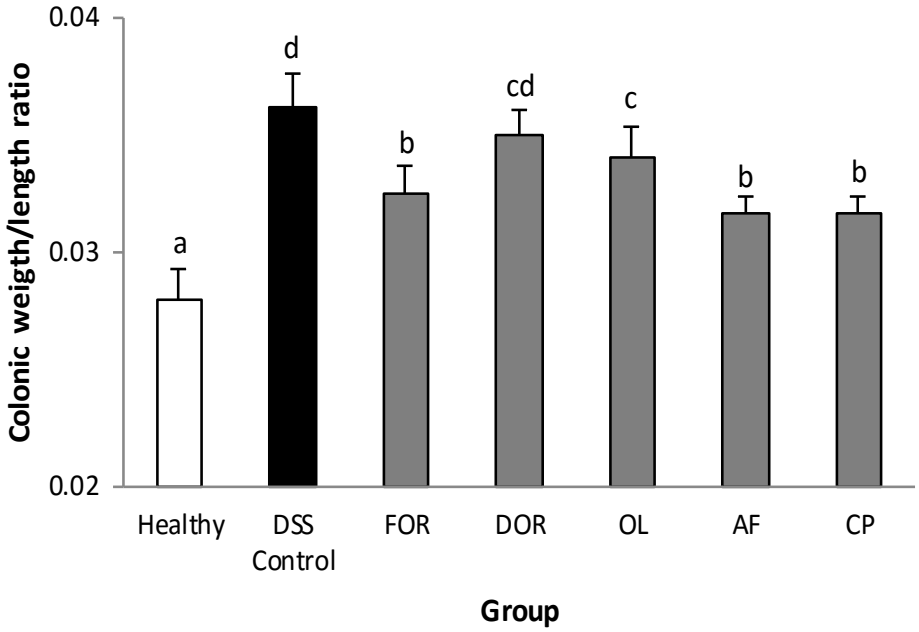
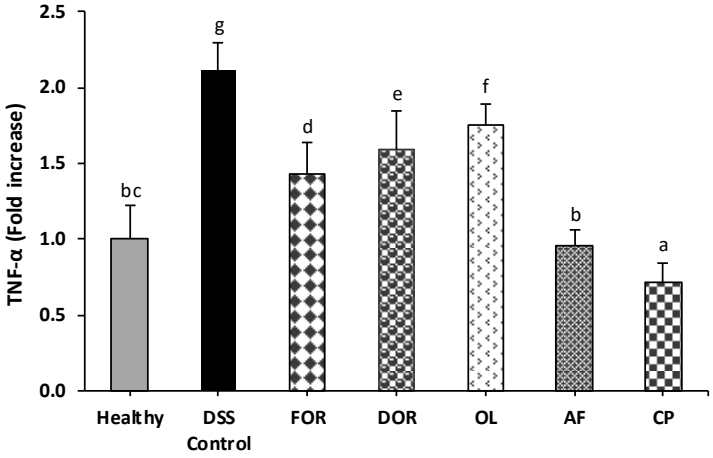
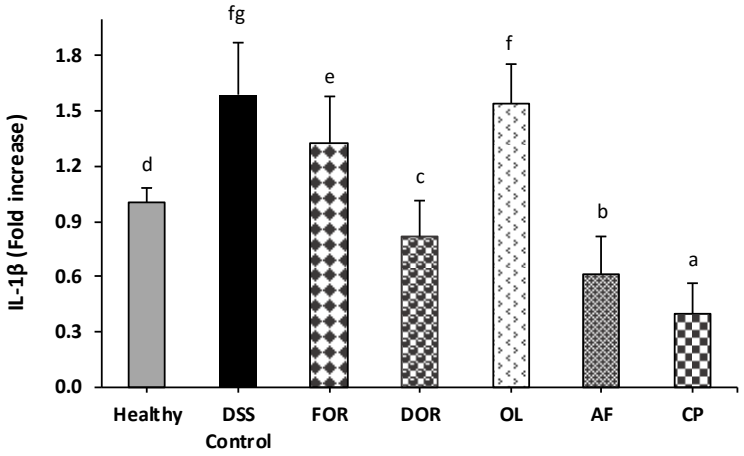


Figure 2. *Pacheco et al.*

A)



B)



C)

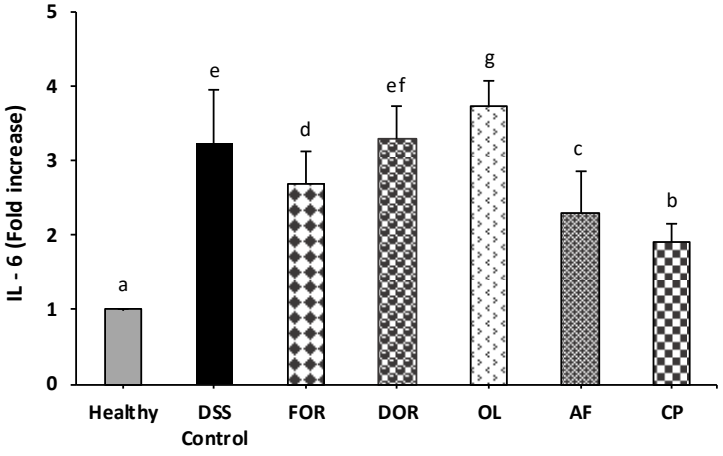
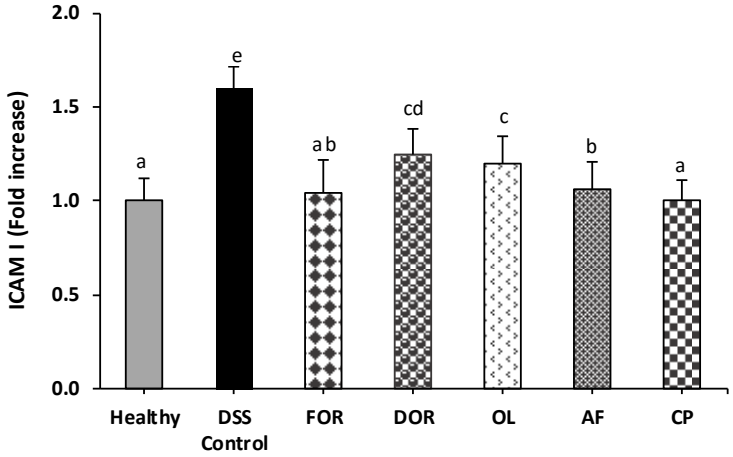
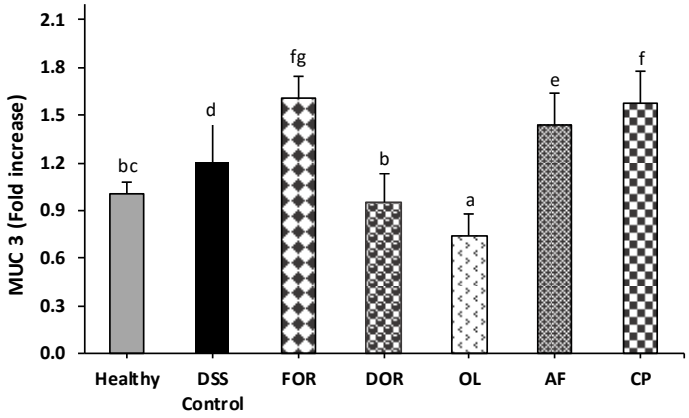


Figure 2. *Pacheco et al.*

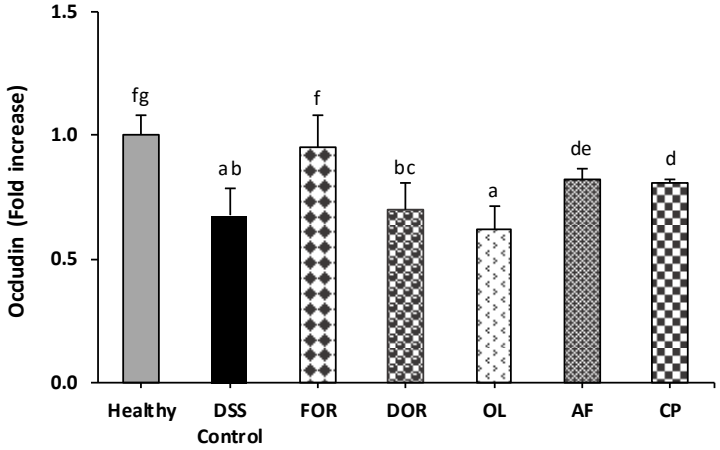
D)



E)

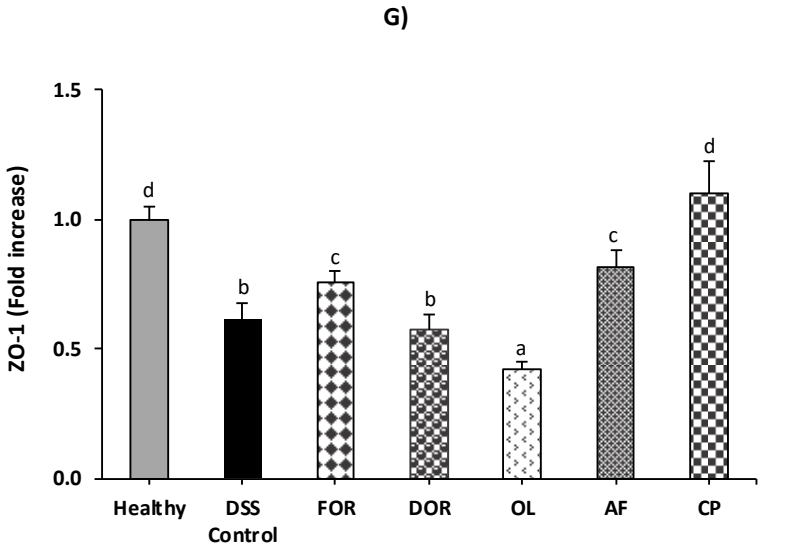


F)



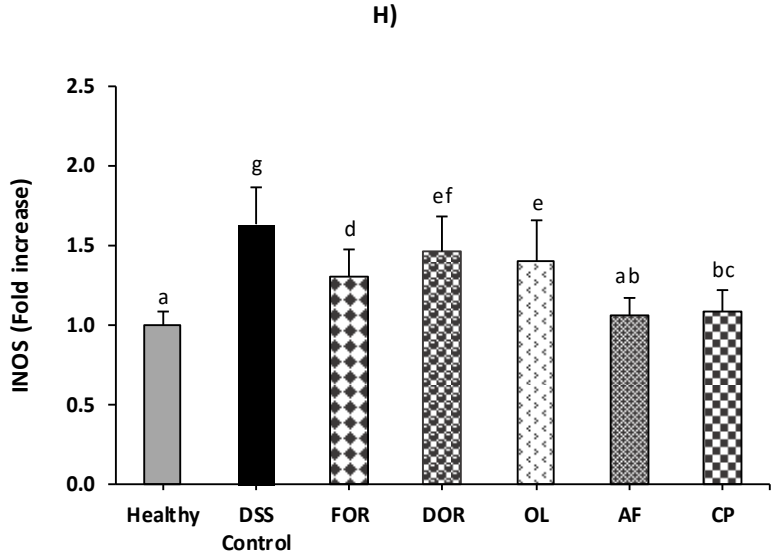
582 **Figure 2**. *Pacheco et al.*

583



584

585



586