

# EFFECTS OF A COCOA DIET ON AN INTESTINAL INFLAMMATION MODEL IN RATS

**Short title:** Cocoa on rat intestinal inflammation

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## **Abstract**

Cocoa is a rich source of fiber and flavonoids with recognized antioxidant and anti-inflammatory potential. The aim of this study was to evaluate the effects of a cocoa-enriched diet on rats with dextran sulphate sodium (DSS)-induced colitis. Wistar rats were fed with either a 5% cocoa diet or standard diet. Colon inflammation was induced by DSS in the drinking water: 5% for 6 days and 2% over the following 9 days. Colitis was assessed by body weight loss, stool consistency and blood presence in stools. A group of animals fed standard diet was treated with quercitrin (1 mg/kg) after colitis establishment. After 2 weeks of DSS treatment, the colon oxidative and inflammatory status and lymphocyte composition from blood and mesenteric lymph nodes (MLN) were assessed. The cocoa-fed group did not exhibit amelioration of clinical colitis but displayed higher antioxidant activity than the colitic reference group by the restoration of colon glutathione content and prevention of lipid peroxidation. The cocoa diet showed anti-inflammatory potential because it down-regulated serum TNF- $\alpha$ , colon iNOS activity and decreased colon cell infiltration. Lymphocyte composition in MLN was not modified by drinking DSS, but there was an increase in the proportion of NK and regulatory T cells in the blood. These changes were not modified by cocoa. In conclusion, cocoa intake may help to inhibit the negative oxidative effects consequent to colitis, although this action is not enough to abrogate the intestinal inflammation significantly.

**Keywords:** Cocoa, flavonoids, dextran sulphate sodium, colitis

## Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) constitute the principal forms of chronic inflammatory bowel disease (IBD). Although the exact etiology of both CD and UC remains unclear, IBD inflammation is thought to result from a complex interaction of genetic and environmental factors that leads to altered immunologic responses to gut microbiota<sup>1</sup>.

Current treatments for IBD include immunosuppressant or biological drugs blocking key molecules of the inflammatory cascade. However, significant side effects have been reported thereby limiting their use<sup>2</sup>. Because of this, the use of complementary or alternative interventions is becoming widespread and an increasing number of studies show the potential of biologically active nutrients, which may play an important role in the immune response modulation of IBD<sup>3</sup>. In this context, since the colon may be subjected to oxidative stress during active inflammation, the incorporation of dietary antioxidants might help to reduce mucosal inflammation<sup>4</sup>. Commonly occurring dietary polyphenols, particularly flavonoids, have been reported to possess remarkable antioxidant and anti-inflammatory capability in experimental conditions<sup>5,6</sup>. Cocoa and cocoa-based products are natural sources of flavan-3-ols such as (-)-epicatechin, (+)-catechin and their oligomers, the procyanidins<sup>7</sup>. Moreover, cocoa is considered an excellent source of dietary fiber, which retains significant amounts of polyphenolic compounds and plays a key role in their transportation through the gastrointestinal tract<sup>8</sup>. Cocoa procyanidins seem to reach the colon intact where they are metabolized by the intestinal microbiota into various phenolic acids<sup>9</sup>. Several studies have demonstrated the antioxidant capacity of cocoa flavonoids and their metabolites<sup>10</sup>. In addition, cocoa has exhibited *in vitro* and *in vivo* anti-inflammatory and regulatory effects on immune cells involved in innate and acquired immunity<sup>11-13</sup>.

As the oral administration of dextran sulphate sodium (DSS) is a widely used and well-characterized model of colitis in rodents and shares clinical and pathological characteristics with human IBD<sup>14</sup>, the aim of this study was to evaluate the effects of a diet containing cocoa on DSS-induced chronic colitis in rats. In particular, we focused on the response of mesenteric lymph nodes and blood lymphocytes and on the assessment of diverse biochemical variables associated with colonic oxidative stress induced by DSS-induced colitis. In addition, the effects of cocoa were compared with those exerted by quercitrin, a glycoside of the flavonoid quercetin with recognized anti-inflammatory activity in the DSS model<sup>6</sup> which was used as a control treatment.

## Materials and methods

### Reagents

Dextran sulphate sodium (DSS) of molecular weight 36-50 kDa was obtained from MP Biomedicals (Illkirch, France). RPMI 1640 medium, glutamine, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from PAA (Pasching, Austria). Quercitrin was obtained from Extrasynthèse (Genay, France). Defatted Natural Forastero cocoa containing 21.85 mg/g polyphenols (Table 1) was provided by Nutrexp SL (Barcelona, Spain). All other reagents, unless otherwise stated, were purchased from Sigma-Aldrich (Madrid, Spain).

**Table 1.** Polyphenol composition of the cocoa used in the study. Individual phenolic compounds and total phenolic content in cocoa powder were determined by HPLC and the Folin-Ciocalteu method respectively, as previously described<sup>15</sup>.

Compound	mg/g
Procyanidin B <sub>2</sub>	1.487
Catechin	0.740
Epicatechin	1.917
Isoquercitin	0.053
Quercitin	0.020
Total polyphenols	21.85

### Animals and diets

Five-week-old female Wistar rats (Harlan, Barcelona, Spain) were housed in cages under controlled conditions of temperature (22±2 °C) and humidity in a 12:12 light-dark cycle. All experimental procedures were reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (reference DMAH3874).

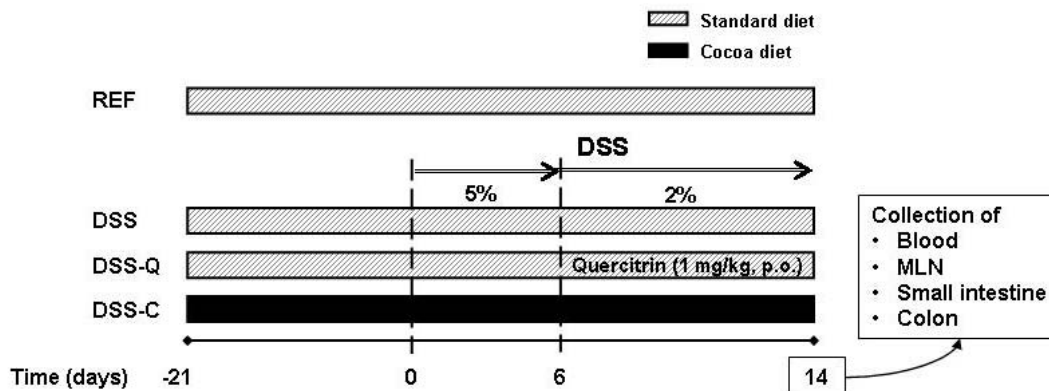
Animals had free access to water and chow. The AIN-93M formulation<sup>16</sup> was used as the standard diet. A diet containing 50 g/kg cocoa was produced from a modification of the AIN-93M formula as previously described<sup>12</sup> and was provided for 3 weeks prior to DSS administration and until the end of the study (Figure 1).

### Induction and assessment of colitis

Colitis was induced by *ad libitum* administration of 5% DSS (w/v) in drinking water for 6 days and 2% DSS for the following 9 days. Animals were randomly assigned to four experimental groups (n=12/group; Figure 1): a group receiving a standard diet and water without DSS (REF); a group fed the standard diet and water with DSS (DSS); a group fed the standard diet and water with DSS that was treated daily with quercitrin (1 mg/kg, p.o.) from the day that the DSS dose was reduced to 2% (DSS-Q); and a group fed the cocoa diet from three weeks prior to DSS drinking and until the end of the study (DSS-C). The dosing

protocol for quercitrin was established according to reported studies showing an amelioration of the DSS-induced inflammatory process<sup>6</sup>.

Clinical assessment of colitis included daily evaluation of body weight, stool consistency (normal, loose or diarrhea), and presence of macroscopic blood in the stools. These variables allowed calculating a disease activity index (DAI) with a 0 to 4 point severity scale<sup>17</sup>. At the end of the study (Figure 1), animals were anesthetized intramuscularly with ketamine (90 mg/kg) and xylazine (10 mg/kg). Blood, mesenteric lymph nodes (MLNs), small intestine and colon were obtained from each animal.



**Figure 1.** Experimental design: three groups (Reference -REF-, DSS and DSS-Quercitrin -DSS-Q-) were fed a standard diet, and a group (DSS-C) was fed a cocoa diet throughout the study from three weeks before colitis induction. DSS was incorporated into the drinking water at 5% and 2% over 6 and 9 days, respectively. Quercitrin was administered by oral route (1 mg/kg/day) to the DSS-Q group from day 6.

### Mesenteric lymph node and blood lymphocyte phenotype

MLNs and blood lymphocytes were isolated as previously described<sup>12</sup>. Lymphocyte phenotype was determined by three-color immunofluorescence staining and flow cytometry analysis. Mouse anti-rat monoclonal antibodies (MAb) used here included: fluorescein isothiocyanate (FITC)-conjugated anti-T-cell-receptor (TCR)  $\alpha\beta$  (R73), CD45RA (OX-33), CD4 (OX-35) and CD25 (IL-2R $\alpha$  chain OX-39) MAb; phycoerythrin (PE)-conjugated anti-CD4 (OX-35) and NKR-P1A (10/78) MAb; peridinin-chlorophyll-protein (PerCP)-conjugated anti-CD8 $\alpha$  (OX-8) MAb (all from BD Biosciences); allophycocyanin (APC)-conjugated anti-Foxp3 (FJK-16a) MAb (eBioscience, Frankfurt, Germany).

Extracellular and intracellular staining was carried out as in previous studies<sup>12</sup>. Flow cytometry analysis was performed on a Cytomics FC500-MPL cytometer (Beckman Coulter, Miami, FL, USA) and data were analyzed by the software Summit V3.1 (Cytomation, Fort Collins, CO, USA). Lymphocyte subsets were defined as B (CD45RA+), Th (CD4+TCR $\alpha\beta$ +), Tc (TCR $\alpha\beta$ +CD8 $\alpha$ +), activated Th (T<sub>act</sub>; CD4+CD25+Foxp3-), and regulatory Th (T<sub>reg</sub>; CD4+CD25+Foxp3+). Results are expressed as a percentage of positive cells in the total lymphocyte population or, in some cases, are shown as a percentage of positive cells in a specific lymphocyte subset (T<sub>act</sub> and T<sub>reg</sub> in Th cells).

### **Blood leukocyte counts**

Total white blood cell counts were determined automatically by means of a Coulter Counter JT hemocytometer (Beckman Coulter) calibrated for rat samples. Differential leukocyte counts were microscopically established in blood smears stained using the May-Grünwald-Giemsa technique.

### **Quantification of TNF- $\alpha$**

Serum TNF- $\alpha$  concentration was quantified using a commercial rat cytokine bead immunoassay (Bender MedSystems GmbH, Vienna, Austria) following manufacturer's recommendations. Analysis was performed using a Cytomics FC500-MPL cytometer (Beckman Coulter) and the FlowCytomix Pro2.2.1 program from Bender MedSystems GmbH.

### **Quantification of S-Ig in small intestine**

The small intestine was excised and carefully rinsed with ice-cold PBS. The distal half of the small intestine was used to obtain the gut wash for S-IgA and S-IgM quantification by ELISA<sup>12</sup>.

### **Assessment of the inflammatory and oxidative status of the colon**

The colon was excised and carefully rinsed with ice-cold PBS. The total weight and length of the colon were determined to establish intestinal wall thickening and colon shortening, respectively. Thereafter, the colon was opened longitudinally and a transverse section of the damaged distal area was kept for histological analysis. The remaining colon was divided into four longitudinal strips that were immediately frozen in liquid N<sub>2</sub> and stored at -80 °C to estimate some inflammatory mediators such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and inducible nitric oxide synthase activity (iNOS). Thiobarbituric acid reactive substance (TBARS) and total glutathione (GSH) content were measured as antioxidant indicators.

A longitudinal colon stripe from each animal was homogenized in 0.1 M sodium phosphate buffer pH 7.4 (200 g/L) containing 1 mM EDTA and 10  $\mu$ M indomethacin. PGE<sub>2</sub> and LTB<sub>4</sub> concentrations were measured in diluted samples (1:10) using enzyme immunoassay kits (Cayman Chemicals, Tallinn, Estonia) according to manufacturer's recommendations. Results were expressed as ng/g of wet tissue.

iNOS activity was measured using a commercial kit (Cayman Chemicals). For this purpose, a second longitudinal colon stripe from each animal was homogenized in the buffer sample provided by the kit, and the assay was performed following manufacturer's instructions. Data were expressed as a conversion percentage of [<sup>3</sup>H] arginine to [<sup>3</sup>H] citrulline.

A third longitudinal colon stripe from each animal was homogenized in 1.15% KCl buffer containing 1% (v/v) butylhydroxytoluene (BHT) at 100 g/L. TBARS concentration was determined as an index of lipid peroxidation, a major indicator of oxidative stress.

Malondialdehyde (MDA) was used as standard curve. Data were expressed as  $\mu\text{mol MDA /g}$  of wet tissue.

A fourth longitudinal colon stripe from each animal was deproteinized by homogenization with 5% trichloroacetic acid in sodium phosphate buffer pH 6.0 (50 g/L), and followed by 3 repeated freeze–thaw cycles. GSH content was quantified using the 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB) recycling assay<sup>18</sup>. DTNB was used as standard and data were expressed as  $\mu\text{mol/g}$  of wet tissue.

### Histological analysis

Colon segments were fixed in 10% buffered formaldehyde. Tissue samples were washed with tap water, dehydrated in increasing concentrations of ethanol (70% for 1 h, 96% for 1 h x3) and embedded in paraffin. Tissue sections were cut (4  $\mu\text{m}$ ) on a microtome (Leitz, Wetzlar, Germany) and stained with the hematoxylin-eosin technique. Histological assessment was carried out in a blind fashion considering cell infiltration and goblet cell lost together with structural changes. Microphotographs were taken with an Olympus BX41 microscope (Olympus, Hamburg, Germany).

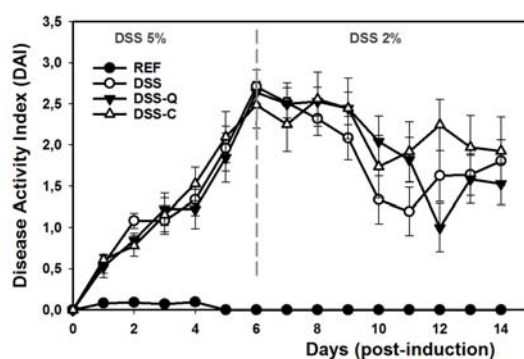
### Statistical analysis

The software package SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data were analyzed using a one-way analysis of variance (ANOVA) followed by the Scheffé's post-hoc significance test after having confirmed normality and equal variance assumptions by the Kolmogorov-Smirnov's and Levene's statistical tests, respectively. Differences were considered statistically significant at  $P$  values of  $< 0.05$ .

## Results

### Effects of cocoa diet on clinical signs of colitis

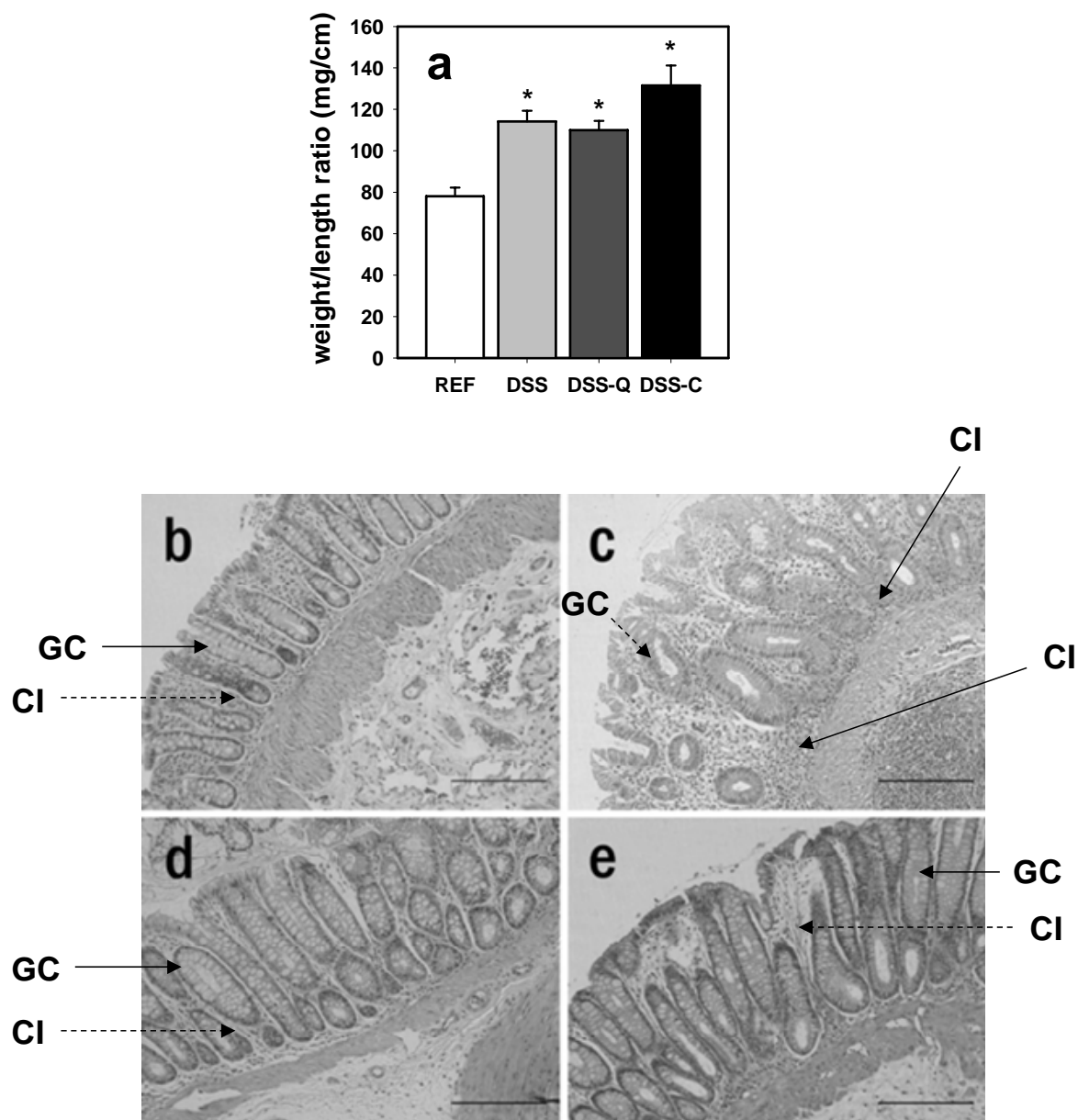
Rats receiving DSS developed colitis, which was assessed by the disease activity index (DAI, Figure 2). DAI increased during the first 6 days, remained stable for the following three days and decreased from day 9. No significant differences were observed among DSS-C, DSS-Q and DSS groups.



**Figure 2.** Clinical time course of DSS-induced colitis. Disease activity index (DAI) was calculated by assessment of body weight, stool consistency and presence of blood in the stools for each animal, using a 0 to 4 severity scale. Values are means, with their standard errors represented by vertical bars (n=12). —●—, Reference; —○—, DSS; —▲—, DSS- Quercitrin; —△—, DSS-Cocoa.

### Effects of cocoa diet on colon architecture after receiving DSS

The analysis of the intestine after 14 days of DSS treatment revealed a higher weight and a shorter length of the colon in the DSS, DSS-Q and DSS-C groups in comparison with that of the REF group. In consequence, the colon weight/length ratio was significantly increased in the three groups ( $P < 0.05$ ; Figure 3a) without significant differences among them. The cocoa diet and the quercitrin treatment did not prevent the changes induced by DSS in the colon architecture. However, the microscopic analysis of the colon sections from animals of the DSS-Q and DSS-C groups showed only a moderate lamina propria mononuclear cell infiltration and the prevention of goblet cell loss in comparison with the DSS group (Figure 3b-3e).



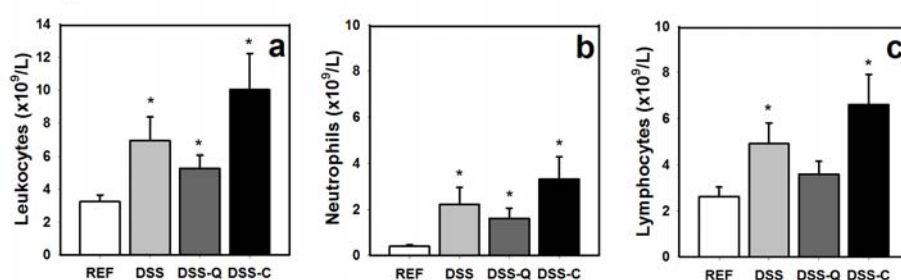
**Figure 3.** Colon architecture after DSS treatment. Colon weight/length ratio (a): Values are means, with their standard errors represented by vertical bars ( $n=12$ ). \* Mean values were significantly different from those of the reference (REF) group ( $P < 0.05$ ; ANOVA followed Scheffé's post-hoc significance test). Micrographs showing histological sections of colonic mucosa at the end of the study from a representative REF rat (b), a DSS control rat (c), a quercitrin-treated rat (d) and a cocoa-fed rat (e) (Scale bar corresponds to 200  $\mu\text{m}$ ).



Continuous arrows indicate presence of either goblet cells (GC) or cell infiltration (CI), and discontinuous arrows indicate their absence.

### Effects of cocoa diet on inflammatory and oxidant indicators from blood and colon

After 14 days of DSS treatment, the colonic inflammatory process was reflected by a marked leukocytosis in the DSS, DSS-Q and DSS-C groups when compared with the REF group ( $P < 0.05$ ; Figure 4a). This increase was due to both neutrophil and lymphocyte counts (Figure 4b and 4c), which were significantly higher in the DSS group than in the REF group ( $P < 0.05$ ) and showed the same increased pattern in animals fed cocoa diet. The leukocytosis in the DSS-Q group was mainly attributed to increased neutrophil counts since lymphocyte counts were similar to those found in the REF group.



**Figure 4.** Blood leukocyte counts after DSS treatment. Number of total leukocytes (a), neutrophils (b) and lymphocytes (c). Values are means, with their standard errors represented by vertical bars (n=12). \*Mean values were significantly different from those of the reference (REF) group ( $P < 0.05$ ; ANOVA followed Scheffé's post-hoc significance test).

Serum TNF- $\alpha$  concentration significantly increased in the DSS group compared to REF animals after 14 days of DSS drinking ( $P < 0.05$ ; Table 2). Similar results were obtained in the DSS-Q group, which was treated with quercitrin. Interestingly, serum TNF- $\alpha$  increase was significantly prevented by the cocoa diet ( $P < 0.05$ ).

The inflammatory response was also studied in colon homogenates by means of PGE<sub>2</sub> and LTB<sub>4</sub> concentrations and iNOS activity (Table 2). PGE<sub>2</sub> concentration was higher in the DSS group ( $P < 0.05$ ) than in the REF group, and neither the cocoa diet nor the quercitrin treatment prevented such increase. Similarly, LTB<sub>4</sub> concentration was increased in the DSS group compared to REF animals and no effect was detected in the cocoa diet group. However, quercitrin treatment tended to decrease the LTB<sub>4</sub> concentration. On the other hand, colon iNOS activity was slightly increased in the DSS and DSS-Q groups compared to that of the REF group, but, interestingly, cocoa-fed animals inhibited iNOS activity in the colon that was even lower than in the REF animals ( $P < 0.05$ ).

With regard to oxidative status in the colon, we determined TBARS as a measure of lipid peroxidation and GSH content as an endogenous antioxidant. Both the quercitrin treatment and the cocoa diet tended to reduce the TBARS produced in the DSS group (Table 2). GSH content was significantly decreased in the DSS group compared to the REF group ( $P < 0.05$ ).

The cocoa diet increased the colon GSH content ( $P < 0.05$  vs. DSS group), which was even higher than in the REF group. GSH content in the DSS-Q group was between that observed in the DSS and REF animals.

**Table 2.** Inflammatory and oxidative status indicators in serum and colon specimens of studied groups.

	REF	DSS	DSS-Q	DSS-C
<b>serum</b> TNF- $\alpha$ (pg/mL)	13.4 $\pm$ 0.6	19.3 $\pm$ 1.8*	18.9 $\pm$ 1.8*	8.4 $\pm$ 1.0*†
<b>colon</b> PGE <sub>2</sub> (ng/g)	29.03 $\pm$ 3.95	46.14 $\pm$ 14.36*	58.75 $\pm$ 9.29*	49.31 $\pm$ 7.77*
<b>colon</b> LTB <sub>4</sub> (ng/g)	0.49 $\pm$ 0.07	0.68 $\pm$ 0.08*	0.59 $\pm$ 0.19	0.64 $\pm$ 0.13
<b>colon</b> iNOS (%)	16.40 $\pm$ 1.84	18.90 $\pm$ 2.99	17.00 $\pm$ 2.03	11.5 $\pm$ 1.75*†
<b>colon</b> TBARS ( $\mu$ mol MDA/g)	2.12 $\pm$ 0.24	2.82 $\pm$ 0.44*	1.97 $\pm$ 0.27	2.26 $\pm$ 0.44
<b>colon</b> GSH ( $\mu$ mol/g)	0.96 $\pm$ 0.08	0.72 $\pm$ 0.06*	0.89 $\pm$ 0.12	1.05 $\pm$ 0.16†

PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; iNOS, inducible nitric oxide synthase activity; TBARS, thiobarbituric acid reactive substance; GSH, total glutathione; REF, reference; DSS, dextran sulphate sodium; Q, quercitrin; C, cocoa.

Data are expressed as mean  $\pm$  SD, n=12.

\* $P < 0.05$  versus the REF group; † $P < 0.05$  versus the DSS group

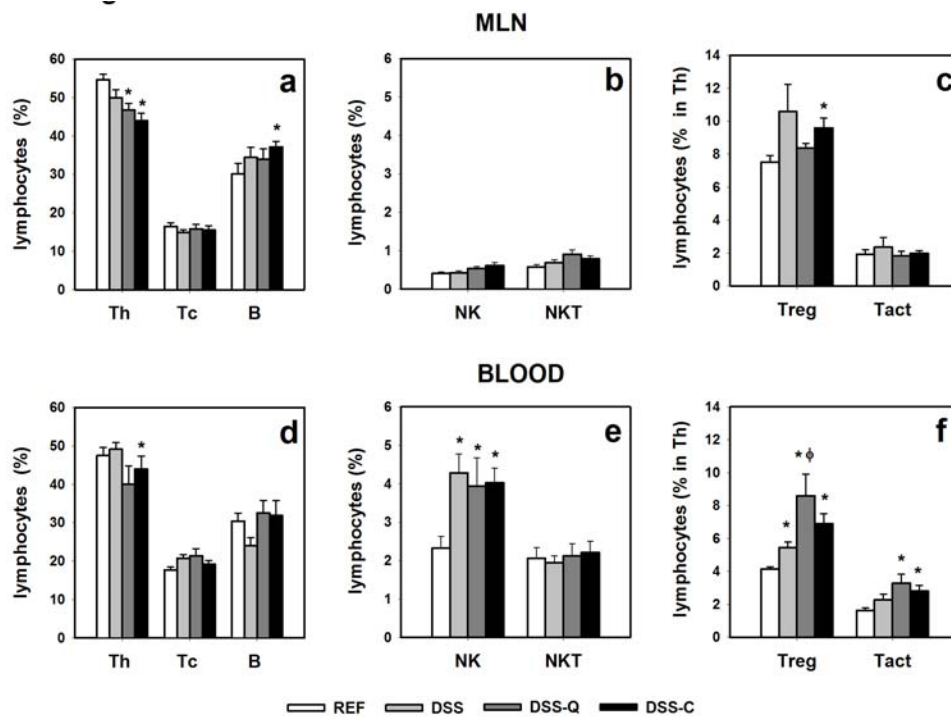
### Effects of cocoa diet on MLN and blood lymphocyte composition

In MLN, the colon inflammation in the DSS group did not modify the percentage of Th, Tc, B, NK, NKT, T<sub>reg</sub> and T<sub>act</sub> lymphocytes compared to the REF group (Figure 5a-5c). However, both the quercitrin-treated and cocoa-fed groups displayed a lower Th cell percentage than the REF group ( $P < 0.05$ ), and the DSS-C group showed a higher B cell proportion ( $P < 0.05$ ) than the REF group.

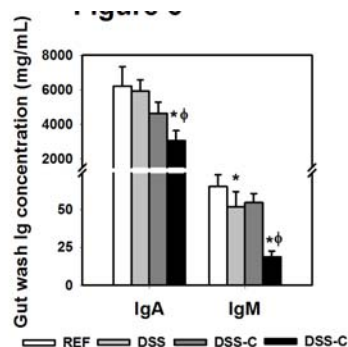
In the blood, DSS administration did not significantly alter the proportion of Th, Tc and B cells (Figure 5d) but did increase the percentages of NK and T<sub>reg</sub> cells, which were higher in the three DSS groups than in the REF group ( $P < 0.05$ , Figure 5e and 5f). The DSS-Q group showed the highest T<sub>reg</sub> proportion in Th and both the DSS-Q and DSS-C groups increased the proportion of T<sub>act</sub> subset in Th cells ( $P < 0.05$ ).

### Effect of cocoa diet on small intestine S-IgA and S-IgM content

In the DSS group, the S-IgA content in the small intestine wash was not modified by the inflammatory process (Figure 6), although there was a reduction in the S-IgM content compared to the REF animals ( $P < 0.05$ ). This reduction was not observed in animals treated with quercitrin. The cocoa diet significantly reduced the amount of both S-IgA and S-IgM in the small intestine wash ( $P < 0.05$ ) in comparison with the REF and the DSS groups.



**Figure 5.** MLN (a-c) and blood (d-f) lymphocyte composition after DSS treatment. Percentages of Th, Tc and B cells (a and d), NK and NKT subsets (b and e), T<sub>reg</sub> and T<sub>act</sub> cells in Th subset (c and f). Values are means, with their standard errors represented by vertical bars (n=12). \*Mean values were significantly different from those of the reference (REF) (□) group ( $P<0.05$ ; ANOVA followed Scheffé's post-hoc significance test). †Mean values were significantly different from those of the DSS (■) group ( $P<0.05$ ; ANOVA followed Scheffé's post-hoc significance test). ■, DSS-Quercitrin group; ■, DSS-Cocoa group.



**Figure 6.** S-IgA and S-IgM concentrations in the small gut wash. Values are means, with their standard errors represented by vertical bars (n=12). \*Mean values were significantly different from those of the reference (REF) (□) group ( $P<0.05$ ; ANOVA followed Scheffé's post-hoc significance test). † Mean values were significantly different from those of the DSS (■) group ( $P<0.05$ ; ANOVA followed Scheffé's post-hoc significance test). ■, DSS-Quercitrin group; ■, DSS-Cocoa group.

## Discussion

This paper assesses the impact of a dietary intervention with cocoa, as a source of antioxidant flavonoids, on the DSS model of rat colitis. In particular, we focused on the intestinal and systemic inflammatory response, including the oxidative status of rats fed cocoa. Cocoa diet has been reported to show anti-inflammatory and immunomodulatory properties<sup>11-13</sup> but there is a scarce number of studies describing the effects of cocoa on IBD. The effects observed were compared with those exerted by quercitrin, with recognized antioxidant and anti-inflammatory properties<sup>6</sup>. The results demonstrate that, in our experimental conditions, cocoa intake and quercitrin treatment do not improve clinical colitis although they certainly contributed to reducing colonic oxidative activity and tissue damage. Moreover, cocoa showed anti-inflammatory potential by down-regulating serum TNF- $\alpha$  concentration and colon iNOS activity and by decreasing colon cell infiltration.

The pathogenesis of IBD involves the recruitment of leukocytes into the intestinal mucosa<sup>19</sup>. Among them, macrophages secrete large amounts of inflammatory mediators and nitrogen and reactive oxygen species that induce oxidative stress<sup>19</sup>. In this line, we found that DSS-induced colitis produced consumption of glutathione and an increased lipid peroxidation in the colon. The cocoa diet avoided the glutathione consumption and partially prevented the lipid peroxidation. Similar results were obtained by quercitrin treatment although neither the cocoa diet nor the quercitrin treatment reduced the clinical development of colitis. In agreement with our results, the antioxidant luteolin incorporated into the diet does not ameliorate and even worsens the DSS-induced colitis in NF- $\kappa$ BEGFP transgenic mice<sup>20</sup>. Moreover, consumption of a lemon verbena infusion rich in polyphenolic compounds does not improve the histopathological changes induced by 4% DSS in rats, despite stimulating the activity of the antioxidant enzyme superoxide dismutase and reducing lipid peroxidation in the colon<sup>21</sup>. However, it has been recently reported that a polyphenol-enriched cocoa extract is able to decrease acute DSS colitis in mice<sup>22</sup>, thus evidencing the promising anti-inflammatory effects of cocoa flavonoids. These results may also suggest that we did not observe a clear colitis reduction because of the lower polyphenol content in the cocoa that we used.

On the other hand, as we have confirmed here, colitis is associated with increases in the inflammatory mediators LTB<sub>4</sub> and PGE<sub>2</sub><sup>23</sup>. However, neither cocoa nor quercitrin treatment could avoid such increases. Another indicator of the inflammatory process in the colon and macrophage infiltrates is the iNOS activity<sup>6</sup>. Although we found no higher iNOS activity in animals receiving DSS, interestingly, the cocoa-fed group showed lower iNOS activity than the DSS group. Similarly, cocoa diet prevented the increase in serum TNF- $\alpha$  concentration, a key molecule in IBD pathogenesis<sup>24</sup>. Both protective effects on iNOS and TNF- $\alpha$  are in line with previous *in vitro* and *in vivo* studies<sup>11,25</sup>, but they could not contribute to attenuate the intestinal inflammatory process.

In order to assess the effects of cocoa on the inflammatory process beyond the colon, cell composition was established in the blood and MLN. There is limited information about the impact of DSS on the cell populations of these compartments in rat. In our study, the inflammatory process did not vary the proportion of Th, Tc, B, NK, NKT, T<sub>reg</sub> and T<sub>act</sub>

lymphocytes in MLN. However, the cocoa diet increased the proportion of B lymphocytes, and both the cocoa diet and the quercitrin treatment reduced the proportion of Th cells in MLN. The results from the cocoa intake by the colitic animals are in line with the effects observed in the MLN of cocoa-fed healthy animals<sup>13</sup>. In any case, the decrease of the proportion of Th cells did not involve clinical recovery, which could be attributed to the fact that Th or B cells may not be essential in DSS colitis, as shown in immunodeficient mice<sup>26</sup>. On the other hand, we found no significant changes in the MLN percentage of T<sub>reg</sub> cells, contrary to recent studies showing a decrease of T<sub>reg</sub> subset in the MLN of mice that received 5% DSS in their drink for 7 days<sup>27</sup>.

In the blood compartment, we found that the intestinal inflammatory process produced neutrophilia and lymphocytosis but the proportions of the main lymphocyte subsets were not modified. However, the NK cell percentage increased in the blood of the DSS animals. This finding is consistent with the fact that depletion of NK cells attenuates the severity of acute colitis in DSS-treated mice<sup>28</sup> and suggests an important role of these cells in colitis. Neither the cocoa diet nor the quercitrin treatment prevented the increase of the blood NK cell proportion, which could be one of the reasons why cocoa-fed animals did not display colitis amelioration in spite of showing antioxidant activity in the colon and reduced serum TNF- $\alpha$ . Regarding the T<sub>reg</sub> subpopulation, the inflammatory process unexpectedly increased the percentage of T<sub>reg</sub> cells in Th lymphocytes in the blood. The T<sub>reg</sub> increase might be seen as an attempt to re-establish intestinal homeostasis under inflammatory conditions.

Although the exact mechanism through which DSS initiates colitis remains unknown, different studies suggest that DSS administration may compromise the gut epithelial barrier function resulting in an increased permeability<sup>14</sup>. The mucosal IgA system contributes to the barrier functionality generating immune protection in a non-inflammatory manner. The cocoa diet significantly decreased the S-IgA and S-IgM in the small gut as previously reported in healthy animals<sup>13</sup>. Although in our study the colonic S-IgA concentration was not assessed, the down-regulatory effect on S-IgA of small intestine may have also occurred in the colon of cocoa-fed animals. In fact, previous studies have reported decreased S-IgA concentration in the faces of healthy animals fed with a diet containing 5% of cocoa<sup>29</sup>. Therefore, it could be suggested that the cocoa diet, although exerting antioxidant and even anti-inflammatory protection, may increase colon permeability by reducing S-IgA content, which may contribute to the inflammatory process. In other words, those beneficial effects of cocoa flavonoids may have been counteracted by a decreased S-IgA protection in the colon.

In conclusion, a diet containing cocoa may help to manage the oxidative stress consequent to colitis, although this action is not enough to reverse the inflammatory process. Cocoa intake shows anti-inflammatory potential and seems to decrease colon cell infiltration. Therefore, cocoa diet may be considered as a promising complementary therapy for IBD.

**Author contributions:** T.P.B carried out the study, performed the statistical analysis and drafted the manuscript. C.R.S., S.R.R and C.C collaborated during the *in vivo* part and the discussion of data. M.C, A.F and F.J.P-C conceived, designed the study and discussed the results.

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