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## Analyzing beneficial effects of nutritional supplements on intestinal epithelial barrier functions during experimental colitis --Manuscript Draft--

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<b>Abstract:</b>	Inflammatory bowel diseases (IBD) including Crohn's disease and ulcerative colitis are chronic relapsing disorders of the intestines causing severe problems in affected individuals such as abdominal cramping, bloody diarrhea and weight loss. Unfortunately, there is no cure yet and treatments aim to alleviate symptoms. Current treatments include anti-inflammatory and immune-suppressive drugs that may cause severe side effects. Thus, the search for alternative treatment options without side effects such as nutritional supplements is warranted. Before application in clinical studies, such compounds need to be rigorously tested for effectiveness and security in animal models. A reliable experimental model is the dextran sulfate sodium (DSS) colitis model in mice that reproduces many of the clinical signs of ulcerative colitis in humans. We recently applied this model to test the beneficial effects of a nutritional supplement containing vitamins C and E, L-arginine and $\omega$ 3-polyunsaturated fatty acids (PUFA). We analyzed various disease parameters and found that this

	<p>supplement was able to ameliorate edema formation, tissue damage, leukocyte infiltration, oxidative stress and production of pro-inflammatory cytokines leading to an overall improvement of the disease activity index. In this article, we explain in detail the correct application of nutritional supplements using the DSS colitis models in C57Bl/6 mice and how disease parameters such as histology, oxidative stress and inflammation are assessed. Analyzing the beneficial effects of different diet supplements may then eventually open new avenues for the development of alternative treatment strategies to alleviate IBD symptoms and/or prolong phases of remission without severe side effects.</p>
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Revised Article Submission JoVE55095R1

Dear Dr. Upponi,

Please find attached our revised manuscript JoVE55095R1 entitled: "Analyzing beneficial effects of nutritional supplements on intestinal epithelial barrier functions during experimental colitis" by Vargas Robles and co-workers. We have addressed all comments of the reviewers and editors as highlighted in the accompanying letter "Answers to Reviewers" using the track changes function of Word.

We hope that you'll find this manuscript now suitable for publication in JOVE.

Kind regards

Dr. Michael Schnoor  
On behalf of all authors

**TITLE:**

**Analyzing beneficial effects of nutritional supplements on intestinal epithelial barrier functions during experimental colitis**

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**SHORT ABSTRACT:**

Current treatments of inflammatory bowel diseases (IBD) aim at alleviating disease symptoms but may cause severe side effects. Thus, alternative strategies are being investigated in animal colitis models. Here, we explain how the beneficial effects of diet supplements on clinical IBD signs are analyzed in such a colitis model.

**LONG ABSTRACT:**

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are chronic relapsing disorders of the intestines. They cause severe problems, such as abdominal cramping, bloody diarrhea, and weight loss, in affected individuals. Unfortunately, there is no cure yet, and treatments only aim to alleviate symptoms. Current treatments include anti-inflammatory and immunosuppressive drugs that may cause severe side effects. This warrants the search for alternative treatment options, such as nutritional supplements, that do not cause side effects. Before their application in clinical studies, such compounds must be rigorously tested for effectiveness and security in animal models. A reliable experimental model is the dextran sulfate sodium (DSS) colitis model in mice, which reproduces many of the clinical signs of ulcerative colitis in humans. We recently applied this model to test the beneficial effects of a nutritional supplement containing vitamins C and E, L-arginine, and  $\omega$ 3-polyunsaturated fatty acids (PUFA). We analyzed various disease parameters and found that this supplement was able to ameliorate edema formation, tissue damage, leukocyte infiltration, oxidative stress, and the production of pro-inflammatory cytokines, leading to an overall improvement in the disease activity index. In this article, we explain in detail the correct application of nutritional supplements using the DSS colitis models in C57Bl/6 mice, as well as how disease parameters such as histology, oxidative stress, and inflammation are assessed. Analyzing the beneficial effects of different diet supplements may then eventually open new avenues for the development of alternative treatment strategies that alleviate IBD symptoms and/or that prolong the phases of remission without causing severe side effects.

**INTRODUCTION:**

Colitis is an inflammatory condition of the colon that can cause diarrhea and abdominal pain. Colitis can be acute, in response to infection or to stress, or it can develop as a chronic disease, such as in ulcerative colitis (UC), which belongs to the group of inflammatory bowel diseases (IBD). Although the clinical signs of UC have been well described, the pathogenesis is still poorly understood<sup>1</sup>. It is accepted among experts that UC is a multifactorial disease, with genetic mutations and aberrant immune responses playing a major role<sup>2</sup>. However, environmental factors, such as style of living and nutrition, also contribute to disease development and progression<sup>3</sup>.

Unfortunately, IBD is not curable, but there are many treatment options that aim to alleviate clinical symptoms. Current treatments include anti-inflammatory drugs, such as sulfasalazine and corticosteroids; immunosuppressants, such as azathioprine; monoclonal antibodies that capture pro-inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), or block adhesion molecules, such as integrins, to reduce excessive leukocyte recruitment; and inhibitors that target kinases to trigger pro-inflammatory pathways, such as Janus kinase (JAK)<sup>4</sup>. Not all patients respond to all treatments, so therapeutic strategies must be individualized<sup>5</sup>. Furthermore, most of these therapeutic drugs interfere with the metabolism and immune responses, often causing severe side effects. For this reason, alternative treatment options have been investigated.

Alternative treatments include probiotics and nutritional supplements, which have been applied in animal models and clinical trials with varying levels of success<sup>6,7</sup>. We recently found that the application of different single nutritional supplements, such as anti-oxidative vitamins or  $\omega$ 3-poly-unsaturated fatty acids (PUFAs), is inferior to the application of a combination of such supplements in alleviating colitis and cardiovascular disease symptoms<sup>8,9</sup>. These studies were performed in mice, so clinical studies must be performed in humans to determine whether these findings will also be applicable to humans. Before clinical studies are initiated, the effectiveness and safety of new treatment options must be evaluated in animal models.

For IBD, the dextran sulfate sodium (DSS) model has been widely used to study the mechanisms of disease development and the beneficial effects of drugs and nutritional supplements<sup>6,10</sup>. In most studies, only an acute disease over a time period of seven days is induced; nevertheless, the clinical signs observed in these animals closely resemble those observed in IBD patients (*i.e.*, bloody diarrhea, weight loss, epithelial dysfunction, and immune cell infiltration)<sup>10</sup>. DSS induces erosions in the mucosa, resulting in barrier dysfunction and in increased intestinal epithelial permeability<sup>11</sup>. The exact mechanism remains unknown. However, a study suggests that DSS interacts with medium chain-length fatty acids to form nano-lipocomplexes that are able to enter epithelial cells and to induce inflammatory signaling pathways<sup>12</sup>. In this article, we describe in detail how colitis is induced and analyzed in mice, how nutritional supplements are applied by gavage to ensure a constant dosage in each animal, and how the effects of such supplements on various colitis symptoms are examined.

## **PROTOCOL:**

All animal experiments have been approved by the Institutional Animal Care and Use Committee of Cinvestav.

### **1.) Preparation of DSS drinking water and the induction of colitis**

1.1) Prepare a 3.5% w/v dextran sulfate sodium (DSS) solution in autoclaved drinking water. DSS dissolves easily and it is not required to filter the final solution.

NOTE: 7 days of DSS treatment induces severe colitis. If inducing mild colitis, 3-4 days of treatment are recommended. As long as the DSS drinking water stays clear, it is not necessary to change the water. However, if it turns turbid, it must be replaced.

1.2) Record the baseline weight of male mice. Ensure that they are 8-12 weeks of age and within a range of 21-25 g bodyweight.

NOTE: The appropriate DSS concentration must be optimized in each laboratory. Results can greatly vary, depending upon the housing conditions. Concentrations between 2.5-5% are usually reported to induce strong colitis in C57Bl/6J WT mice<sup>10</sup>. DSS from different companies and different lots should be tested, as results may also vary with different sources of DSS.

1.3) Provide water *ad libitum* and replace it with fresh 3.5% DSS water if necessary.

## **2.) Application of nutritional supplements by gavage**

2.1) Prepare a “feedable” solution of the desired nutritional supplements in an appropriate solvent. For this study, we used a mixture containing 200 mg/kg L-arginine, 83 mg/kg vitamin C, 46 mg/kg vitamin E, 77 mg/kg eicosapentaenoic acid (EPA), and 115 mg/kg docosahexaenoic acid (DHA) in a 1:1 solution of water and safflower oil.

2.2) Fill a 1-mL syringe connected to a gavage needle (15 G x 50 mm) with the nutritional supplement mixture. Wipe the outside of the gavage needle to remove any compound outside of the needle and to ensure proper dose application.

2.3) Restrain the mouse by grasping the base of the tail with one hand and by firmly gripping a skin fold at the back of the neck with the thumb and forefinger of the other hand. Place the tail between the third finger and the base of the thumb of the same hand that holds the neck.

2.4) While maintaining the mouse in an upright position, insert the needle into the left side of the animal's mouth and carefully follow the roof of the mouth to locate the esophagus. If no resistance is encountered, advance the needle towards the stomach.

NOTE: Verify that the animal is breathing properly before proceeding.

2.5) Administer the mixture slowly, remove the tube by slowly pulling out the syringe, and release the mouse. Apply nutritional supplements once daily by gavage during the course of the colitis experiment.

## **3.) Determination of the disease activity index**

NOTE: The disease activity index is the combination of the scores for weight loss, perianal bleeding, and stool consistency, which are obtained daily<sup>13</sup>.

3.1) Measure the bodyweight daily and assign a score of 0 to 4 according to the weight loss percentage (0: 0%, 1: 1-5%, 2: 5-10%, 3: 10-20%, and 4: >20%).

NOTE: Bodyweight loss is determined by calculating the difference in percent between the basal weight before the induction of DSS-colitis and the actual weight.

3.2) To determine the level of perianal bleeding, collect a fresh stool sample and use the provided applicators to apply a thin smear on a slide from a guaiac fecal occult blood test kit. Use a fresh applicator for each sample.

3.2.1) Close the cover on the front and open the window on the back of the slide. Apply two drops of the developer solution to the back at the sample location.

3.2.2) Read the results after 30 s. Any trace of blue color is positive for occult blood. Assign a score of 0 to 4 (0: none, 0.5-2.5: positive guaiac fecal occult blood test (depending upon the signal strength), and 3-4: gross bleeding).

NOTE: If blood in the stool is visible, the guaiac fecal occult blood test does not have to be performed, and a score of 3, 3.5, or 4 is assigned, depending upon the amount of visible blood.

3.2) Determine stool consistency by observing a fresh stool sample. Assign a score of 0 to 4 (0: normal/solid, 0.5-2.5: pasty stool, and 3-4: diarrhea).

#### **4.) Determining intestinal epithelial permeability *in vivo* by Evans blue assay**

4.1) Anesthetize the animals by injecting them intraperitoneally with a mixture of ketamine (100 mg/kg of bodyweight) and xylazine (13 mg/kg of bodyweight) diluted in saline solution (0.9% NaCl), and assess the depth of anesthesia by monitoring the pinch withdrawal reflex.

4.2) Place the anesthetized animals in a supine position and perform a laparotomy to expose the intestines.

4.3) Locate the cecum and make a small incision in the proximal segment of the colon *ascendens* (ideally, immediately adjacent to the cecum).

4.4) Insert a feeding needle (G22) and secure it with a ligature using a common silk thread. Carefully flush abundant PBS through the tube to rinse out all feces from the colon. Instill Evans blue solution (1.5% w/v in PBS) into the colon until it reaches the anus.

4.5) Incubate the Evans blue for 15 min. Wash out the dye by flushing the tube with abundant PBS until the perianal washout is clear.

4.6) Euthanize the animals by cervical dislocation.

4.7) Excise the colon and rinse it again with abundant PBS. Rinse once with 1 mL of 6 mM N-acetylcysteine in PBS to eliminate any dye sticking to the colonic mucus.

4.8) Cut the colon longitudinally and rinse it once more with PBS and 1 mL of 6 mM N-acetylcysteine.



4.9) Record the weight and length. To extract the Evans blue dye, place the colon in 2 mL of N,N-dimethylformamide overnight at room temperature with gentle agitation. Measure the dye concentration spectrophotometrically at 610 nm.

## **5.) Tissue collection**

5.1) Anesthetize the animals by injecting them intraperitoneally with a mixture of ketamine (100 mg/kg of bodyweight) and xylazine (13 mg/kg of bodyweight) diluted in saline solution (0.9% NaCl), and assess the depth of anesthesia by monitoring the pinch withdrawal reflex.

5.2) Euthanize the animals by cervical dislocation.

5.3) Place the animals in a supine position and perform a laparotomy to expose the abdominal cavity.

5.4) Using scissors, dissect the entire colon and record its length.

5.5) Flush the colon carefully several times with chilled PBS to remove the feces. Record the tissue weight and prepare for the following experiments, as described in steps 5.6-5.8.

5.6) For RNA isolation and myeloperoxidase (MPO) assays, cut off an appropriately-sized tissue sample (100 mg is usually sufficient), place it inside a tube, and snap-freeze it in liquid nitrogen. Store the tissues at -80 °C for future use.

5.7) For immunofluorescence staining and oxidative stress determination (see below), cut a small colon sample (0.5-1 cm) and submerge it in small aluminum cups filled with optimal cutting temperature (OCT) compound. Place the cups on dry ice and let them freeze slowly, in order to avoid bubble formation. Frozen tissue samples can be stored at -80 °C for future use.

5.8) For Swiss rolls<sup>14</sup>, open the colon longitudinally and roll it up, with the mucosa facing outwards, using fine-tipped forceps. Carefully place it inside an embedding cassette and continue with step 6.1.

Note: DSS induces damage in the colon. However, damage distribution varies from the proximal to the distal colon, with most damage usually seen in the distal colon and rectum. Thus, we recommend analyzing the histology either in the distal colon or in Swiss rolls of the entire colon.

## **6.) Analysis of colon histology by hematoxylin-eosin staining**

### **6.1) Tissue preparation**

6.1.1) For the histological analysis, submerge either the Swiss rolls or the small tissue pieces from certain colon regions of control and treated mice in 5 mL of 10% formaldehyde for 48 h at room temperature (RT) to achieve proper fixation.

NOTE: All further steps in step 6 are carried out at RT, unless otherwise indicated.

6.1.2) Wash them with tap water for 18 h.

6.1.3) Dehydrate them with 15 mL of 70% ethanol for 1 h, 96% for 1 h, and absolute ethanol for 1 h. Repeat each step with fresh alcohol before proceeding to the next concentration.

6.1.6) Continue the dehydration in a mixture of 7.5 mL of absolute ethanol and 7.5 mL of absolute xylene for 1 h. Repeat once before passing the samples to 15 mL of absolute xylene for 1 h. Repeat this step once with fresh xylene.

## **6.2) Embedding colon tissue samples in paraffin**

6.2.1) Immerse the samples in 50 mL of liquid paraffin for 17 h. Repeat this step once but for only 3 h.

6.2.2) Immerse the samples in cubes (plastic, square mold, size: 22 x 22 x 22 mm) in 8-10 mL of liquid paraffin.

6.2.3) Allow the paraffin with the colon tissue samples to solidify for 24 h at RT.

## **6.3) Cutting tissue cross-sections using a microtome**

6.3.1) Cut colon samples using a microtome, according to the manufacturer's instructions, at a thickness of 5  $\mu$ m.

6.3.2) Collect the cuts in a water bath (1 L) containing 0.3% gelatin. This prevents the folding of tissue cross-sections. Recover the tissue sections and mount them on glass slides.

## **6.4) Hematoxyline and eosin staining of tissue cross-sections**

6.4.1) Deparaffinize the samples for 18 h at 60 °C in an incubator. For this purpose, use a glass slide rack with a handle.

6.4.2) After deparaffinization, immerse the slides in 70 mL of absolute xylene for 5 min. Repeat this step once with fresh xylene.

6.4.3) Transfer the samples into 70 mL of absolute alcohol for 1 min. Repeat this step once with fresh alcohol.

6.4.4) Incubate the colon tissue samples in 70 mL of ethanol 96% for 1 min. Repeat this step once with fresh alcohol.

6.4.5) Wash the samples in tap water twice for 2 s.

6.4.6) Incubate the tissues in Harris hematoxylin for 7 min.

6.4.7) Wash the samples in tap water twice for 2 s.

6.4.8) Incubate the samples in acidic alcohol (70 mL of ethanol 70% and 700 µL of 1 M hydrochloric acid) for 7 s.

6.4.9) Wash the samples in tap water twice for 2 s.

6.4.10) Incubate the tissue samples in 70 mL of lithium carbonate (1 g of lithium carbonate in 100 mL of distilled water) for 7 s.

6.4.11) Wash the samples in tap water twice for 2 s.

6.4.12) Incubate the samples in 70 mL of ethanol 80% for 1 min.

6.4.13) Transfer the samples into 70 mL of Eosin-Y for 15 s.

6.4.14) Incubate them in 70 mL of 96% ethanol for 1 min. Repeat this step once with fresh alcohol.

6.4.15) Incubate the tissue samples in 70 mL of absolute ethanol (ethyl alcohol absolute anhydrous) for 1 min. Repeat this step once with fresh alcohol.

6.4.16) Incubate the samples in 70 mL of a mixture of 35 mL of absolute alcohol and 35 mL of absolute xylene for 1 min.

6.4.17) Transfer the samples into 70 mL of absolute xylene for 1 min. Repeat this step for 5 min in 70 mL of fresh absolute xylene.

6.4.18) Add 80 µL of synthetic resin to the colon tissue samples, cover them with coverslips, and let them dry for 24 h at RT. Finally, analyze the samples using bright-field microscopy<sup>8</sup>.

## **7.) Analyzing junction composition by immunofluorescence**

7.1) Prepare the tissue as described in step 5.7 and cut 8 µm-thick cross-sections using a cryostat.

7.2) Fix and permeabilize colon cross-sections with 96% ethanol at -20 °C for 20 min.

NOTE: All subsequent incubations should be carried out at RT, unless otherwise stated, in a humid dark box to prevent drying and fluorochrome fading.

7.3) Air-dry samples, and then rinse them 3 times for 5 min each with 1X PBS

7.4) Incubate the samples with blocking solution (PBS containing 0.01% Tween and 2% BSA) for 2 h.

- 7.5) Remove the blocking solution and rinse it with PBS–0.01% Tween for 10 min.
- 7.6) During this time, dilute the primary antibodies to the desired concentrations in PBS–0.01% Tween.
- 7.7) Remove the washing solution, apply the antibody solution from the previous step, and incubate overnight at 4 °C in a humidified box.
- 7.8) Remove the antibody solution and wash 3 times with PBS–0.01% Tween for 5 min each and once with deionized water for 10 min, with very gentle agitation.
- 7.9) While washing, centrifuge fluorochrome-conjugated, species-specific secondary antibodies at 14,000 x g for 20 min at 4 °C.
- 7.10) Dilute the secondary antibodies without precipitates as indicated by the provider in PBS–0.01% Tween, apply them, and incubate for 1 h at RT.
- 7.11) Repeat step 7.8 and remove the washing solution as completely as possible.
- 7.12) Pipette 4 µL of anti-fading agent over each sample on the slide.
- 7.13) Cover the samples with a coverslip.
- 7.14) Air dry for 1 h.
- 7.15) Seal slide with nail polish. Slides can be stored at -20 °C until further analysis.
- 7.16) Analyze on a confocal laser microscope<sup>8</sup>.

## **8. Determination of oxidative stress by oxidation of ethidium**

- 8.1) After 7 days of DSS colitis, prepare tissue as described in step 5.7 and cut colon sections in a cryostat at a thickness of 5 µm. Mount cross-sections onto glass slides treated with poly-L-lysine solution and incubate the samples with 5 µM of dihydroethidium (DHE) in water at 37 °C for 30 min in the dark.
- 8.2) Wash the samples with PBS (pH 7.6) three times for 15 min each with gentle agitation at RT. Air dry the samples and add a drop of mounting medium to protect the fluorescence. Observe the fluorescence of oxidized ethidium on a laser-scanning confocal microscope (40x magnification, 568 nm wavelength).

## **9.) Analysis of leukocyte recruitment by MPO assay**

- 9.1) After 7 days of DSS colitis, collect colon tissue and homogenize the samples (200-400 mg) with 1 mL of hexadecyltrimethylammonium (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0) using a homogenizer on ice.

9.2) Rinse the tip of the homogenizer twice with 1 mL of HTAB buffer to include all tissue in the lysate and sonicate it five times at 40% amplitude for 10 s each. Freeze-thaw in liquid nitrogen three times.

9.3) Centrifuge at 14,000 x g for 15 min and collect the supernatant. Prepare 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution by mixing ABTS, 5 mL of sodium citrate (1 M in water), 0.1 mL of hydrogen peroxide, and 45 mL of bi-distilled water.

9.4) Mix 0.1 mL of each supernatant with 0.1 mL of ABTS solution in a 96-well flat-bottomed plate. After 10 min, measure the absorbance at 460 nm using a spectrophotometer.

## **10.) Evaluating the expression of pro-inflammatory cytokines by PCR**

### **10.1) Tissue homogenization**

10.1.1) Homogenize 50 to 100 mg of colon tissue samples in 1 mL of an acid guanidinium thiocyanate-phenol-chloroform mixture using a power homogenizer.

10.1.2) Incubate it at room temperature for 15 min.

### **10.2) Phase separation**

10.2.1) Add 0.2 mL of chloroform and shake vigorously for 30 s.

10.2.2) Incubate at 4 °C for 30 min.

10.2.3) Centrifuge at 12,000 x g for 60 min at 4 °C.

10.2.4) Transfer the upper aqueous phase into a fresh tube (~500 µL).

### **10.3) RNA precipitation and resuspension**

10.3.1) Add 0.5 mL of isopropyl alcohol and incubate the samples at 4 °C for 60 min.

10.3.2) Centrifuge at 12,000 x g for 30 min at 4 °C.

10.3.3) Remove the supernatant completely.

10.3.4) Add 1 mL of ethanol 75% and vortex.

10.3.5) Centrifuge at 12,000 x g for 30 min at 4 °C.

10.3.6) Remove the supernatant.

10.3.7) Allow the remaining ethanol to air-dry for 3-5 min.

NOTE: It is important not to let the RNA pellet dry completely, as this will greatly decrease its solubility.

10.3.8) Re-dissolve the pellet in 0.3 mL of diethylpyrocarbonate (DEPC)-treated water. Immediately transcribe the RNA samples into cDNA (more stable, see step 10.5) or store at -80 °C.

#### **10.4) RNA quantification**

10.4.1) Dilute 1 µL of RNA with 99 µL of DEPC-treated water.

10.4.2) Read the OD at 260 nm and 280 nm using a UV/VIS spectrophotometer to determine sample concentration and purity.

#### **10.5) cDNA synthesis**

10.5.1) Mix RNA samples (1-5 µg) with 1 µL of Oligo(dT)<sub>12-18</sub> (0.5 µg/µL) and DEPC-treated water (total volume of 12 µL) in RNase-free sterile tubes.

10.5.2) Incubate at 70 °C for 10 min.

10.5.3) Add 2 µL of 10X PCR Buffer, 1 µL of 50 mM MgCl<sub>2</sub>, 1 µL of 10 mM dNTP mix, and 2 µL of 0.1 M dithiothreitol (DTT).

10.5.4) Incubate at 4 °C for 5 min.

10.5.5) Add 1 µL of reverse transcriptase and incubate at 42 °C for 5 min.

10.5.6) Incubate at 70 °C for 15 min.

10.5.7) Incubate at 4 °C for 15 min. cDNA can be stored at -20 °C until further use.

#### **10.6) PCR**

10.6.1) Mix 2.5 µL of 10X PCR Buffer, 1.5 µL of 25 mM MgCl<sub>2</sub>, 0.5 µL of 10 mM dNTP mix, 0.25 µL of Taq DNA polymerase, 0.25 µL of each primer (10 µM) and cDNA (100 ng), and sterile water to a total volume of 25 µL.

10.6.2) Run the samples on a 96-well thermal cycler. To prevent amplification of genomic DNA, forward and reverse primers should be located in different exons: IL1 $\beta$ -FW: GCAACTGTTCTGAACTCAACT and IL1 $\beta$ -RE: TCTTTTGGGGTCCGTCAACT; IL6-FW: CCTTCCTACCCCAATTTCCAA and IL6-RE: AGATGAATTGGATGGTCTTGGTC; KC-FW: TGTCAGTGCCTGCAGACCAT and KC-RE: CCTGAGGGCAACACCTTCA; and Actin-FW: TATCCACCTTCCAGCAGATGT and

Actin-RE: AGCTCAGTAACAGTCCGCCTA. Use the following PCR conditions: 95 °C for 5 min followed by 30 cycles at 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s, plus a final extension for 10 min at 72 °C.

## REPRESENTATIVE RESULTS:

### *Diet supplements can protect from DSS colitis*

Anti-inflammatory and anti-oxidative diet supplements, such as vitamin C, vitamin E, the nitric oxide (NO)-source L-arginine, and  $\omega$ 3-polyunsaturated fatty acids ( $\omega$ 3-PUFAs), have been used with varying success in animal models to alleviate signs of inflammatory diseases<sup>3,6,15</sup>. Malnutrition, oxidative stress, and the production of pro-inflammatory cytokines can trigger both acute and chronic colitis<sup>3</sup>. In a previous study, we proved that a combination of micronutrients showed protective effects against DSS-induced colitis *in vivo*. Here, we provide detailed protocols on how these effects were measured<sup>8</sup>. First, we analyzed the effects of nutritional supplements on disease progression by determining the disease activity index (DAI), consisting of the three parameters of weight loss, stool consistency, and perianal bleeding. Representative results showed that DSS treatment led to a continuously-increasing DAI, as expected, reaching a maximum of  $8.2 \pm 0.46$  on day seven (Fig. 1A). Anti-inflammatory diet supplementation delayed the onset of colitis, but at later time points, when colitis was very strong, the protective effect was marginal and no longer statistically significant, suggesting that diet supplements are only effective when the colitis is not yet too strong. Importantly, dietary intervention could prevent the DSS-induced increase in intestinal epithelial permeability, which is an important feature of colitis (Fig. 1B). DSS colitis led to reduced colon lengths, and this shortening was also ameliorated by the anti-inflammatory and anti-oxidative diet supplement (Fig. 1C). Thus, clinical symptoms of colitis can indeed be alleviated by changes in diet composition.

### *Colon tissue damage and disassembly of TJ and AJ during colitis is attenuated by anti-inflammatory and anti-oxidative diet supplements*

To analyze why colitis progression was less severe in animals receiving a diet supplement, tissue morphology was investigated after hematoxylin/eosin (H/E) staining of paraffin-embedded distal colon tissues. Control samples showed a normal morphology (Fig. 2A, upper panel), whereas animals treated with 3.5% DSS showed typical signs of colitis (*i.e.*, leukocyte infiltration, edema formation, crypt dysplasia, and ulcerations; Fig. 2A, middle panel). Of note, parallel treatment with diet supplementation clearly alleviated colitis-induced morphological changes, with an overall morphology that was more comparable to the control group (Fig 2A, bottom panel). These observations were very similar to what has been reported for co-treatments with probiotic bacteria<sup>13</sup> and clearly show that changes in diet can counteract the development of colitis.

We knew already that colitis-induced intestinal epithelial permeability could be counteracted by diet supplementation. Increased permeability is a hallmark of IBD, and it is in large part caused by the disassembly of the stabilizing adherens junctions (AJ) and tight junctions (TJ). Destabilization of epithelial cell contacts by the internalization of junctional proteins is induced by pro-inflammatory cytokines, which are abundantly produced during colitis<sup>16</sup>. We found, through the immunofluorescent staining of distal

colon tissue, that the TJ molecule zonula occludens-1 (ZO-1) and the AJ molecule E-cadherin were internalized during DSS-induced colitis and that co-localization of these proteins was partly lost at crypt epithelial cell contacts (Fig. 2B). Importantly, internalization of E-cadherin and ZO-1 was reduced when DSS-treated mice were co-treated with an anti-inflammatory and anti-oxidative diet supplement (Fig. 2B). Overall, crypt and junction structures were more comparable to controls when the diet supplement was applied, suggesting that the observed protective effect was at least in part due to the preservation of tissue architecture.

*Dietary intervention can counteract inflammatory neutrophil recruitment, oxidative stress, and the production of pro-inflammatory cytokines*

Another hallmark of IBD is uncontrolled neutrophil infiltration. Recruited neutrophils contribute to tissue damage by producing and releasing reactive oxygen species (ROS) and proteases<sup>17</sup>. Using an MPO activity assay, we found strong neutrophil recruitment in the colon in response to DSS, which was counteracted by diet supplementation (Fig. 3A).

Oxidative stress is another important feature of colitis, caused by the release of ROS from recruited neutrophils. Thus, we analyzed the production of ROS in colon cross-sections. Fig. 3B demonstrates that DSS treatment strongly increased oxidative stress. Of note, changes in diet completely prevented DSS-induced oxidative stress (Fig. 3B), which is most likely a consequence of reduced neutrophil recruitment (Fig. 3A).

Pro-inflammatory cytokines and chemokines are strongly produced by various cell types during colitis<sup>18</sup>. Such inflammatory mediators contribute to neutrophil recruitment and junction protein internalization—features that we already knew could be attenuated by changes in diet. Analysis of mRNA expression by RT-PCR revealed that DSS increased the expression of IL1 $\beta$ , IL-6, and keratinocyte-derived chemokine (KC), as expected (Fig. 3C), and that anti-inflammatory diet supplementation diminished this DSS-induced increase (Fig. 3C). These data imply that diet supplements can indeed serve to counteract clinical signs of IBD.

**FIGURE LEGENDS:**

**Figure 1. Nutritional supplements can alleviate disease activity index, intestinal epithelial permeability, and colon shortening.** (A) The disease activity index (DAI, values from 0-12) consists of the three parameters of weight loss, stool consistency, and perianal bleeding and was recorded daily in the experimental groups of control (ctrl), colitis, and colitis + nutritional supplement (colitis+suppl.). The control group did not show any signs of colitis, resulting in a DAI of 0 throughout the experimental period. n = 5 per group. (B) Intestinal epithelial permeability was measured by Evans blue leakage in the abovementioned experimental groups and was expressed as the absorption at 620 nm per g of tissue. n = 8 per group. All data are shown as the mean  $\pm$  the standard deviation of the mean (SDM). \*p<0.05; \*\*p<0.01; \*\*\*p<0.001. Statistics were calculated by one-way ANOVA. (C) Representative images of whole colons of the respective experimental groups after 7 days of treatment. The scale on the left is in cm.



**Figure 2. Tissue morphology and junction architecture are better preserved during colitis when applying nutritional supplements.** (A) 5- $\mu$ m paraffin cross-section of tissue biopsies from the distal colons of the respective experimental groups were stained with hematoxylin/eosin and analyzed for typical signs of colitis, such as edema formation (arrows), leukocyte infiltration (arrowheads), and ulceration (\*). Overall tissue morphology of the colitis+suppl. group more resembles that of the control than the colitis group, proving an overall protective effect against DSS colitis. Images are representative of 3 independent tissue preparations per group. Images were taken using a 10x objective. Bar = 100  $\mu$ m. (B) 8- $\mu$ m colon cryo-sections of the respective groups were stained with antibodies against ZO-1 (green) and E-cadherin (red). Co-localization (yellow in the merged images; arrows) of E-cadherin and ZO-1 at crypt epithelial cell contacts, which is lost during colitis, is mostly preserved in the colitis + suppl. group. Images are representative of 3 independent tissue preparations. Bar = 50  $\mu$ m.

**Figure 3. Neutrophil recruitment, oxidative stress, and the production of pro-inflammatory cytokines can be reduced by nutritional supplements.** (A) Myeloperoxidase (MPO) activity was measured to analyze the amount of leukocyte recruitment into colon tissues in the experimental groups: control (ctrl), colitis, and colitis + nutritional supplement (colitis+suppl.).  $n = 5$  per group;  $*p < 0.05$ . Data are shown as the mean  $\pm$  SDM. Statistics were calculated by one-way ANOVA. (B) The fluorescence of oxidized ethidium, depicted in red as the measure of oxidative stress in 8- $\mu$ m cryo-sections of colon tissues, was recorded by laser confocal microscopy. Images are representative of 3 independent tissue preparations. Bar = 100  $\mu$ m. (C) The production of the pro-inflammatory cytokines IL1 $\beta$  and IL-6 and the chemokine KC was determined by semi-quantitative RT-PCR in a 1.5% agarose gel (black and white were reverted for the sake of clarity).  $\beta$ -actin served as the housekeeping gene. Representative images of three independent cDNA preparations are shown.

## DISCUSSION:

In order to use nutritional supplements in clinical studies, the benefits and safety of such supplements must be carefully evaluated *in vivo* in animal studies. In the case of colitis, several appropriate animal models that resemble the clinical signs of IBD have been established, including chemical models using DSS, TNBS, or acetic acid; knock-out (KO) models such as IL10-KO; and immune-cell-mediated colitis using adoptive T-cell transfer<sup>19-21</sup>. The DSS model of colitis is a rapid and reliable method of inducing colitis that resembles many disease symptoms of human IBD and has been used in a plethora of studies investigating the molecular mechanisms of colitis<sup>22</sup>. Moreover, DSS colitis is reversible and thus also allows for the study of tissue healing after the colitogenic stimulus has been removed. The provided protocol describes in detail the DSS colitis model that has been previously optimized in our laboratory<sup>8</sup>.

A disadvantage is that the optimization of the DSS concentration must be done in every laboratory, since environmental factors greatly affect disease development and progression<sup>23</sup>. Additionally, DSS must be in a molecular weight range of 40-50 kDa to induce colitis. For the unexperienced researcher, it will be important to develop an eye

for an unbiased assessment of the DAI parameters of stool consistency and perianal bleeding. If the assessment is performed by an unexperienced researcher, it is recommendable to have a colleague confirm the assessment, ideally in a blinded fashion.

To analyze the intestinal epithelial permeability for macromolecules, three major methods have been used throughout the literature: the Evans blue dye assay, as described here; the instillation of 4-kDa FITC-dextran in an intestinal loop; and the feeding of 4-kDa FITC-dextran<sup>13,24,25</sup>. In the latter assay, FITC-dextran is fed by gavage, and the amount of FITC dextran that crossed the epithelium and leaked into the blood stream is measured fluorometrically from serum samples. In this assay, the permeability of the entire gastrointestinal tract is measured, since the tracer passes all the way from the esophagus to the rectum and most of the tracer will pass before reaching the colon. In the loop model, permeability is measured in a confined intestinal region used for the loop (usually the small intestine)<sup>25</sup>. In contrast, the Evans blue assay has the advantage that only epithelial permeability in the colon is measured, since the tracer is directly instilled into the entire colon. Thus, using this assay, we can conclude that the colonic epithelium is protected by the diet supplement.

It is always important to record the colon weight and length after extraction and before using the tissues for other assays, because some data (e.g., leaked Evans blue in the permeability assay) are expressed per g of tissue. Furthermore, the weight-to-length ratio is an independent read-out of tissue damage<sup>26</sup>. In general, tissue morphology is analyzed using paraffin-embedded tissues stained with hematoxylin and eosin, as described here. Paraffin embedding has the advantage that the tissue morphology is much better conserved compared to other embedding methods. This allows for the unequivocal identification of different cell types within the mucosa (*i.e.*, immune cells, epithelium, goblet cells, etc.) and the analysis of tissue alterations during colitis, such as edema formation, leukocyte infiltration, and epithelial apical erosions. On the other hand, paraffin has the disadvantage that immunofluorescent staining can only be performed after time-consuming epitope recovery. Therefore, we always prepare different frozen tissue biopsies embedded in OCT compound. Such tissue samples can be easily sectioned using a cryostat and show low levels of autofluorescence. We use ethanol for fixation by dehydration because neither does it interfere with actin filaments (as methanol does), nor does it trigger autofluorescence (as PFA does). Using these techniques, we found that an anti-inflammatory and anti-oxidative diet supplement can improve tissue morphology and junction architecture during colitis (compare Fig. 2).

Excessive neutrophil recruitment is a critical event during colitis that is induced by the production of chemoattractants in response to inflammation<sup>17</sup>. Neutrophil recruitment is a physiological event because neutrophils contribute to the removal of the inflammatory cue and to subsequent wound healing. However, if this innate immune response is not properly controlled and terminated, neutrophils in the mucosa can contribute to tissue damage by releasing proteases and reactive oxygen species. Neutrophils contain MPO, which can be easily measured and quantified using a colorimetric assay, where the amount of MPO is directly proportional to the number of neutrophils. Fig. 3A shows that

neutrophil recruitment increases during colitis and that dietary intervention can prevent this excessive and uncontrolled immune response. In accordance with the abundant neutrophil presence in the mucosa, oxidative stress increases during colitis (Fig. 3B).

Our diet supplementation clearly protects the mucosa from oxidative stress, which is most likely a consequence of reduced neutrophil recruitment. As mentioned, neutrophils are attracted by chemokines and the production of chemokines is induced by pro-inflammatory cytokines that are secreted by various cell types during colitis. Thus, we assumed that reduced neutrophil recruitment is a consequence of reduced cytokine and chemokine production. Fig. 3C demonstrates that the pro-inflammatory cytokines IL-1 $\beta$  and IL-6, as well as the chemokine KC, are upregulated during DSS-induced colitis. The diet supplement reversed the levels of IL-6 back to control levels and ameliorated the increased production of IL-1 $\beta$  and KC. Of note, KC is the most important chemoattractant for neutrophils in mice.

Thus, it is tempting to speculate that the observed preservation of tissue morphology is due to reduced neutrophil infiltration, which is, in turn, a consequence of the reduced production of KC. Analyzing RNA production in DSS-treated tissues by RT-PCR is problematic when residual DSS is present in the isolated RNA. This is because DSS inhibits both reverse transcriptases and Taq-polymerases. If no amplification can be achieved, it will be necessary to further purify the RNA samples by LiCl-mediated precipitation, as described elsewhere<sup>27</sup>.

In summary, we describe in detail different methods for the analysis of tissue damage during DSS colitis and how this damage can be ameliorated by dietary supplements. Knowledge gained from such animal experiments can justify the establishment of patient cohorts to investigate the protective effects of the analyzed supplements in human IBD. Data obtained from clinical studies may then open new avenues to develop alternative treatment strategies for the management of IBD.

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#### **DISCLOSURES:**

The authors have nothing to disclose.

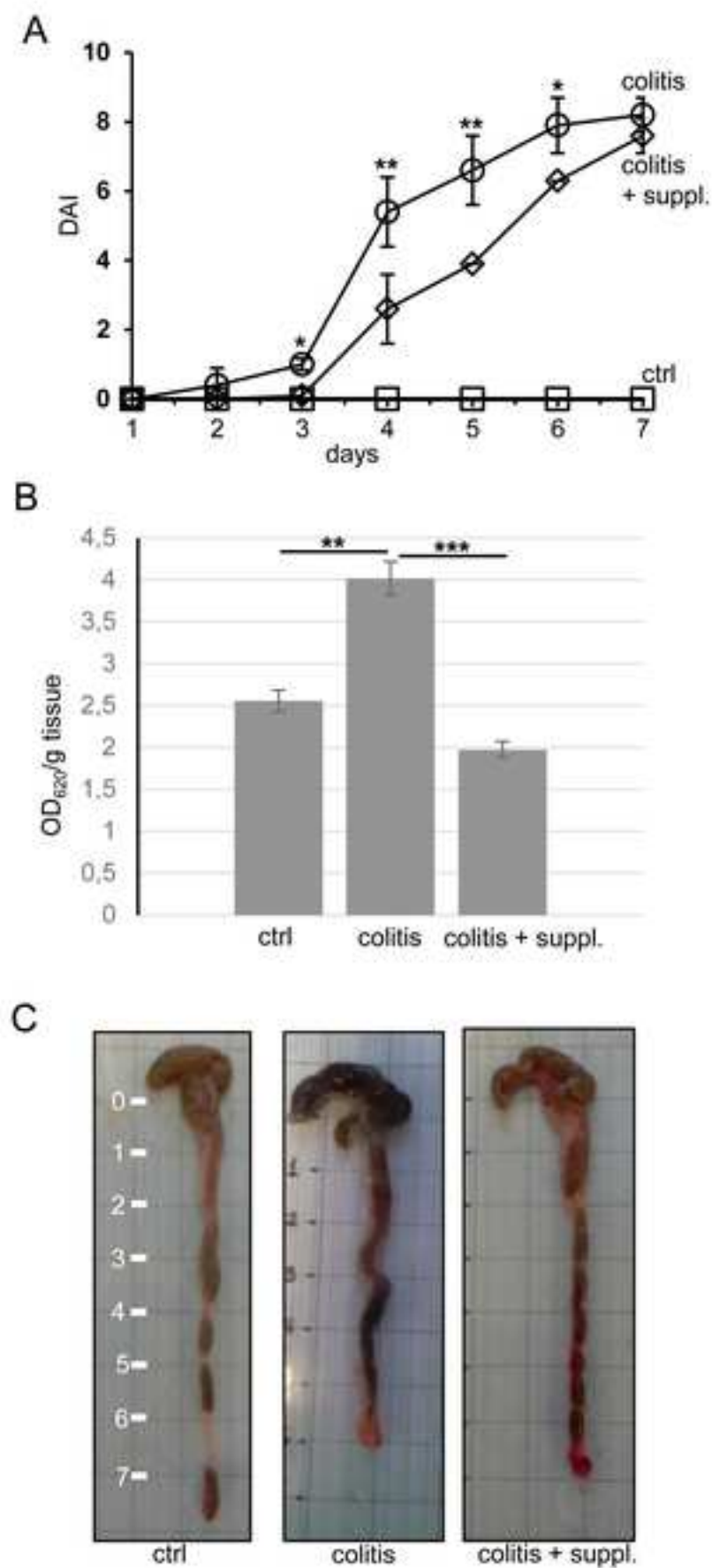
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Figure 1



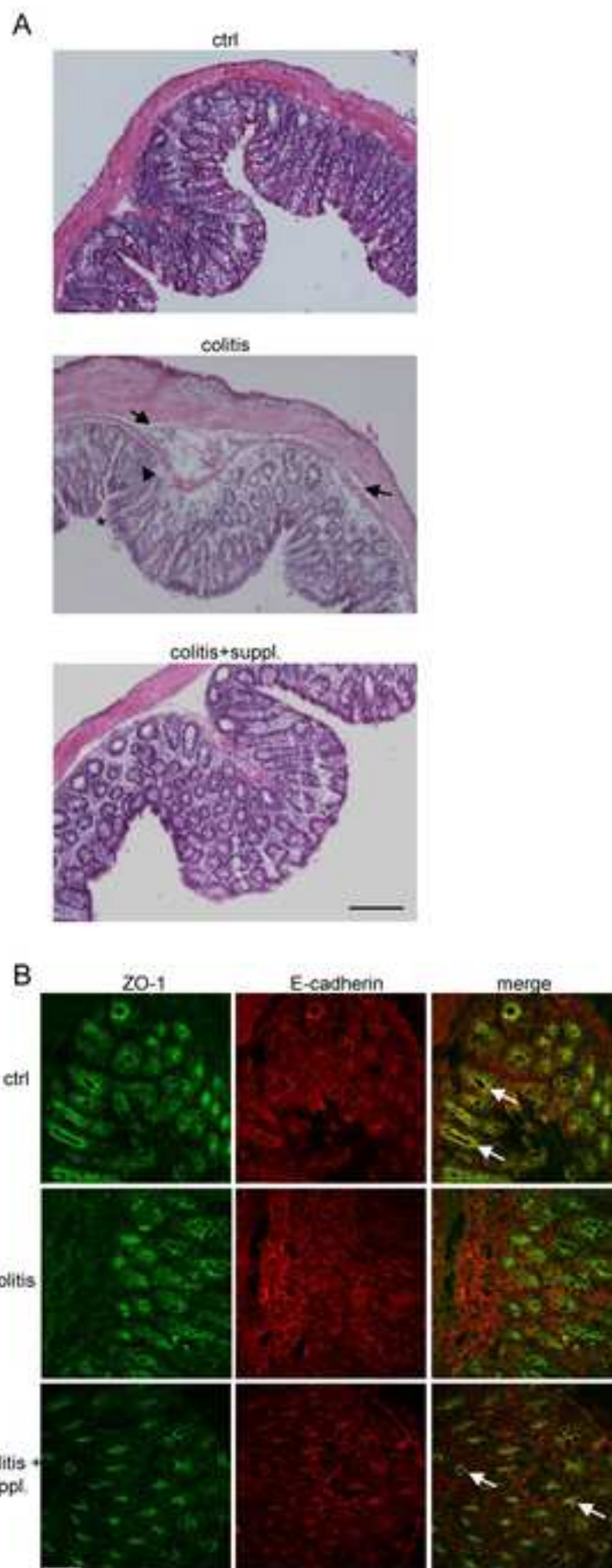
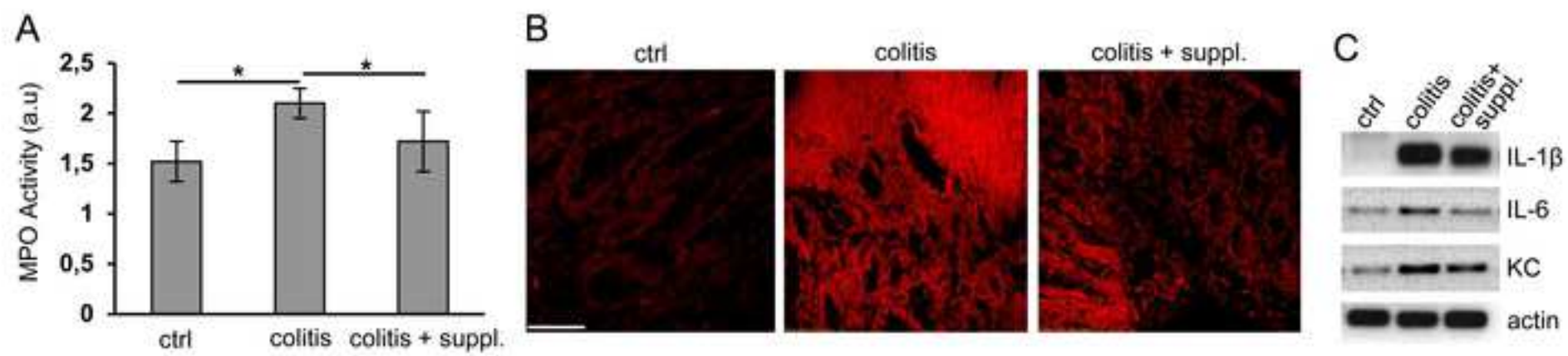
**Figure 2**

Figure 3





Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
0.3% of gelatin	Bioxon	158	
10% formaldehyde	J.T. Baker	2106-03	
96-well plate with flat bottom	Corning	3368	
Absolute ethanol	J.T. Baker	9000-03	
Absolute xylene	J.T. Baker	9490-03	
ABTS	Sigma Aldrich	H5882	
Bovine Serum Albumin	Sigma-Aldrich	9048-46-8	
ColoScreen	Helena Laboratories	5072	
Corabion (Kindly provided by Merck, Naucalpan, Mexico)	Merck		
Dextran Sulfate Sodium Salt	Affymetrix	9011-18-1	(M.W. 40,000-50,000)
Dihydroethidium	Life Technology	D11347	
Eosin-Y	J.T. Baker	L087-03	
Evans blue	Sigma-Aldrich	314-13-6	
Feeding needle	Cadence Science Inc.	9921	
Glass slide rack with handle	Electron Microscopy	70312-16	
Glass Slides	Corning	2947	
Harris hematoxylin	Sigma-Aldrich	H3136	
Hexadecyltrimethyammonium (HTAB)	Sigma Aldrich	H6269	
Histosette (Embedding cassette)	Simport	M498.2	
Hydrochloric acid 1 M	J.T. Baker	9535-62	
Hydrogen peroxide	Sigma Aldrich	H3410	
Ketamine	PiSA Agropecuaria	Q-7833-028	
Liquid paraffin	Paraplast	39501-006	
Lithium carbonate	Sigma-Aldrich	2362	
N,N-dimethylformamide	J.T. Baker	68-12-2	
N-acetylcysteine	Sigma-Aldrich	A9165	
Plastic cubes	Electron Microscopy	70181	
Poly-L-Lysine Solution	Sigma-Aldrich	25988-63-0	
Prism 5 statistical software	GraphPad Software	Prism 5	
Saline Solution 0.9% NaCl	CS PiSA	Q-7833-009	
Sodium citrate	Sigma-Aldrich	W302600	
Synthetic resin	Poly Mont	7987	

Taq DNA polymerase	Invitrogen	11615-010
Tissue-tek. O.C.T Compound	Sakura Finetek	4583
Tuberculine Syringe	BD Plastipak	305945
Tween 20	Sigma-Aldrich	9005-64-5
VECTASHIELD Antifade Mounting Medium with DAPI	Vector Laboratories	H-1200
Xylazine	PiSA Agropecuaria	Q-7833-099



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Dear Dr. Upponi, Dear Reviewers,

Please find below a point-by-point description of the changes made to the manuscript in response to your constructive comments. All changes in the manuscript have been made using the "track changes" function of Word.

**Editorial comments:**

•**NOTE:** *Please download this version of the Microsoft word document (File name: 55095\_R1\_062916) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.*

Answer: This version has been downloaded und used to make the required changes.

•*There is an instance of unnecessary branding: 10.1.1 - Trizol*

Answer: Instead of Trizol, we use the name of the solution as provided by the company: acid guanidinium thiocyanate-phenol-chloroform mixture.

•*Formatting:*

-*Please use italics for Latin phrases (in vivo, in vitro, etc.).*

Answer: The text has been checked and all latin phrases are now in italics.

-*Please include spaces between numbers and units.*

Answer: The text has been checked and where missing, spaces have been added.

•*Length exceeds 2.75 pg of highlighted material and must be reduced accordingly.*

The highlighted text has been shortened.

•*Grammar: Please copyedit the manuscript for numerous grammatical errors. Such editing is required prior to acceptance, and should be performed by a native English speaker.*

Answer: The text has been copyedited as requested.

-*6.4.8, 7.4 – missing parenthesis*

Answer: Parentheses have been added.

•*Visualization: Section 6.4 is still discontinuous. We will not be able to film the section in detail unless additional steps are highlighted with a decrease in other highlighted material.*

Answer: Highlighting section 6 has been modified. It is now more continuous and includes the entire tissue preparation but omits the actual staining protocol. However, since hematoxylin/eosin stainings are standard protocols published in a plethora of research papers, we think it will be

sufficient to state in the video that the prepared tissue can be used for hematoxylin/eosin stainings according to standard protocols as described in the manuscript in section 6.4”.

•*If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.*

Answer: All figures shown in the manuscript have not been published before.

•*JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.*

Answer: DOI was missing for reference 14 where it has been manually included.

## **Reviewers' comments:**

### **Reviewer #1:**

#### *Minor Concerns:*

1. *It would be helpful to have more details on preparation of the DSS solution. What company provides the best DSS for such experiments? Do you recommend filtering the final DSS solution? What is duration of the experiment (days of DSS treatment)? How many times the DSS solution should be replaced with a fresh one during this time period?*

Answer: We used DSS from Affymetrix. However, other companies such as ICN and Sigma also provide DSS. As with the DSS concentration, the ideal source of DSS has to be tested as well in each laboratory and caution needs to be paid when lots change. DSS dissolves easily in water and it is not necessary to feed sterile DSS, so we do not filter the solution. 7 days of DSS treatment induces severe colitis. If mild colitis needs to be analyzed, 3-4 days of treatment are recommended. As long as the DSS drinking water stays clear, it is not necessary to change the water; if it turns turbid, it needs to be replaced. These informations have been added to section 1.

2. *It is not clear wheter the nutritional supplement should be administered just once, or every day during DSS colitis.*

Answer: The nutritional supplement should be applied daily by gavage as described in our previous article (Vargas Robles, 2016, Ox Med Cell Longev). This has been clarified in section 2.5 of the protocol: “Apply nutritional supplements once daily by gavage during the course of the colitis experiment.”

### **Reviewer #2:**

#### *Minor Concerns:*

1) *It would be good to go a little more into detail how DSS is inducing a colitis-like phenotype. This topic is only glanced over in the introduction.*

Answer: More information has been added to the introduction on page 3: “DSS induces erosions in the mucosa, resulting in barrier dysfunction and increased intestinal epithelial permeability. The exact mechanism remains unknown; however, a study suggests that DSS interacts with medium-chain-length fatty acids to form nano-lipocomplexes that are able to enter epithelial cells and induce inflammatory signaling pathways.” References 11 and 12 have been added for the interested reader.

2) *Page 2, lines 85/86. Three times "well" sounds awkward*

Answer: We agree. Lines 85-88 on page 2 now read: “Although the clinical signs of UC have been well described, the pathogenesis is still poorly understood.<sup>1</sup> It is accepted among experts that UC is a multifactorial disease, with genetic mutations and aberrant immune responses playing a major role.”

3) Page 2: *Is the feeding of supplements a one time application or done more often?*

Answer: Compare comment of reviewer 1. It should be applied daily and this has been clarified in section 2.5.

4) Page 2, 2.2 *Somewhat confusing, the syringe is described as 1ml, 27G x 13 mm. The latter two numbers do not describe the syringe but probably a needle, which I assume is replaced by the feeding needle.*

Answer: Sorry for the confusion. The sentence now reads: Fill a 1 ml syringe connected to a gavage needle (15 G x 50 mm) with the nutritional supplement mixture.

5) Page 7 line 287 ...*mount THEM on*

Answer: Thank you. This has been corrected (now line 303).

6) Page 10, line 444 *STORE*

Answer: Thank you. This has been corrected (now line 462).

### **Reviewer #3:**

#### *Minor Concerns:*

1. Page 9, 9.2 *homogenizer should be "homogenate"*

Answer: We actually wash off remaining tissue from the tip of the homogenizing device to make sure that all tissue is included for sonication. This has been clarified in section 9.2.

2. Page 3, 1.2: *DSS range should be changed to 2.5-5%*

Answer: The range has been changed as requested in section 1.2.

3. *Western Blot analysis of tissue should be added to methodology. In the example Fig 3C the text states mRNA data however immunoblotting is presented. Both could be shown.*

Answer: Figure 3C actually represents PCR bands from an agarose gel where black and white have been reverted. This has been clarified in the figure legend. We agree that Western blots are means of analyzing protein production. However, since chemokines and cytokines are rather secreted proteins, analysis by ELISA with commercial kits is more frequently being used. Since we did not use Western blots neither in this nor in our previous paper, and given that Western blot is a very standardized technique; we do not think that it will be critical to include a detailed description of a Western blot protocol here.

4. *For immunofluorescence studies other methods of fixation should be discussed and what is the advantage of ethanol fixation.*

Answer: We have included a sentence in the discussion explaining the use of ethanol for fixation on page 15: “We use ethanol for fixation by dehydration because neither does it interfere with actin filaments (as methanol does) nor does it trigger autofluorescence (as PFA does)”.



*5. For tissue processing and analysis, tissue localization ie. proximal versus distal colon should be added and discussed as the effects of DSS could vary along the colon.*

Answer: We agree. In the figure legend, we mentioned that the images were taken from distal colon tissues. We also added this information in the results section on page 12. The images shown in figure 2A were taken from distal colon areas. This information has been added to the figure legend. To analyze the overall colon damage, “swiss rolls” of the entire colon should be prepared as described in section 5.8. A note has been added to section 5.8 to point this out.

*6. For both MPO and ROS assays time points should be specified and the temporal differences should be discussed.*

Answer: We clarified in sections 8.1 and 9.1 that tissues were collected after 7 days of DSS colitis. We did not perform time courses but such inflammation parameters usually increase over time.

*7. Page 14 permeability assays; as has been shown by Parkos et.al in a number of recent manuscripts direct application of FITC dextran into ligated ileal (could be extended to proximal colon) loops is another localized way to measure intestinal permeability. Should be added to discussion.*

Answer: Thank you. We have included such a paper of the Parkos group and described this method in the discussion on page 15, lines 643-654.

We hope that our revisions meet your expectations and that the manuscript is now suitable for publication in “JoVE”.

Sincerely

A handwritten signature in black ink, appearing to read 'Michael Schnoor'.

Michael Schnoor (on behalf of all authors)