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SHORT COMMUNICATION

A Mineral Extract from red Algae Ameliorates Chronic Spontaneous Colitis in IL-10 Deficient Mice in a Mouse Strain Dependent Manner

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Inflammatory bowel disease is an urgent public health problem with a high incidence in developed countries. Alterations of lifestyle or dietary interventions may attenuate the disease progression and increase the efficacy of current therapies. Here we tested the effect of chronic supplementation with a mineral extract from red marine algae – rich in calcium (34%), magnesium, phosphorus, selenium and other trace minerals – in a clinically relevant model of spontaneous enterocolitis, interleukin (IL)-10$^{-/-}$ mice. The mineral extract was administered in the drinking water of *Il10*$^{-/-}$ mice on C57BL/6 J and BALB/c strain backgrounds for 25 weeks commencing from 3 to 4 weeks of age. The mineral extract ameliorated the spontaneous development of colitis and severity of disease in *Il10*$^{-/-}$ mice on a C57BL/6 J background. Mineral extract-treated *Il10*$^{-/-}$ C57BL/6 J strain mice had significantly reduced mortality, circulating levels of serum Amyloid A and reduced colonic tissue damage. In contrast, comparable treatment of *Il10*$^{-/-}$ mice on a BALB/c background with the mineral extract did not alter the course of colitis. These data demonstrate that chronic supplementation with a natural mineral extract selectively ameliorates spontaneous mild–moderate colitis in *Il10*$^{-/-}$ mice on a C57BL/6 J, but does not attenuate more moderate–severe colitis in BALB/c strain animals. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: colitis; interleukin-10 deficient mice; *Lithothamnion spp*.; mineral extract.

Abbreviations: CD, Crohn's Disease; DAI, Disease Activity Index; ELISA, Enzyme-linked immunosorbent assay; H&E, Hematoxylin & Eosin; IBD, Inflammatory Bowel Disease; IFN-γ, Interferon-γ; IL, Interleukin; MPO, Myeloperoxidase; PMA/I, Phorbol 12-Myristate 13-Acetate *plus* Ionomycin; SAA, Serum Amyloid A; Th, T helper cell type; TNF-α, Tumor Necrosis Factor-α; UC, Ulcerative Colitis.

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD), two major chronic inflammatory disorders of uncertain etiology. The development of new therapeutics has targeted the inflammatory cytokines associated with IBD including the TNF pathway, with an aim to resolve the inflammation in the gut. One area of active investigation in developing new strategies is how alterations in the diet of IBD patients can alter the course of the disease (Cabre and Domenech, 2012). In this context, the use of probiotics/prebiotics is one of the most widely explored approaches to alter colon pathology, including alterations in the composition of the gut microbiome to modulate the inflammatory state of the colon (DuPont and DuPont, 2011). In this study, we investigated the effect of chronic dietary supplementation of mice with a mineral extract from *Lithothamnion spp* in a model of chronic spontaneous colitis. Previously, the same extract has been tested in man, with supplementation of the diet of patients with osteoarthritis of the knee leading to no adverse effects (Frestedt *et al*., 2008). In this study, the effect of continual prophylactic diet supplementation with mineral extract was evaluated in the interleukin (IL)-10 deficient ($^{-/-}$) mice, which develop spontaneous chronic enterocolitis that shares histopathological features with human CD (Berg *et al*., 1996). The age of onset of disease and the severity of colitis in *Il10*$^{-/-}$ mice is dependent on both the mouse background strain and the environmental conditions in which animals are housed (Berg *et al*., 1996). Due to this phenotype, *Il10*$^{-/-}$ mice are routinely used as a model to test the efficacy of new drugs or therapeutic strategies for human IBD (Sollid and Johansen, 2008). In this study, we compared the efficacy of dietary supplementation with mineral extract on the development of spontaneous colitis in *Il10*$^{-/-}$ mice on both the C57BL/6 J and BALB/c backgrounds, which represent respective experimental models of mild–moderate and moderate–severe colitis.

MATERIALS AND METHODS

Animals. Female *Il10*$^{-/-}$ mice on a C57BL/6 J and BALB/c background (Jackson Labs, Bar Harbor, USA) were housed under Specific Pathogen-Free conditions. The mating system adopted for the breeding of *Il10*$^{-/-}$ mice was homozygote × homozygote. Experiments were performed in compliance with Irish Department of Health and Children regulations and were approved by the Trinity College BioResources ethical review board.

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Supplementation with mineral extract in drinking water. The mineral extract (Aquamin) was obtained from the red marine algae *Lithothamnion corallioides*, harvested under approved license off the coasts of Ireland and Iceland and provided by Marigot Ltd (Cork, Ireland). It contains 34% calcium, 1% magnesium and measurable levels of 72 other traces of minerals. Drinking water was supplemented with 36.75 mg mineral extract per ml of drinking water. The addition of Aquamin to drinking water did not affect daily water consumption of mice. Fresh Aquamin-supplemented water was provided every week.

Disease Activity Index (DAI). DAI was calculated for each mouse weekly as described (Saunders *et al*., 2010). The maximum DAI score was 12 based on assigning a 1–4 scoring system for each parameter: score 0, no weight loss, normal stool and no blood; score 1, 1–3% weight loss; score 2, 3–6% weight loss, loose stool (a loose stool was defined as the formation of a stool that readily becomes paste upon handling) and blood visible in stool; score 3, 6–9% weight loss; and score 4, >9% weight loss, diarrhea and gross bleeding.

Colon histology. At autopsy, the presence of tumors in the colonic mucosa of mice was macroscopically quantified. A ~1 cm of the distal colon was removed and fixed in 10% formalin-saline. Sections (5 μm) were stained with hematoxylin and eosin. Histology scoring was performed in a blinded fashion, independently by two observers. Sections were graded using a cumulative score ranging from 0 to 3–4 (Saunders *et al*., 2010). An arbitrary maximum combined score of 10 was determined from the severity of inflammatory cell infiltration (Score 0, none; Score 1, slight-dispersed cell infiltrate; Score 2, moderate-increased cell infiltrates forming occasional cell foci; Score 3, severe-large areas of cell infiltrates causing loss of tissue architecture), extent of injury (Score 0, none; Score 1, mucosal; Score 2, mucosal and submucosal; Score 3, transmural) and crypt damage (Score 0,none; Score 1, basal 1/3 damaged; Score 2, basal 2/3 damaged; Score 3, only surface epithelium intact; Score 4, loss of entire crypt and epithelium). Colon histology sections were checked for the presence of adenocarcinomas.

Myeloperoxidase (MPO), colon cytokines and serum Amyloid A (SAA) levels. Colons were homogenized in a buffer containing 1 X PBS, 2% foetal bovine serum and 0.5% cetyltrimethylammonium bromide. MPO activity was detected using *O*-phenylenediamine as substrate and data interpolated from a MPO standard curve (Sigma-Aldrich, UK). Cytokines (IL-1β, TNF-α, IL-6 and IL-17) in colon homogenates were detected by ELISA kit (BD Biosciences, and R&D Systems, USA), according to manufacturer's instructions. Blood from mice was recovered at termination and serum isolated for the detection SAA by ELISA kit (Life Diagnostics Ltd).

Spleen cytokine analysis. Single cell suspensions were prepared from spleens and 1×10^6 cells/ml cultured in RPMI-1640, supplemented with 10% FCS, 2 mM L-glutamine, and 50 U/ml penicillin plus 50 μg/ml streptomycin. Cells were stimulated with 2.5 ng/ml phorbol 12-myristate 13-acetate (PMA) plus 250 ng/ml ionomycin (Sigma-Aldrich, UK) or 0.5 μg/ml anti-CD3 (clone 145-2C11) plus 4 μg/ml anti-CD28 monoclonal antibodies (clone 37.51; BD Biosciences, USA). Supernatants were harvested for the detection of cytokines (IL-2, TNF-α, IFNγ, IL-4 and IL-17) by ELISA.

Statistical analysis. Results are presented as mean ± SEM or SD. The Kaplan–Meyer method was used to evaluate survival differences. The Mann–Whitney non-parametric test was used for the analysis of DAI and histology data. P value < 0.05 was considered significant (unpaired Student's t test).

RESULTS AND DISCUSSION

Il10$^{-/-}$ mice were placed on a diet with mineral extract supplementation in their drinking water for 25 weeks commencing from 3–4 weeks of age, an age that precedes any evidence of intestinal inflammation. This prophylactic regime spans the progression of mild–moderate colitis in C57BL/6 J strain, to moderate–severe colitis in BALB/c strain *Il10*$^{-/-}$ mice. Development of spontaneous colon pathology was assessed by body weight, DAI and mortality. There were no differences between animals receiving water only (untreated) and mineral extract-treated *Il10*$^{-/-}$ C57BL/6 J strain mice in body weight gain (data not shown). However, the DAI score, the cumulative score of the presence of soft stools, faecal blood, rectal prolapse and death showed a marked delay in disease onset in mineral extract-treated mice (Fig. 1A). Importantly, there was a significant ($P < 0.01$) decrease in mortality in mice receiving mineral extract over the 25 weeks compared to untreated animals (Fig. 1B). After 25 weeks, SAA levels, a parameter correlated with the clinical disease in IBD patients (Niederau *et al*., 1997), were found to be significantly lower ($P < 0.05$) in mineral extract-treated mice relative to untreated animals (Fig. 1C). Histology showed that *Il10*$^{-/-}$ C57BL/6 J mice treated with mineral extract exhibited decreased ($P < 0.01$) colon damage (evaluated as inflammatory cell infiltration, tissue injury and crypt disruption) compared to controls (Fig. 1D,E). In addition, MPO enzymatic activity, a marker of tissue inflammation, was significantly ($P < 0.05$) decreased in colon homogenates from mineral extract-treated *Il10*$^{-/-}$ C57BL/6 J mice relative to untreated animals (Fig. 1 F).

The colitis seen in *Il10*$^{-/-}$ mice is associated with increases in pro-inflammatory cytokines in the colon (Berg *et al*., 1996). Levels of pro-inflammatory cytokines associated with the pathogenesis of IBD, including IL-1β, TNF-α, IL-6 and IL-17, were measured in colon homogenates by ELISA. While the levels of IL-1β in the colons of both groups of IL-10 deficient mice were markedly elevated relative to wild-type mice, *Il10*$^{-/-}$ mice treated with mineral extract-treated had a significant ($P < 0.05$) reduction in colon IL-1β levels compared to untreated *Il10*$^{-/-}$ mice (Fig. 1G). In contrast, there were no significant differences in colon levels of TNF-α, IL-6 and IL-17

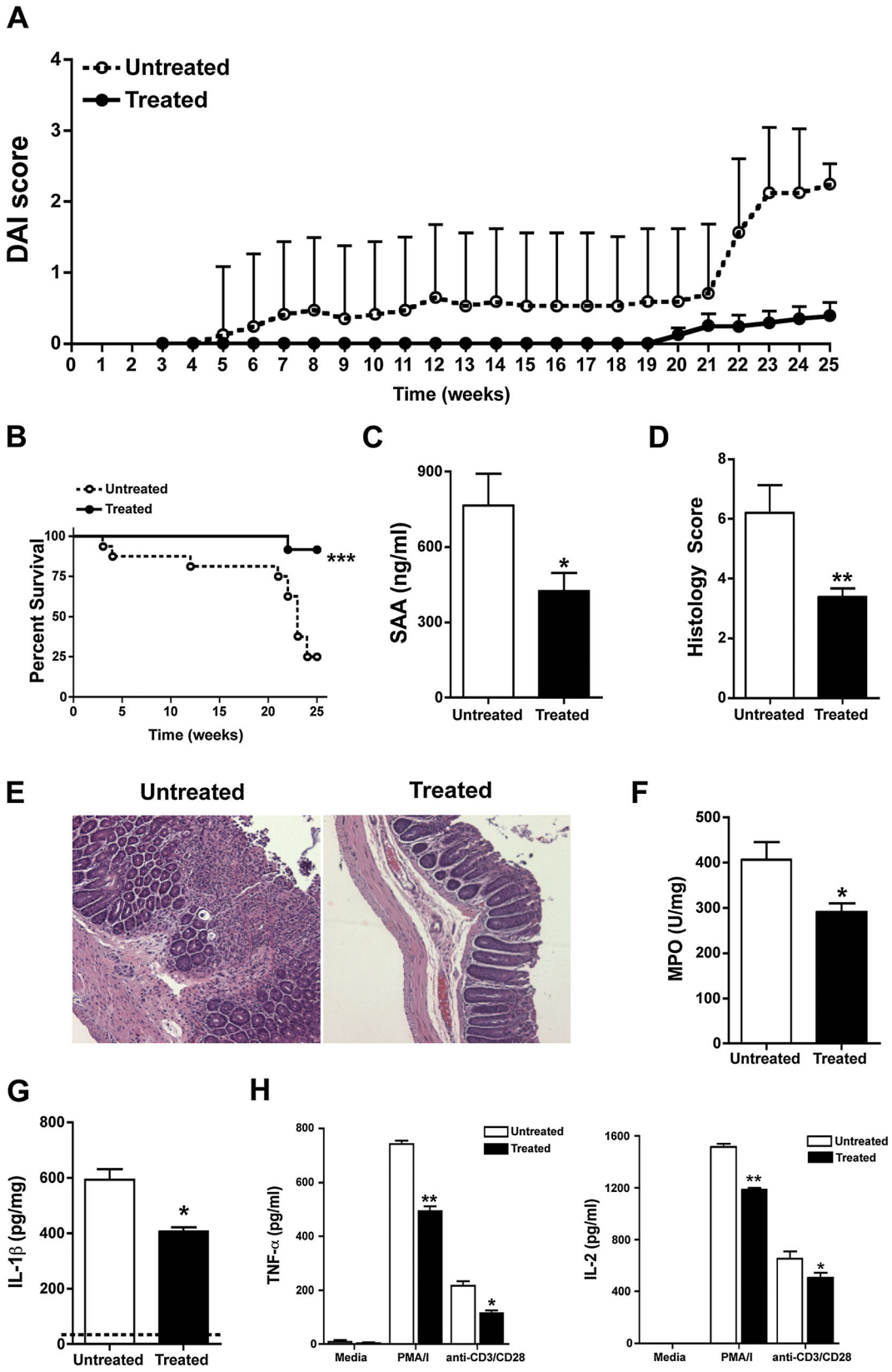

Figure 1. *Mineral extract supplementation reduces spontaneous colitis in Il10*$^{-/-}$ *mice on a C57BL/6 J strain background*. C57BL/6 J *Il10*$^{-/-}$ mice were treated from 3–4 weeks of age onwards, with normal or mineral extract supplemented drinking water and monitored for 25 weeks. Mice were monitored for Disease Activity Index (DAI) (A) and mortality (B). At the termination of the study, serum Amyloid A (SAA) (C), colon histology (D, E), and myeloperoxidase (MPO; F) were evaluated. Representative images of histopathology in colons from an untreated (score ~6), and mineral extract-treated (score ~2) mice (E). Levels of IL-1β were detected in colon homogenates (G). The dotted line is representative of IL-1β levels in wild type C57BL/6 J mice. Detection of TNF-α and IL-2 in supernatants from spleen cells recovered from mice and stimulated *in vitro* with PMA/I or anti-CD3/CD28 mAb (H). Data are expressed as mean ± SEM (n = 6–18). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ *vs* control (untreated) using an unpaired Student's *t* test with Welch correction applied as necessary; the Kaplan–Meyer method was used to evaluate survival differences between mineral extract and untreated groups. The Mann–Whitney non-parametric test was used to analyze the histology data. Colon cytokines are expressed as pg *per* mg of colon protein (mean ± SEM, n = 5–18). Cytokines detected in splenocyte supernatants are expressed as pg *per* ml of supernatant (mean ± SD, n = 4). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

between the two groups of mice (data not shown). It is not clear why the effects on colon pro-inflammatory cytokine expression, were specific to IL-1β. However, IL-1β expression is a major inflammatory hallmark in the initiation and maintenance of colitis (Coccia *et al.*, 2012). The colitis that develops in *Il10*$^{-/-}$ mice is also associated with an increased incidence of colorectal adenocarcinomas (Berg *et al.*, 1996). We did note reduced frequencies of adenocarcinomas in the colons of *Il10*$^{-/-}$ C57BL/6 J strain mice treated with mineral extract (data not shown). In other studies the same mineral extract has been reported to reduce polyp formation and inflammation in the gastro-intestinal tract of mice on a high-fat diet (Aslam *et al.*, 2010). It is not known whether the mineral extract directly attenuated the propensity of *Il10*$^{-/-}$ mice to chronically develop adenocarcinomas, or if this is an indirect consequence of a reduction in colon inflammation and/or altered composition of the gut microbiome.

To address the effect of chronic supplementation with mineral extract on systemic immunity, we examined cytokine production from spleen cells polyclonally activated with PMA/I, or treated with anti-CD3/CD28 antibodies for T cell activation. Splenocytes from *Il10*$^{-/-}$ mice exposed to mineral extract had significantly reduced production of TNF-α and IL-2 ($P < 0.01$ and $P < 0.05$, respectively) following activation with PMA/I or anti-CD3/CD28 (Fig. 1H). In contrast, there was no difference between the two groups of mice in the production of IFN-γ, IL-4 and IL-17 (data not shown). Collectively, these data strongly suggest that the mild–moderate spontaneous colitis that develops in *Il10*$^{-/-}$ mice on a C57BL/6 J background is attenuated by chronic supplementation with the mineral extract added to the drinking water.

Using the same regime as used in *Il10*$^{-/-}$ C57BL/6 J strain mice, mineral extract supplementation was also evaluated in *Il10*$^{-/-}$ mice on a BALB/c background, which are more susceptible to colitis and develop, in absence of IL-10, a more severe disease than C57BL/6 J mice (Berg *et al.*, 1996). Supplementation of *Il10*$^{-/-}$ BALB/c strain mice with mineral extract did not alter the course of the development of colitis, as assessed by body weight (data not shown), mortality, DAI, SAA levels and colon histology (Fig. 2). Although the maximum DAI scored in C57BL/6 J strain is comparable to BALB/c mice, the degree (%) of mortality in BALB/c mice was higher than in C57BL/6 J mice. In *Il10*$^{-/-}$ mice, the mouse strain-specific differences in the activity of the mineral extract tested may reflect therapeutic efficacy in milder forms of colitis, as develops in C57BL/6 J, but not in the more aggressive and severe colon inflammation that occurs in the BALB/c strain.

In this study, we have shown that chronic dietary supplementation with a natural mineral-rich extract from marine algae can ameliorate mild to moderate disease, but had no effect in more severe disease, in a mouse model of spontaneous colitis. Due to its complex and multi-component nature, it is difficult to identify the exact mechanism of action and/or the single component

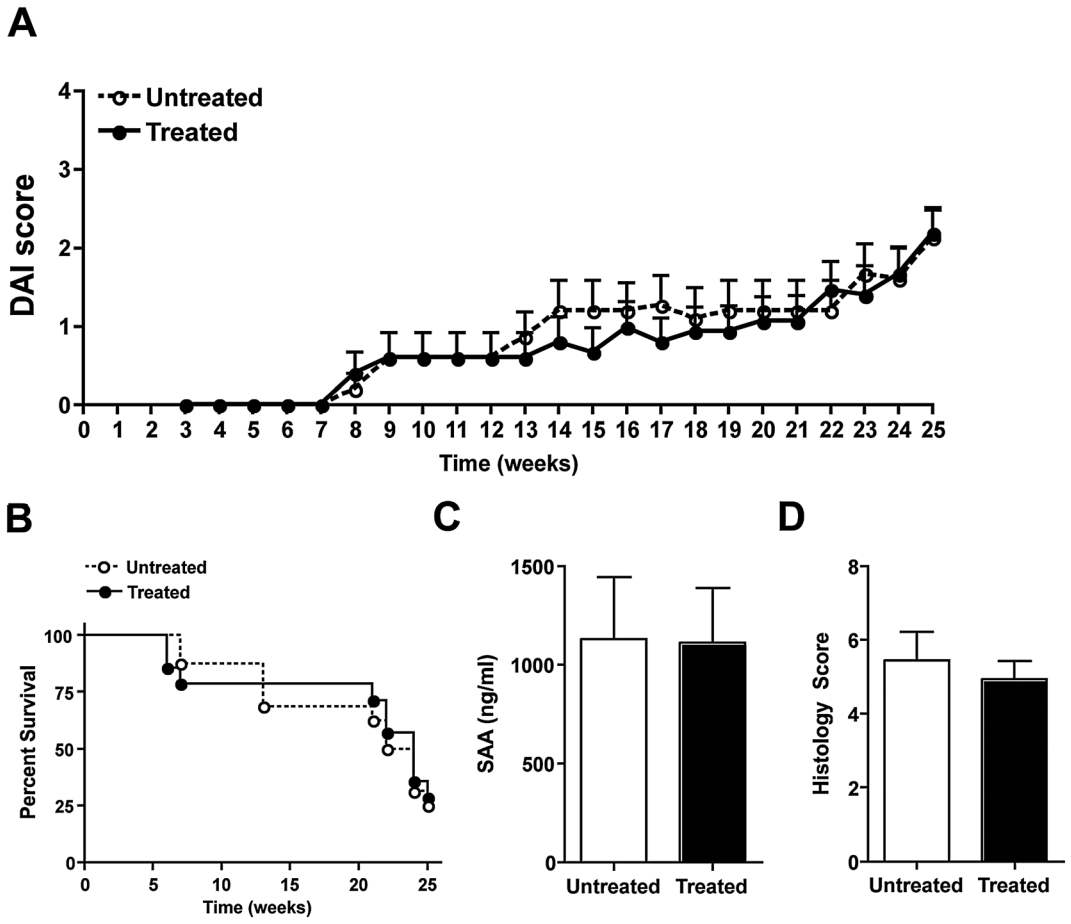

Figure 2. *Mineral extract supplementation does not alter development of colitis in Il10*$^{-/-}$ *mice on a BALB/c background*. BALB/c *Il10*$^{-/-}$ mice were treated with water or mineral extract in the drinking water from 3–4 weeks of age onwards and monitored for 25 weeks. A) DAI score, B) Survival, C) SAA levels and D) Histology score were quantified. Data are expressed as mean ± SEM (n = 6–15).

responsible for the effects of the mineral extract. The high concentration of calcium, known to suppress colon inflammation, present in the mineral extract may play an important role in its mechanism of action, as speculated by others previously (Aslam *et al*., 2010). However, further experiments are required to elucidate how the mineral extract attenuates colon inflammation, and modulates systemic immunity and intestinal homeostasis. Such studies would also need to address the effects of the mineral extract on the integrity of the intestinal barrier function and also whether it alters the gut microbiome. Here we show that in a mouse model of mild colitis mineral extract may attenuate physiological and inflammatory processes that may lead to experimental colitis. The underlying protective mechanisms, once elucidated, could be used for the development of additional strategy aimed to slow down the IBD progression or to prolong the effective time window of current therapies.

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Conflict of interest

The authors state no conflict of interest.

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