

Combined Enterococcus faecium SF68®– Butyrate–Folate Supplementation Restores Intestinal Barrier Integrity and Epithelial Metabolic Function in Experimental DSS-Induced Colitis

Clelia Di Salvo

University of Pisa

Giulia Valdiserra

University of Pisa

Letizia Campigli

University of Pisa

Vanessa D'Antongiovanni

University of Pisa

Nunzia Bernardini

University of Pisa

Lorenzo Flori

UniCamillus, Saint Camillus International University of Health Sciences

Lara Testai

University of Pisa

Laura Benvenuti

University of Pisa

Carolina Pellegrini

University of Pisa

Matteo Fornai

matteo.fornai@unipi.it

University of Pisa

Luca Antonioli

University of Pisa

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Abstract

Inflammatory bowel diseases (IBD) are characterized by intestinal inflammation, epithelial barrier damage, dysbiosis and impaired epithelial metabolism. Since current therapies mainly target inflammation without fully restoring intestinal homeostasis, novel therapeutic approaches aimed at addressing multiple pathogenic mechanisms may represent a useful strategy. Here, we evaluated the efficacy of a mixture combining *Enterococcus faecium* SF68® (SF68), butyrate and folate in an experimental model of dextran sulfate sodium (DSS)-induced colitis, investigating both its ability to prevent disease onset and to promote recovery during established inflammation. In this setting, disease activity index (DAI), colonic cytokine levels, including tumor necrosis factor (TNF) and interleukin (IL)-10, intestinal epithelial barrier integrity [lipopolysaccharide-binding protein (LBP) plasma levels, tight junction proteins (ZO-1, occludin, claudin-1) expression], short-chain fatty acid (SCFA) transporters expression (SMCT1, MCT1, MCT4) and citrate synthase activity, a marker of mitochondrial metabolic function, were assessed. In the preventive protocol, the mixture exhibited anti-inflammatory properties, as shown by the reduction in TNF levels, together with the increase in colonic IL-10 levels. In addition, the mixture reduced circulating LBP levels, increased ZO-1 and occludin expression, outperforming SF68 alone. Conversely, in the curative protocol, the therapeutic effects were more limited, although improvements in DAI and selected inflammatory and barrier-related parameters were still observed. Moreover, the combined formulation uniquely increased MCT4 expression and restored citrate synthase activity, indicating recovery of epithelial mitochondrial oxidative metabolism and butyrate utilization. Overall, these findings demonstrate that the combination of *Enterococcus faecium* SF68®, butyrate and folate simultaneously targets inflammation, intestinal epithelial barrier dysfunction and metabolic impairment, providing broader efficacy than SF68 treatment alone and representing a promising adjunctive strategy to current IBD therapies.

1. Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic relapsing–remitting disorders characterized by dysbiosis, increased intestinal permeability, and loss of immune tolerance toward commensal microbiota [1]. Despite the enrichment of the IBD pharmacological armamentarium, current treatment strategies achieve only partial efficacy and fail to provide long-term remission [2, 3]. Of note, increasing evidence indicates that durable remission depends not only on suppressing intestinal inflammation but also on restoring epithelial barrier function and host–microbiota homeostasis.

Beyond the well-established contribution of intestinal epithelial barrier (IEB) dysfunction to IBD pathogenesis, several clinical studies, including the ERICA trial, demonstrated that structural and functional IEB defects may persist even after remission and represent strong predictors of disease relapse [4, 5]. In addition, patients responding to anti-TNF therapy often exhibit long-lasting dysbiosis, which has also been associated with an increased risk of disease recurrence, highlighting that anti-inflammatory strategies, despite being effective in controlling intestinal inflammation, fail to restore both

IEB integrity and microbiota homeostasis [6]. Moreover, increased intestinal permeability has been identified as a plausible mechanism underlying the persistence of bowel symptoms, including abdominal pain and diarrhoea, experienced by 18–29% of IBD patients, despite mucosal healing and inflammatory remission [7].

Collectively, these findings suggest that durable disease control requires therapeutic strategies that combine suppression of inflammation with restoration of epithelial barrier integrity and intestinal homeostasis. The IEB is increasingly recognized as a dynamic interface integrating physical protection, immune regulation and epithelial metabolism. Consequently, therapeutic strategies targeting only inflammation are unlikely to fully restore intestinal homeostasis [8].

Although probiotics and microbiota-derived metabolites such as butyrate have shown promising anti-inflammatory and barrier-protective properties [9], their clinical efficacy remain inconsistent, likely because they target only selected aspects of IBD pathophysiology [10]. Beyond its immunomodulatory properties, butyrate represents the primary oxidative substrate for colonocytes, and impaired epithelial butyrate utilization has recently emerged as a hallmark of IBD-associated metabolic dysfunction [11]. Whether simultaneously targeting microbiota composition, epithelial barrier integrity and epithelial metabolism provide broader therapeutic benefits than single-component interventions remain largely unexplored.

Building on our recent findings showing that a formulation containing *Enterococcus faecium* SF68® (SF68), butyrate and folate improved IEB integrity and butyrate utilization in experimental irritable bowel syndrome, we investigated whether this combined strategy could also counteract epithelial dysfunction in inflammatory colitis [12].

We hypothesized that simultaneously targeting microbial composition, epithelial barrier integrity and epithelial metabolic function would provide broader protection than probiotic therapy alone, ultimately restoring intestinal homeostasis through coordinated modulation of inflammation, butyrate metabolism and mitochondrial function. Unlike previous studies focusing on individual interventions, the present work investigates a multi-target strategy designed to simultaneously restore epithelial barrier integrity, microbial-derived metabolite utilization and epithelial metabolic competence, three tightly interconnected processes increasingly recognized as key determinants of disease persistence and relapse.

2 Materials and Methods

2.1 Animals

C57BL/6 male mice (20–22g body weight), seven-week-old, were purchased from ENVIGO S.r.l (San Pietro al Natisone UD, Italy) and used during the entire duration of the study. They were housed five in a cage in a temperature-controlled room on a 12-hour light cycle at 22–24°C with 50%–60% humidity and not used for at least one week before the beginning of the experimental procedures. Mice were fed with

standard laboratory chow and tap water *ad libitum*. All animal procedures were conducted in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and complied with the ARRIVE guidelines. The experiments have been approved by the Ethical Committee for Animal Experimentation of the University of Pisa and by the Italian Ministry of Health (Authorisation No. 176/2022-PR). All efforts to reduce and minimize the number of animals and their suffering were carried out.

2.2 Animal model of DSS-induced colitis and experimental design

To induce the experimental model of colitis, mice were fed with 2.5% dextran sulfate sodium (DSS) (W/V, molecular weight, 36,000–50,000 Da, MP Biomedicals, LLC; Solon, OH, USA) dissolved in drinking water. Two different protocols were established to evaluate the efficacy of the supplementation containing *Enterococcus faecium* SF68® (hereafter referred to as SF68), butyrate and folate (from now on named “mixture”) in preventing the colitis onset (“*Preventive Protocol*”) as well as in promoting recovery during established inflammation (“*Curative Protocol*”) (See Fig. 1). In the *Preventive Protocol*, 2.5% DSS was administered for 7 consecutive days, while the mixture was given daily by oral gavage starting 7 days before the beginning of DSS intake and until the end of the experiment. In contrast, in the *Curative Protocol*, 2.5% DSS was administered for 5 consecutive days, while the mixture was given daily by oral gavage starting from the third day of DSS administration and until the end of the experiment. Mice were randomly assigned to the experimental groups of the two study protocols, with group sizes ranging from 6 to 8 animals. In both protocols, animals were sacrificed at day 14. The daily mixture contained the three components at the following doses: SF68: 10^8 CFU/mice/day, butyrate: 0.6 mg/kg and folate: 0.9 µg/mice, established based on previous experiments [12]. Another combined strategy, already available in the market, containing *Bifidobacterium*, butyrate and fructo-oligosaccharides (4.3 mg/mice/day) was used in the study as a comparator. SF68, the mixture and the comparator were dissolved in methylcellulose before administration. SF68 was kindly provided by Cerbios-Pharma SA. At the end of the study, the animals were anaesthetized and euthanized. Blood and tissue samples were collected and stored at -80°C for further analysis.

2.3 Evaluation of Disease Activity Index (DAI) score

At day 14, animals were assessed for the Disease Activity Index (DAI), used as composite score evaluating disease severity based on body weight loss, stool consistency, and rectal bleeding. In particular, a sub score was assigned to each of the 3 indicators (percentage of body weight loss, rectal bleeding, and stool consistency), as described in Table 1. For each animal, DAI was obtained by summing the three sub scores with a maximum total score of 12. DAI assessment was conducted by three blinded scientists.

Table 1

Disease Activity Index (DAI) scoring criteria. Body weight loss, rectal bleeding, and stool consistency were scored on a scale from 0 to 4. The final DAI score is represented as the sum of the three subscores, with higher scores indicating greater disease severity.

Body Weight Loss (%)	Rectal Bleeding	Stool consistency
< 1%: 0	0: negative	0: normal
1–5%: 1	1: slight bleeding	1: mild soft
5–10%: 2	2: moderate bleeding	2: soft
10–20%: 3	3: bleeding	3: gross
> 20%: 4	4: gross rectal bleeding	4: watery diarrhea

2.4 Quantification of colonic interleukin (IL)-1 β , IL-6, IL-10 and TNF levels

Colonic IL-1 β , IL-6, IL-10 and TNF levels, were measured using a commercial ELISA kit (88–7013A-88, 88-7064-88, 88-7105-88, 88-7324-86, respectively, all purchased from Invitrogen, USA), as previously described [13, 14]. Briefly, colonic tissues were thawed, weighed, and homogenized in PBS (0.4 ml/20 mg of tissue) at 4°C, and then centrifuged for 5 min at 12000 rpm. Aliquots of 100 μ L were used to perform the assay. IL-1 β , IL-6, TNF and IL-10 levels were expressed as picograms per millilitres (pg/mL) of colonic extract.

2.5 Quantification of plasma lipopolysaccharide binding protein (LBP) levels

Lipopolysaccharide binding protein (LBP) levels in plasma were measured using a commercial ELISA kit (ab213876, Prodotti Gianni, Milan, Italy), as previously described [15]. For the procedure, blood samples were centrifuged for 5 minutes at 4000rpm and supernatants were collected. Aliquots (100 μ L) were used for the assay. LBP levels were expressed as ng/mL of plasma.

2.6 Western blot assays

Colonic tissues were lysed as previously reported [16]. Tissues were weighed and then homogenized in lysis buffer (50 mg in 400 μ L), using a polytron homogenizer (QIAGEN, Milan, Italy). Homogenates were centrifuged at 12000rpm for 30 minutes at 4°C and supernatants were employed for the assay. Bradford test was performed to quantify total proteins. Subsequently, proteins were separated onto a pre-cast 4%-20% polyacrylamide gel (Mini-PROTEAN TGX gel, Biorad) and transferred to PVDF membranes (Trans-Blot TurboTM PVDF Transfer packs, Biorad). Membranes were blocked then with 3% BSA diluted in Tris-buffered saline (TBS, 20 mM Tris-HCl, PH 7.5, 150 mM NaCl) with 0.1% Tween 20. Primary antibodies against β -actin (A3854, Sigma-Aldrich), occludin (ab216327, Abcam), zonulin-1 (ab96587, Abcam), claudin-1 (PA-520754, Invitrogen), monocarboxylate Transporter 1 (MCT1) (PA-576687,

Invitrogen), sodium-coupled monocarboxylate transporter 1 (SMCT1) (BS6106R, Bioss Antibodies) and monocarboxylate Transporter 4 (MCT4) (PA-5106683, Invitrogen) were used. Secondary antibodies were bought from Abcam (anti-mouse ab97040 and anti-rabbit ab6721). Protein bands were revealed with ECL reagents (Clarity Western ECL Blotting Substrate, Biorad). Subsequently, iBright Analysis software was used to perform the densitometry analysis.

2.7 Assessment of Citrate Synthase Activity in intestinal tissue

Intestinal samples were homogenized in Tris buffer (100 mM, pH 8.2) containing 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 100 μ M) and acetyl-coenzyme A (100 μ M). Protein concentration was determined prior to the assay, and 1 μ g of total protein per well was loaded into 96-well plates.

The enzymatic reaction was initiated by the addition of oxaloacetic acid (500 μ M). Absorbance was monitored at 412 nm at 37°C at 30-second intervals for 15 min using a microplate reader (EnSpire, PerkinElmer, Waltham, MA, USA).

Citrate synthase activity was calculated by comparison with a calibration curve generated using purified citrate synthase of known activity (MedChemExpress, USA). Enzymatic activity was expressed as mU/mL.

2.8 Statistical analysis

The results are presented as mean $\pm$ standard error of the mean (S.E.M.). One-way ANOVA was used to assess statistical significance, followed by Tukey's post hoc tests. Significant differences were obtained with P values < 0.05. All statistical procedures were performed using GraphPad Prism 10.0 software (GraphPad Prism, San Diego, CA, USA).

3 Results

3.1 Effect of SF68 alone and the mixture on disease activity index (DAI) and macroscopic parameters as preventive and curative strategy

In both preventive and curative protocols, animals with colitis showed a significant increase in DAI score together with a reduction in colonic length and an increase in spleen weight, although not significant, highlighting how DSS model can reliably mimic IBD features (Fig. 2A-B). In the preventive protocol, the administration of SF68 alone or in combination with butyrate and folate did not exert any beneficial effects, whereas in the curative protocol, both SF68 and the mixture, but also the comparator, significantly reduced DAI levels in comparison with the DSS group (Fig. 2A-B). No effect was recorded on spleen weight and colonic length parameters in the curative protocol.

3.2 Effect of SF68 alone and the mixture on interleukin (IL)-1 β , IL-6, IL-10 and tumor necrosis factor (TNF) colonic levels

IBD patients are characterized by elevated intestinal levels of several pro-inflammatory cytokines, involved in the initiation and maintenance of the chronic inflammatory response [17]. Accordingly, in both preventive and curative protocol, DSS animals displayed a significant increase in TNF and IL-6 colonic levels, along with increase in IL-1 β , although not significant, and a reduction in IL-10 levels, as compared with control (Fig. 3A-B). In the preventive protocol, the supplementation with SF68, the mixture and the comparator significantly reduced TNF colonic levels (Fig. 3A). Of note, only the mixture was able to normalize IL-10 colonic levels (Fig. 3A). In the curative protocol, the reduction of TNF colonic levels mixture-mediated was the only remarkable effect (Fig. 3B).

3.3 Effect of SF68 alone and the mixture on IEB permeability: lipopolysaccharide binding protein (LBP) and tight junction expression at a colonic level

IBD patients showed an increased IEB permeability as well as a reduction of tight junction proteins expression, multiprotein complexes able to regulate the selective passage of ions and solutes through the IEB [18, 19]. In the same way, colitis animals from both protocols showed a raise in plasmatic levels of LBP (Fig. 4A-B), indirect index of intestinal permeability [20], as well as a reduction in ZO-1, occludin and claudin-1, although not always significant, in comparison with the control group (Fig. 4A-B).

The administration of both SF68 alone and in combination with folate and butyrate as a preventive strategy induced a significant decrease of LBP levels along with an increase of occludin expression in comparison with the DSS group. Of note, only the mixture displayed an ability to increase ZO-1 colonic expression in this therapeutic setting (Fig. 4A). No variations were recorded for claudin-1 (Fig. 4A).

Regarding the curative protocol, both SF68 and the mixture confirmed the abovementioned positive effect on IEB, as shown by the decreased LBP levels and the augmented occludin expression (Fig. 4B). Of note, only the mixture was able to increase claudin-1 colonic expression in comparison with the DSS group (Fig. 4B). Interestingly, the comparator did not show such beneficial effect in both protocols (Fig. 4A-B).

3.4 Effect of SF68 alone and the mixture on colonic SCFA transporter expression

IBD is associated with impaired butyrate epithelial uptake, likely due to reduced expression of the SCFA transporters [21].

Accordingly, in both protocols DSS animals were characterized by a reduction in SMCT1 and MCT1 colonic expression, reflecting the hypothesis of the reduced butyrate uptake in a colitis context (Fig. 5A). Both SF68 and the mixture normalized SMCT1 and MCT1 colonic expression in comparison with the DSS group, while only the mixture was effective in increasing MCT4 levels in the two experimental settings (Fig. 5A-B).

3.5 Effect of SF68 alone and the mixture on mitochondrial functionality

Since defective butyrate utilization may depend on impaired mitochondrial oxidative metabolism in intestinal epithelial cells, citrate synthase activity in intestinal tissues, a commonly used indicator of mitochondrial functionality, integrity and oxidative metabolism, was also assessed. As expected, DSS animals from both preventive and curative protocols were characterized by a significant reduction in citrate synthase activity in comparison with controls (Fig. 6A-B). Of note, only the mixture as a preventive strategy significantly restored enzyme activity as compared with the colitis group, while SF68 did not exert any positive effect (Fig. 6A-B).

4. Discussion

The increasing burden of inflammatory bowel diseases (IBD), together with their chronic and relapsing nature, highlights the need for integrated therapeutic strategies able to induce long-term remission [22]. However, current therapies often encounter primary and secondary loss of efficacy over time, since they primarily target inflammation, without restoring dysbiosis as well as intestinal epithelial barrier (IEB) dysfunctions, recently recognized as predictive markers of disease relapse [5, 22]. For these reasons, integrating epithelial barrier-restoring strategies with conventional anti-inflammatory therapies may represent a novel approach toward disease modification.

Based on these premises, the present study was aimed at evaluating the efficacy of a mixture, containing *Enterococcus faecium* SF68® (SF68), butyrate and folate in an experimental model of colitis induced by the administration of dextran sulfate sodium (DSS). Although *Enterococcus faecium* and butyrate alone have already shown anti-inflammatory and gut-barrier protection properties in the context of colitis [23, 24], the favourable effect of their combination *plus* folate has not been investigated in this setting yet. Our findings provide evidence supporting the rationale for simultaneously targeting epithelial barrier function and metabolism as complementary therapeutic objectives in IBD.

In particular, the present study employed two complementary DSS-induced colitis protocols to investigate whether a formulation containing SF68, butyrate and folate could prevent disease onset or promote recovery during established inflammation.

Consistent with the well-established features of DSS-induced colitis, both experimental protocols developed a robust inflammatory phenotype, as evidenced by increased disease activity index (DAI), colon shortening, and elevated colonic levels of TNF, IL-6 and IL-1 β , confirming the suitability of the model for the evaluation of novel therapeutic strategies for colitis. [25]. Interestingly, the therapeutic efficacy of the tested mixture differed according to disease stage. While preventive administration did not significantly improve disease severity, treatment initiated during established colitis reduced DAI, indicating that the formulation may exert greater benefits once epithelial damage and inflammation have already developed. This observation is in agreement with previous evidence showing that the efficacy of

probiotic-based interventions is strongly influenced by the inflammatory status and the disease phase of the host [26]. Despite the limited effect on DAI in the preventive protocol, both the mixture and SF68 significantly reduced colonic TNF levels, suggesting that the immunomodulatory effects may precede measurable improvements in disease severity. Notably, only the combined formulation restored IL-10 levels, a key anti-inflammatory cytokine produced mainly by regulatory T (Treg) cells and essential for limiting intestinal inflammation [27]. This additional effect may reflect the contribution of butyrate, which has been widely shown to promote Treg differentiation and IL-10 production in experimental colitis [24].

Importantly, in the curative setting only the mixture retained its ability to reduce TNF levels, whereas SF68 alone no longer significantly modulated its release. This finding suggests that controlling established intestinal inflammation may require a broader therapeutic strategy simultaneously targeting multiple pathogenic mechanisms, including immune responses and microbiota-related dysfunctions, rather than relying on probiotic supplementation alone. In line with this interpretation, previous experimental and clinical studies have shown that multi-component formulations combining probiotics with complementary bioactive compounds exert superior anti-inflammatory effects in comparison with single-component interventions [28, 29].

In the IBD scenario, the perpetuation of an inflammatory response, mostly mediated by IL-1 β , plays a central role in driving IEB damage by directly disrupting tight junction proteins and promoting epithelial cell death, thus increasing intestinal permeability [30]. Accordingly, in both experimental schemes, colitis mice were characterized by increased LBP plasmatic levels, widely accepted as an indirect marker of intestinal permeability, along with a reduction of occludin and ZO-1 colonic expression. Treatment with SF68 partially improved IEB dysfunctions, as shown by the reduction of LBP levels and increased occludin expression. However, the mixture exerted a broader and more consistent effect than the probiotic alone especially in the preventive setting, where it also enhanced ZO-1 colonic expression. In addition, only the mixture was able to restore claudin-1 levels in the curative protocol. Our results showing the prominent efficacy of the mixture in reinforcing IEB are likely driven by the integration of butyrate within the supplementation, endowed with several complementary properties, including its anti-inflammatory ability [31] as well as its direct effect in supporting epithelial integrity and promoting tight junction assembly [32].

Furthermore, IBD is commonly associated with a general decrease in short-chain fatty acid (SCFA)–producing bacteria, especially the butyrate-synthesizing ones such as *Roseburia* and *Faecalibacterium prausnitzii* [33]. In addition, several studies indicated that IBD patients are characterized by altered butyrate metabolism [34], along with a downregulation of its transporter (MCT1) within the colonic mucosa [21]. Taken together, these alterations may impair epithelial butyrate utilization, thus compromising its beneficial effects within the intestinal environment [31]. Consistently, our colitis mice exhibited a decreased MCT1 and SMCT1 colonic expression compared with controls, demonstrating how the disruption of butyrate homeostasis represents a hallmark of an inflammatory colitis. In this context, preventive treatment with SF68 and the mixture normalized SMCT1 and MCT1, although not always in a significant way, in both settings. Notably, only the mixture was able to

upregulate MCT4 colonic levels. Our finding showed that the combined strategy exerts a more pronounced effect than SF68 alone in restoring butyrate transport. Moreover, the integration of butyrate in the mixture may provide a direct source of this metabolite for epithelial cells, which, together with the restoration of SCFA transporters, could further contribute to the recovery of butyrate homeostasis.

Furthermore, IBD patients, particularly those with UC, exhibit mitochondrial dysfunction, often referred as “mitochondriopathy”, regardless duration or age of the disease onset [35]. This condition is characterized by a reduction in epithelial mitochondrial genes involved in energy production pathways [35], together with a functional decreased activity of mitochondrial chain complexes II, III, and IV in the colonic mucosa [36]. These alterations may further exacerbate IEB dysfunction and inflammation and are not directly targeted by the currently available therapeutic approaches.

Consistent with this concept, restoration of epithelial butyrate transport should ultimately translate into improved intracellular oxidative metabolism, as butyrate represents the primary energy substrate for colonocytes. Accordingly, restoration of epithelial metabolic function emerged as another distinctive feature of the combined formulation. Indeed, citrate synthase activity, used as an indicator of mitochondrial integrity, functionality and oxidative capacity, was markedly reduced in DSS mice, confirming the presence of impaired epithelial mitochondrial metabolism. In this context, administration of the mixture, but not SF68 alone, significantly restored citrate synthase activity, suggesting an improvement in intestinal mitochondrial function. This evidence underscores the superiority of the combined strategy over the single approach in promoting intestinal butyrate homeostasis by improving both SCFA transport and mitochondrial metabolic capacity.

Several limitations should be acknowledged in this study. First, DSS-induced colitis mainly reproduces UC and therefore does not encompass the entire complexity of human IBD. Second, microbiota composition was not directly assessed, preventing mechanistic correlations between microbial changes and epithelial metabolic recovery. Third, although mitochondrial function was evaluated through citrate synthase activity, additional analyses of mitochondrial respiration would further strengthen the proposed mechanism.

In conclusion, the present findings demonstrate that the combined formulation provides broader protective effects than SF68 alone by simultaneously targeting inflammation, IEB integrity and metabolic function. Rather than acting solely as an anti-inflammatory intervention, the formulation appears to restore epithelial functional competence by improving both SCFA transport and mitochondrial oxidative metabolism. These coordinated effects may underlie the recovery of barrier integrity and intestinal homeostasis observed in the present study. Overall, our findings support this combined strategy as a promising barrier-targeted adjunct to current IBD therapies, warranting further investigation as a potential approach to promote durable mucosal recovery and reduce disease relapse.

Declarations

Conflicts of interest:

L.A. is a scientific consultant for Johnson & Johnson and Eli Lilly Italy. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships.

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Author Contribution

Study design and funding acquisition: LA. Data collection: CDS, GV, LC, LB,VD,LF. Prepared initial draft: CDS. Revision of the manuscript: NB, CP, LT, MF, LA. All authors approved the final version.

Data Availability

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

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Figures

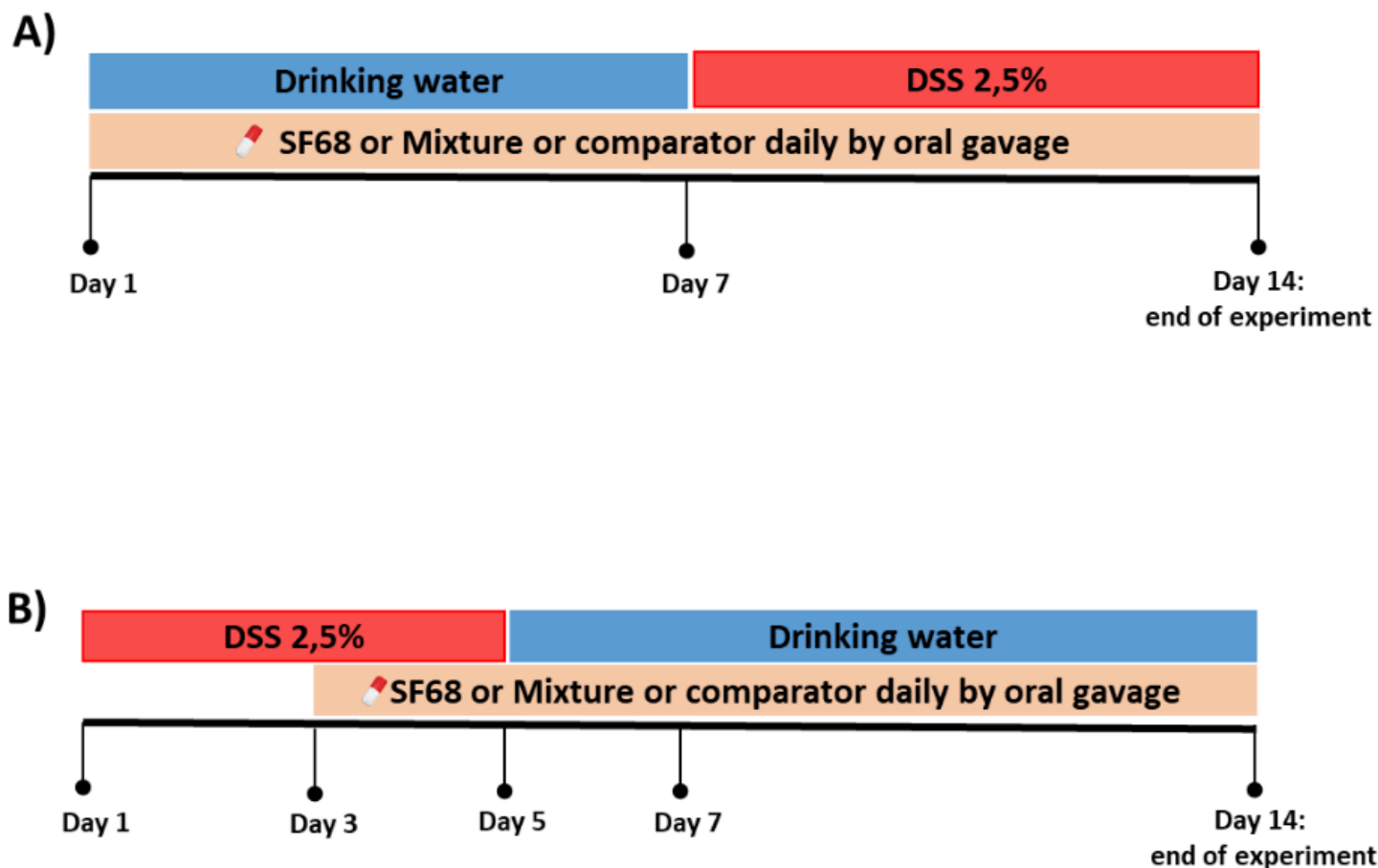


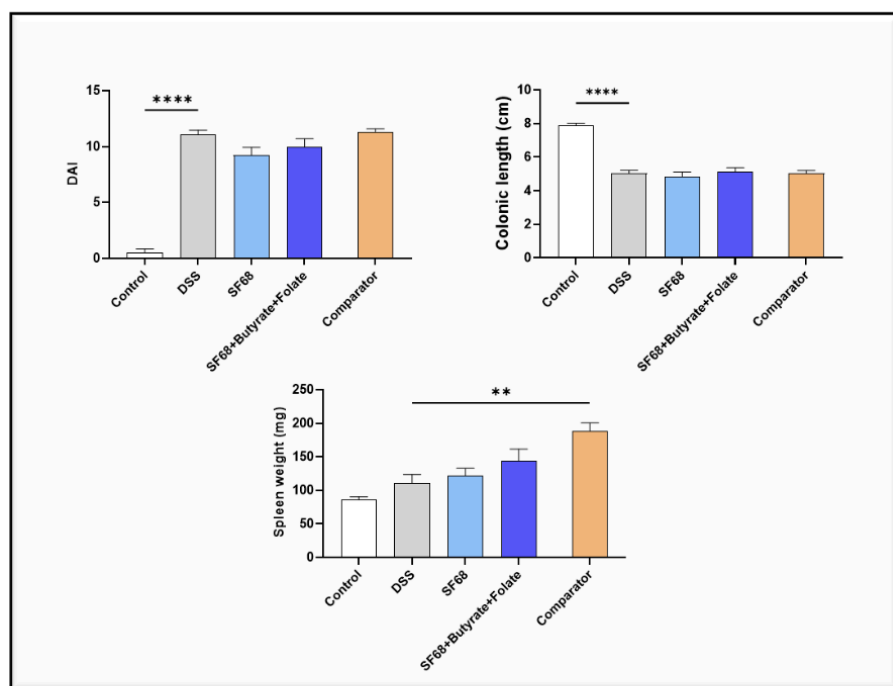
Figure 1

Representative scheme of the two experimental protocols. **A) Preventive Protocol:** 2,5% DSS was given daily in drinking water for one week. The daily administration of SF68, mixture and comparator started seven days before 2,5% DSS beginning and continued until day 14. **B) Curative Protocol:** 2,5% DSS was given daily in drinking water for five days and the daily administration of SF68, mixture and comparator started at day three and continued until day 14. In both protocols, animals were sacrificed at day 14.

Abbreviations: DSS: dextran sulfate sodium.

A)

Preventive protocol



B)

Curative protocol

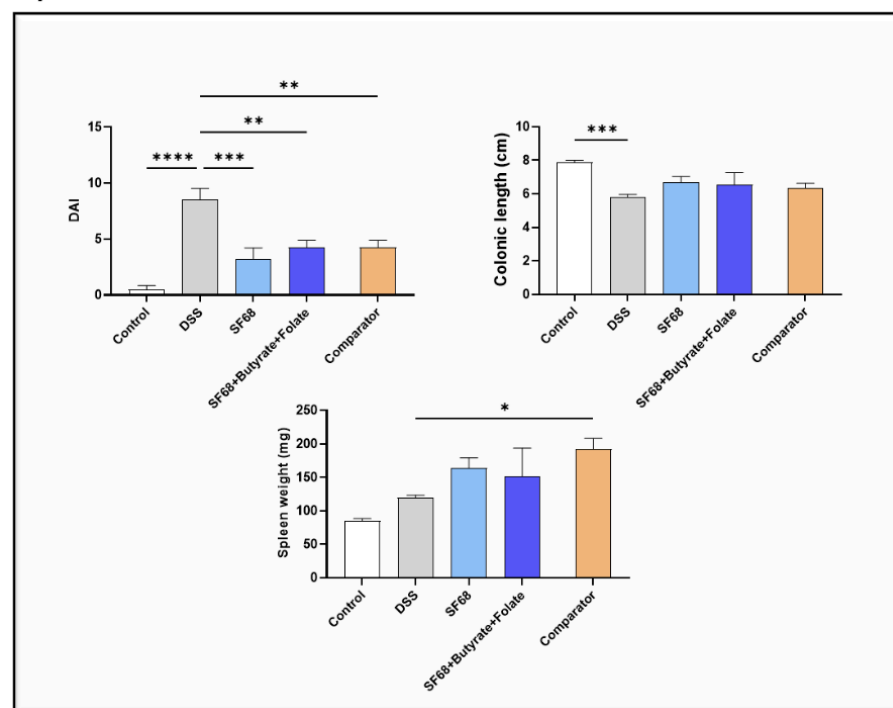


Figure 2

Effects of SF68, the mixture, and the comparator on DAI, colonic length, and spleen weight in the **(A)** preventive protocol and **(B)** curative protocol (day 14). Data are presented as mean $\pm$ SEM (preventive protocol, $n = 6-8$ mice per group; curative protocol, $n = 4-6$ mice per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Abbreviations: DSS, dextran sulfate sodium; DAI, disease activity index.

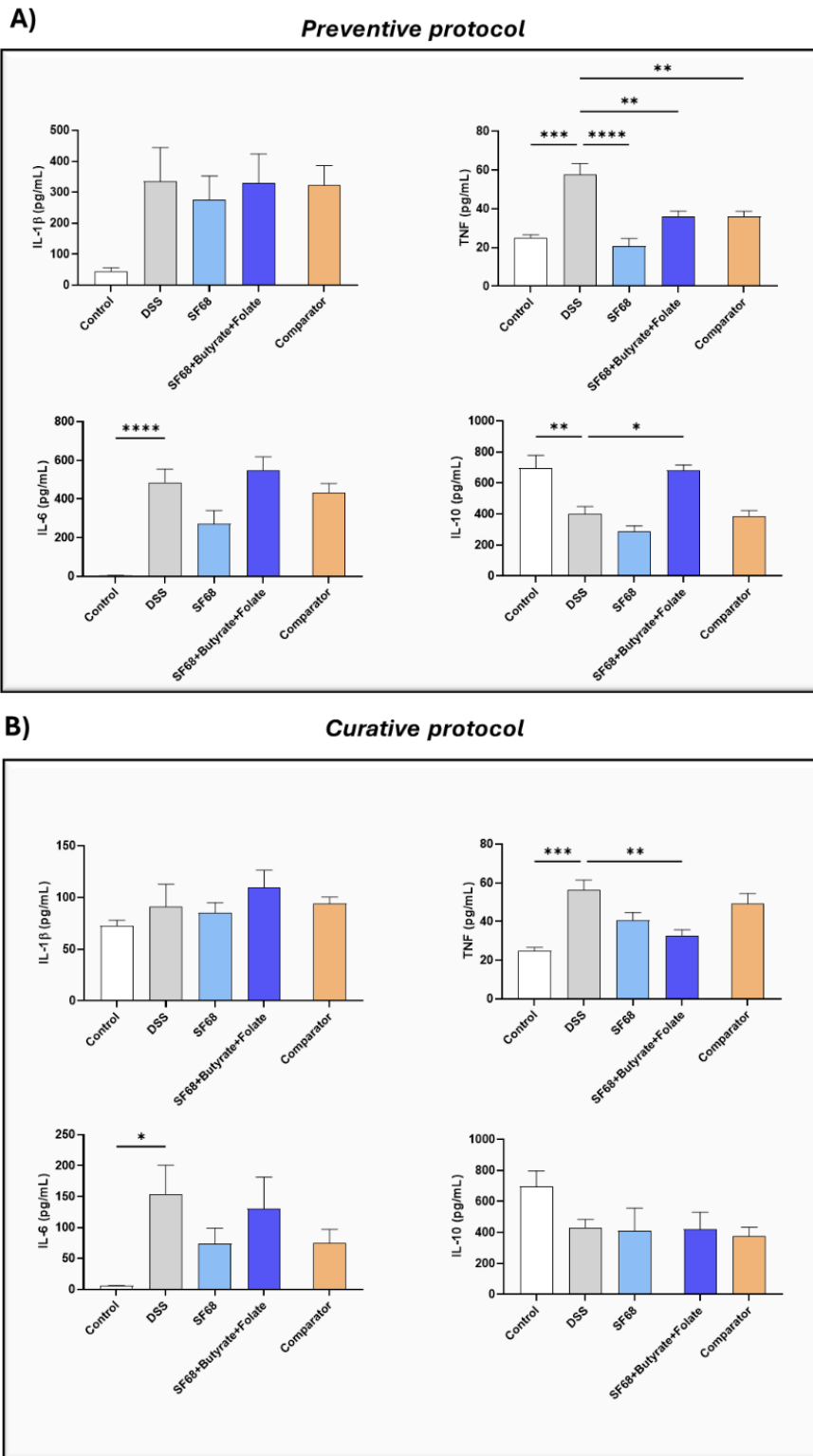


Figure 3

Effects of SF68, mixture and comparator on IL-1 β , TNF, IL-6, IL-10 colonic levels in **A)** preventive protocol and **B)** curative protocol. Each column represents the mean $\pm$ SEM (preventive protocol, n = 4–7 mice per group; curative protocol, n = 4–6 mice per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test results: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Abbreviations: DSS: dextran sulfate sodium; IL-, interleukin; TNF, tumor necrosis factor.

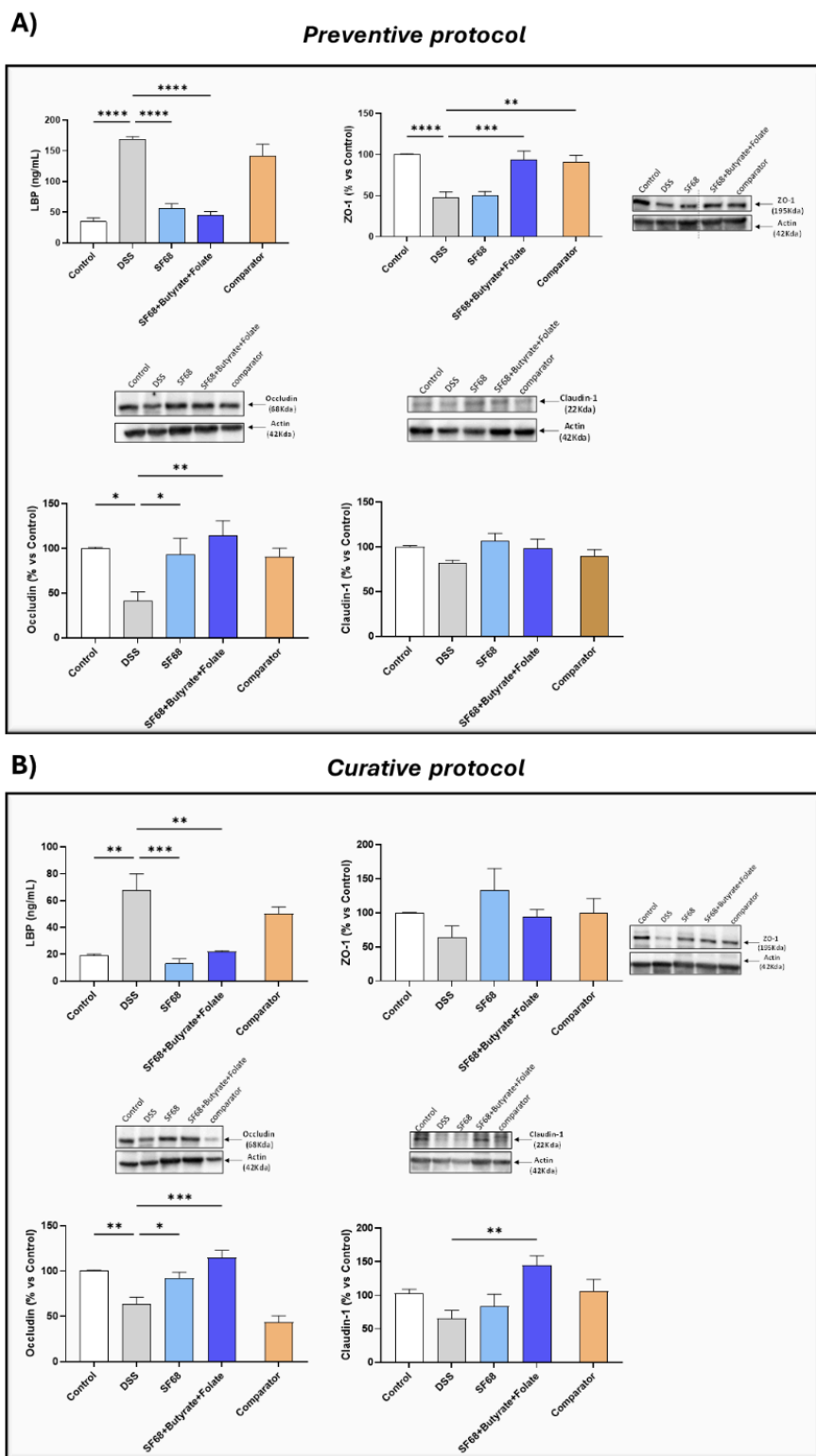


Figure 4

LBP plasmatic levels, representative blots and densitometric analysis of the expression of ZO-1, occludin and claudin-1 in colonic tissues from control and DSS mice, treated or not with SF68, mixture and comparator in **A)** preventive protocol and **B)** curative protocol. Cropped gel for ZO-1 from preventive protocol. Each column represents the mean $\pm$ SEM (preventive protocol, n = 4–6 mice per group; curative protocol, n = 4–6 mice per group). Statistical analysis was performed using one-way ANOVA followed by

Tukey's post hoc test results: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Abbreviations: DSS: dextran sulfate sodium; LBP, lipopolysaccharide binding protein, ZO-1, zonulin-1.

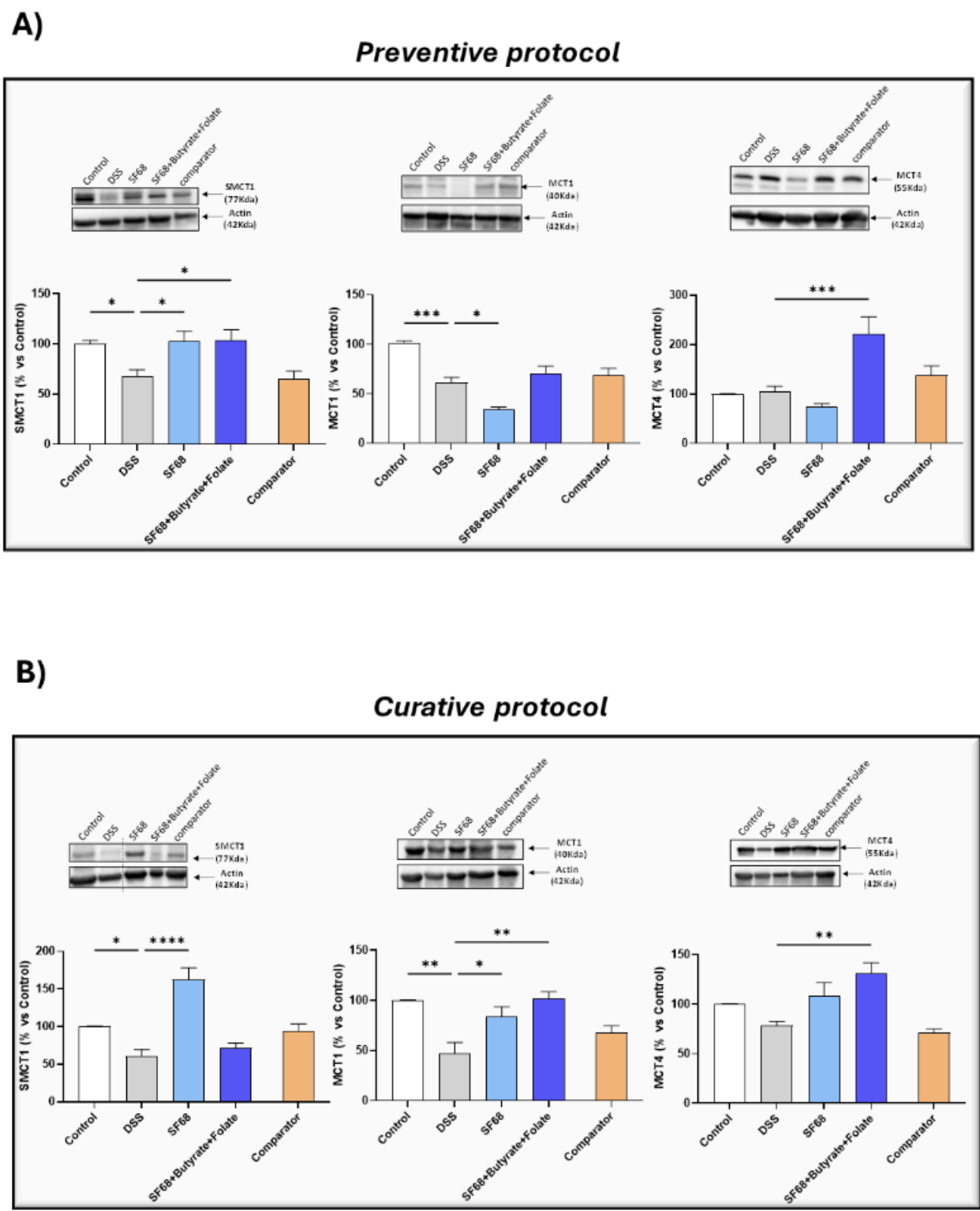


Figure 5

Representative blot and densitometric analysis of SMCT1, MCT1, MCT4 from control and DSS mice, treated or not with SF68, mixture and comparator in **A)** preventive protocol and **B)** curative protocol.

Cropped gel for SMCT1 from curative protocol. Each column represents the mean $\pm$ SEM (preventive protocol, n = 4–7 mice per group; curative protocol, n = 4–5 mice per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test results: * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Abbreviations: DSS: dextran sulfate sodium; SMCT1, sodium-coupled monocarboxylate transporter 1, MCT1, monocarboxylate transporter 1; MCT4, monocarboxylate transporter 4.

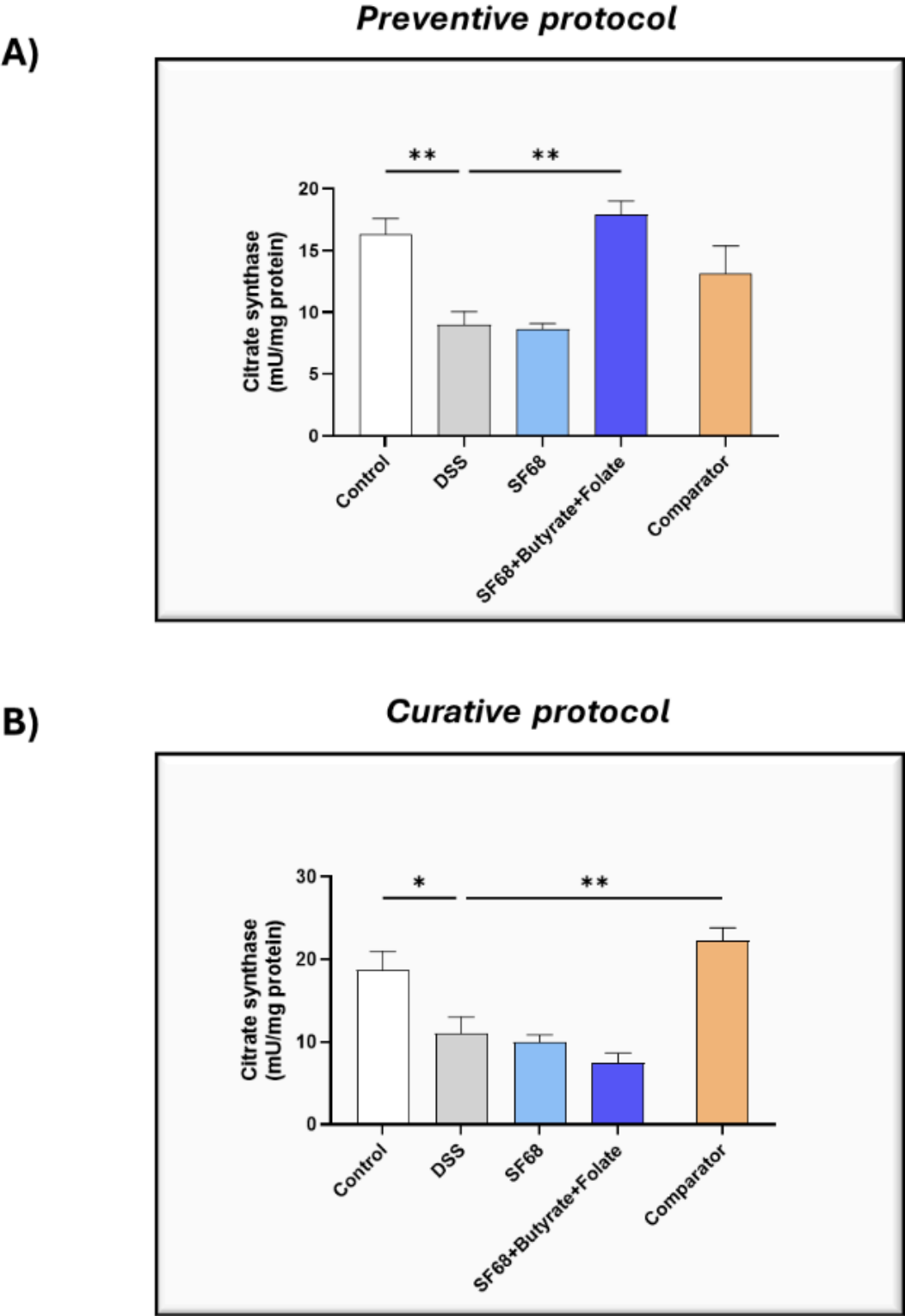


Figure 6

Citrate synthase activity from control and DSS mice, treated or not with SF68, mixture and comparator in **A)** preventive protocol and **B)** curative protocol. Each column represents the mean $\pm$ SEM (preventive protocol, n = 5–6 mice per group; curative protocol, n = 4–5 mice per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test results: * p < 0.05, ** p < 0.01. *Abbreviations:* DSS: dextran sulfate sodium.