An orally administered butyrate-releasing derivative reduces neutrophil recruitment and inflammation in dextran sulphate sodium-induced murine colitis

Raffaele Simeoli[4,5], Giuseppina Mattace Raso[4], Claudio Pirozzi[4], Adriano Lama[4], Anna Santoro[4], Roberto Russo[4], Trinidad Montero-Melendez[5], Roberto Berni Canani[6], Antonio Calignano[4], Mauro Perretti[5] and Rosaria Meli[4]

[4]Department of Pharmacy, University of Naples Federico II, Naples, Italy; [5]Centre for Biochemical Pharmacology, The William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London, London, United Kingdom, [6]Department of Translational Medicine–Pediatric Section and European Laboratory for the Investigation of Food Induced Diseases, University of Naples Federico II, Naples, Italy.

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Short title: Butyrate derivative reduces colitis in mice

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*To whom correspondence should be addressed: Rosaria Meli, via D. Montesano, 49-80131, Naples, Italy, +39-081-678413, fax +39-081-678403; E-mail: meli@unina.it; Raffaele Simeoli, Wolfson CARD, King's College London, Guys' Campus, London Bridge SE1 1UL, United Kingdom, +44 7539007146; E-mail: raffaele.simeoli@kcl.ac.uk

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Abstract

Background and purpose: Butyrate has shown benefits in inflammatory bowel diseases (IBD). However, its oral administration is infrequent due to rancid smell and unpleasant taste. The efficacy of a more palatable butyrate-releasing derivative, N-(1-carbamoyl-2-phenylethyl) butyramide (FBA), was evaluated in a mouse model of colitis induced by dextran sodium sulphate (DSS).

Experimental approach: Male 10-week-old BALB/c mice received DSS (2.5%) in drinking water (for 5d) followed by DSS-free water for 7d (DSS group). Oral FBA administration (42.5 $mg \cdot kg^{-1}$) started 7d before DSS as preventive (P-FBA), or 2d after DSS as therapeutic (T-FBA), and both treatments lasted at 19d. One DSS-untreated group received only tap water (CON) for totally 4 groups.

Key results: FBA treatments reduced colitis symptoms and colon damage. P-FBA and T-FBA significantly decreased polymorphonuclear cell infiltration score compared to the DSS group. FBA revert the imbalance between pro- and anti-inflammatory cytokines (reducing inducible NOS protein expression, chemokine (C-C motif) ligand 2 and IL-6 transcripts in colon and increasing TGF-β and IL-10). Morever, P-FBA and T-FBA limit neutrophil recruitment (by expression and localization of the neutrophil granule protease Ly-6G), restore deficiency of butyrate transporter and improve intestinal epithelial integrity, preventing tight-junction impairment (zonulin-1 and occludin). FBA, such as its parental compound sodium butyrate, inhibits histone deacetylase-9 and restores H3 histone acetylation, exerting an anti-inflammatory activity through NF-κB inhibition and PPAR-γ up-regulation.

Conclusions and implications: FBA reduces inflammatory intestinal damage in mice indicating its potential as post-biotic derivative, to overcome the limits of sodium butyrate's oral administration.

Key words: DSS colitis, short chain fatty acids, postbiotic, Annexin A1, HDAC-inhibitor, NF-κB, PPAR-γ, tight-junctions.

LIGANDS
Annexin I
Butyrate
CCL2
IL-6
IL-10
TGF-b

TARGETS	
Other protein targets[a]	**Enzymes**[d]
TNF-α	HDAC9
GPCRs[b]	iNOS
FFA2 receptor	MPO
FPR1	**Nuclear hormone receptors**[e]
FPR2	PPAR-γ
Transporters[c]	
MCT-1	

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d,e] Alexander et al., 2015a,b,c,d,e).

Abbreviations used: Anx-A1 and *Anxa1*, Annexin A1; *Ccl2*, chemokine (C-C motif) ligand 2; CON, control; DSS, dextran sodium sulphate; *Ffar2*, free fatty acid receptor 2; *Fpr1/2*, formyl peptide receptor 1 and 2; GPR43, G-protein coupled receptor 43; HDAC9, histone deacetylase 9; IBD, inflammatory bowel disease; *Il6* and *Il10*, interleukin 6 and 10; Ly-6G and *Ly6g*, lymphocyte antigen 6; MCT-1, monocarboxylate transporter; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; iNOS, inducible nitric oxide synthase; *Ocln*, occludin; P-B, sodium butyrate as preventive treatment; P-FBA, N-(1-carbamoyl-2-phenylethyl) butyramide as preventive treatment; PMNs, polymorphonuclear cells; PPAR-γ, peroxisome proliferator-activated receptor gamma; SCFAs, short-chain fatty acids; *Slc16a1*, solute carrier family 16 (monocarboxylic acid transporters), member 1; *Tgfb*, transforming growth factor-β; T-B, sodium butyrate as therapeutic treatment; T-FBA, N-(1-carbamoyl-2-phenylethyl) butyramide as therapeutic treatment; *Tnf*, tumour necrosis factor-α; Tregs, regulatory T cells; TJ, tight junction; *Tjp1*, tight junction protein 1; UC, ulcerative colitis; ZO-1, zonula occludens 1.

Introduction

Host-microbial homeostasis requires appropriate immune regulation within the gut mucosa, preventing uncontrolled immune responses against the beneficial commensal microbiota, which could potentially lead to inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) (Geuking et al., 2014). Several studies indicate that products of bacterial metabolism, such as short-chain fatty acids (SCFAs), may modulate the immune response of the host (McDermott & Huffnagle, 2014). In particular, butyrate produced by intestinal microbial fermentation of undigested resistant starches and dietary fibres, is absorbed by the colonic cell and extensively metabolized, constituting the main source of energy. Many intestinal and extra-intestinal effects are ascribed to butyrate (Canani et al., 2011), showing its possible therapeutic indications in gastroenterology. To date, several studies have evaluated butyrate effectiveness in several animal model of UC (Mishiro et al., 2013; Vieira et al., 2012). In humans few studies have been performed probably due to low compliance with the oral route (for its rancid taste) or rectal enemas administration (for its cumbersome application to the patient and irritability due to acid property). Moreover, rectal administration of butyrate or mixture of SCFAs did not show beneficial effects or displayed only trends towards clinical amelioration (Breuer et al., 1997; Hamer et al., 2010; Scheppach et al., 1992; Steinhart et al., 1996; Vernia et al., 2003). The discrepancy in human studies using enemas may be due to differences in treatment, duration, use of butyrate alone or mixture of SCFAs enemas, and use of several concentrations and volumes of these mixtures. Conversely, other studies reported that fermentable dietary fiber supplementation, which resulted in increased fecal butyrate levels, was effective in maintaining remission in UC, revealing a significant improvement in clinical and inflammatory aspects (Fernandez-Banares et al., 1999; Hanai et al., 2004; Wedlake et al., 2014).The importance of butyrate supplementation has been demonstrated by the impaired butyrate metabolism in intestinal

inflamed mucosa of patients affected by IBD (De Preter et al., 2012). In fact, data show that butyrate deficiency results from the reduction of butyrate uptake by the inflamed mucosa due to down regulation of the monocarboxylate transporter (MCT)-1 expressed on the apical membrane of intestinal epithelium (Thibault et al., 2007). Particularly, the reduction of the intracellular availability of butyrate in colonic cells may decrease its protective effects toward cancer in IBD patients (Thibault et al., 2010).

GPR43 is a G-protein-coupled receptor expressed in colonic epithelium, adipose tissue and immune cells (Bindels et al., 2013) and together with GPR109A are considered the main butyrate targets involved in suppression of colonic inflammation and carcinogenesis (Singh et al., 2014). Moreover, butyrate modulates histone acetylation, as histone deacetylase (HDAC) inhibitor, and alters host epigenome, leading to its epigenetic mechanism (Canani et al., 2011; Hamer et al., 2008).

On the basis of all its characteristics, butyrate can be considered a postbiotic being a non-viable bacterial metabolic product obtained from probiotic microorganisms that have biologic activity in the host. The purpose of this study was to investigate the efficacy of more palatable butyrate-releasing compound, the N- (1-carbamoyl-2-phenyl-ethyl) butyramide (FBA), in dextran sodium sulphate (DSS)-induced colitis model as innovative post-biotic derivative. Our hypothesis is that FBA similarly or better than its parental compound (sodium butyrate) is able to reduce colon imflammation and colitis symptoms by decreasing neutrophils recruitment and the production/release of pro-inflammatory mediators in the colonic mucosa following DSS exposure. The mechanisms behind these effects could be related to the restoration of butyrate transporter, PPAR-γ and tight junctions in colon tissue together with the HDAC9/NF-κB axis inhibition.

Methods

Reagents and diet. Prof. Antonio Calignano provided FBA (International application patent with publication number WO2009130735), whose synthesis and characterization was previously reported (Mattace Raso et al., 2013). FBA is stable to acids and alkalis and capable of releasing butyric acid at small and large bowel level in a constant manner over time. Interestingly, FBA does not present the unpleasant odour of butyrate and, being tasteless, overcomes the poor palatability of butyrate that often reduces the therapy compliance. All chemicals, included sodium butyrate, were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The standard laboratory unpurified diet was purchased from Harlan Teklad. The 2018 Teklad Global Protein Rodent Diet contained the following: water, 120 $g \cdot kg^{-1}$; protein, 185 $g \cdot kg^{-1}$; fat, 55 $g \cdot kg^{-1}$; fiber, 45 $g \cdot kg^{-1}$; ash, 60 $g \cdot kg^{-1}$; minerals, 0.13 $g \cdot kg^{-1}$ (containing 50 mg ferrous $Fe \cdot kg^{-1}$ and 44 mg $Mn \cdot kg^{-1}$); and vitamin mix, 0.52 $g \cdot kg^{-1}$ (containing 81 mg vitamin $E \cdot kg^{-1}$).

Induction of colitis and treatments. Experimental colitis, was induced in 10-week-old BALB/c AnNHsd male mice (25±2 g) (Harlan-Corezzano, Italy) by 2.5% wt:vol DSS (36-50 kDa, MP Biomedical) in drinking water *ad libitum* from day 7 until 12 followed by DSS-free water from day 13 until day 19 (end of experimental protocol). Mice were randomly divided into the 4 following groups (n=10 per group): 1) control mice (CON group); 2) mice receiving DSS (DSS group); 3) DSS-fed mice receiving FBA as preventive therapy (P-FBA); 4) DSS-fed mice receiving FBA therapeutically (T-FBA). Untreated CON and DSS groups received tap water by gavage as vehicle.

In addition, we decided to set up two further groups of DSS-fed mice treated with its parental compound sodium butyrate (B): 5) DSS-fed mice receiving sodium butyrate as preventive therapy (P-B); 6) DSS-fed mice receiving sodium butyrate therapeutically (T-B). FBA and

sodium butyrate were given daily. Sodium butyrate (B, 20 mg·kg^{-1}) or FBA (42.5 mg·kg^{-1}, the equimolecular dose of B) was administered by gavage, and the treatment started 7 d before (preventive) or 2 d after (therapeutic) DSS challenge, continuing for all experimental period. We used the parental compound in the evaluation of body weight, disease activity index, colon length, histopathologic score, survival rate and mechanistic studies to confirm the similar profile of FBA as butyrate-releasing derivative. Mortality rate was assessed during the entire experimental time (from day 1 to day 19) and a Kaplan-Meier survival curve was calculated (Supplemental Figure 1). All procedures involving animals and their care were conducted in conformity with international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept) and were approved by the Institutional Committee on the Ethics of Animal Experiments (CSV) of the University of Naples "Federico II" and by the Ministero della Salute under protocol no. 0022569-P-20/12/2010. At day 19, following an overnight fast, animals were killed by an intraperitoneal injection of a mixture of ketamine/xylazine followed by cervical dislocation.

Evaluation of experimental colitis. In all mice, weight, presence of blood, and gross stool consistency were determined daily as previously described (Dieleman et al., 1997). Each score was determined as follows: 1) change in weight (0: weight loss <1% compared to the starting weight, 1: weight loss between 1 and 5%, 2: weight loss between 6 and 15%, 4: weight loss >15%); 2) stool blood (0: negative, 2: positive, 4: gross bleeding); and 3) stool consistency (0: normal, 2: loose stools, 4: diarrhea) as previously described (Cooper et al., 1993). Briefly, the disease activity index was determined by combining the scores from these 3 categories and dividing that number by 3.

Histological analysis and scoring of colon sections. At day 19, after mice euthanasia, tissues were collected and colon length was measured. Distal sections were stored in formalin 10% or O.C.T for histological and immunofluorescent analyses. Following H&E staining, colon sections were analyzed in blinded manner for the evaluation of the histopathological score as previously described (Chang et al., 2014).

Real-time PCR. Total RNA isolated from colon was extracted using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. cDNA was synthesized using a Maxima First Strand cDNA Synthesis Kit (Fermentas) from 2 μg total RNA. PCRs were performed with Bio-Rad CFX96 Connect Real-time PCR System and software (Bio-Rad Laboratories). The primer sequences for target genes and PCR conditions are reported in Supplemental Table 1.

Serum TNF-α determination. At day 19, mice were euthanized and blood was collected by cardiac puncture. Sera were obtained by centrifugation at 1500 x g at 4°C for 15 min, and stored at -70°C. TNF-α levels (pg/ml) were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA) kits for mice from BD Pharmingen, according to the manufacturer's instructions.

Western blotting. Colon tissue was homogenized and protein lysates were subjected to SDS-PAGE as already described (Simeoli et al., 2015). The filters were probed with primary antibody overnight. To evaluate nuclear factor-κB (NF-κB) activation and histone H3 acetylation, NF-κB p50 (Cell Signaling Technology), Acetyl-H3 and H3 (EMD Millipore) were measured in nuclear extracts. iNOS (Cayman Chemicals) protein expression was evaluated in whole colon lysates. The blot was developed using enhanced chemiluminescence

detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and the immune complex visualized by Image Quant. The protein bands were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad Laboratories). Western blots for Lamin A and GAPDH (Sigma-Aldrich) were performed to ensure equal sample loading in nuclear and whole lysates, respectively.

Measurement of myeloperoxidase (MPO) activity. Proximal colonic tissues were homogenized according to (Bradley et al., 1982). The homogenates were assessed for myeloperoxidase (MPO) activity as previously described (Smith & Castro, 1978). MPO was expressed as units/mg protein with 1unit hydrolyzing 1 μmol H_2O_2/min.

Immunofluorescence analysis of Ly-6G and Annexin A1. Colon samples for immunofluorescence were embedded in O.C.T. (PelcoCryo-Z-T, Ted Pella Inc), and cryosectioned (10 μm thick). Tissue sections were then fixed in 4% paraformaldehyde for 10 min at room temperature (RT). To examine co-localization of Anx-A1(Annexin A1) with Ly-6G (lymphocyte antigen 6), sections were blocked and then incubated with a monoclonal antibody anti Ly-6G FITC (BD Biosciences) and a rabbit anti-AnxA1 antibody overnight at 4°C. Sections were then incubated with Alexa-Fluor® 546 goat anti-rabbit IgG (Invitrogen) for AnxA1 1h at RT. After incubation with secondary antibody, sections were incubated with DAPI to visualize nuclei. Fluorescence was visualized with Olympus BX51 fluorescence microscope (Olympus) equipped with a DS-QiMc monochromatic camera (Nikon) and X-Cite® Series 120Q Xenon lamp. NIS-Elements BR3.1 software (Nikon) was used for all analyses. Merge images were performed with ImageJ® software. Two negative controls were used: slides incubated with or without primary antibody. Images were recorded at identical

gain settings, and performed in duplicate in non-serial distant sections. Four image fields were taken of each section.

Immunofluorescence analysis of Occludin and Zonula occludens (ZO)-1. Colon segments were fixed in 10% formalin and embedded in paraffin. 7 µm sections were deparaffinised in decreasing ethanol percentage and antigens were unmasked. After antigens retrieval, sections were permeabilized in Tris-buffered saline (TBS) plus 0.1% Triton X-100. After non specific background blocking sections were incubated with anti-occludin or anti-ZO-1 (1:50 for occludin Santa Cruz Biotechnology and 1:100 for ZO-1 Invitrogen). Sections were probed with secondary Alexa Fluor® 488 antibody (1:200, Invitrogen Corporation). Slides were visualized on a fluorescence microscope and images were stored digitally with Leica software. Two negative controls were used: slides incubated with or without primary antibody. The quantitative measurements of immunofluorescence analysis for Occludin and ZO-1 was performed and revealed by Integrated Pixel Intensity using an ImageJ® software.

Statistical analysis. All data are presented as means ± SEMs. The statistical analyses were performed with the use of Graph-Pad Prism (Graph-Pad Software). For all the experimental data, we evaluated group differences with one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. For the analysis of the body weight changes during the entire experimental period, we used two-way ANOVA followed by Tukey multiple comparison test, setting as variables the treatment and the time. Survival study was analyzed using the Kaplan-Meier log-rank test. Statistical significance was set at $P<0.05$.

Results

FBA similarly to butyrate reduces disease activity index and colon tissue damage. The oral treatment with P-FBA and T-FBA significantly reduced the development of colitis evaluated by disease activity index (DAI) at day 12 and 18 compared to DSS group (Figure 1A). A similar effect was observed even when DSS-mice were treated with butyrate as preventive or therapeutic therapy.

Beneficial effects of FBA more then butyrate were evident also in the excised colon samples, revealing an improvement in the tissue shortening induced by DSS (Figure 1B). Control colon sections stained with H&E showed intact epithelium, well-defined crypt length, no edema and neutrophil infiltration in mucosa and *submucosa*, and no ulcers or erosions (Figure 1C). In contrast, colon tissue from DSS group showed severe inflammatory lesions throughout the mucosa and loss of crypt architecture. Both FBA and butyrate were able to protect colonic mucosa structure, ameliorating mucosa integrity and crypt structure and improving epithelial surface compared to DSS group (Figure 1C). The beneficial effects mediated by FBA and butyrate either as preventive and therapeutic schemes, were also confirmed by evaluation of histopathological score performed on distal colon sections stained with H&E (Figure 1D).

The mortality rate and body weight were also monitored during all the experimental period (Supplemental Figure 1 and 2).

Effects of FBA on neutrophil infiltration in colonic mucosa. We determined the expression and localization of the neutrophil granule protease Ly-6G in colon tissue by fluorescence microscopy (Figure 2A). To verify if protective effect of FBA was also associated with modulation of pro-resolving factors, such as Annexin A1, double-staining

immunofluorescence was performed, revealing high Anx-A1 levels in neutrophils, confirmed by a marked co-localization with Ly-6G in colon of mice with active disease. Both FBA schemes were able to counteract neutrophil infiltration induced by DSS-challenge reducing the Ly-6G and Anx-A1 staining (Figure 2A). This effect was also quantitatively demonstrated since polymorphonuclear cells (PMNs) infiltrating score was lower in P-FBA and T-FBA colonic mucosa compared to DSS group (Figure 2B). Similarly, FBA, either as preventive or therapeutic treatment, contrasted DSS-mediated effects reducing *Anxa1* mRNA transcripts (Figure 2C). Furthermore, in both FBA-treated groups, activity of MPO, a lysosomal hemoprotein found in the azurophilic granules in neutrophils (Figure 2D), and *Ly6g* mRNA levels (Figure 2E) were significantly less than in the DSS group.

Effect of FBA on neutrophil markers and GPR43 expression in colonic mucosa. Butyrate can influence chemotaxis of immune cells through GPR43 receptor (Bindels et al., 2013). Therefore, we analysed mRNA levels of this receptor in colon. As depicted in figure 3A, FBA, especially when used as therapeutic, was able to increase significantly mRNA levels for *Ffar2* (free fatty acids receptor-2, which encode for GPR43) compared to control and DSS groups. In contrast, FBA, either as preventive or therapeutic treatment, counteracted DSS-mediated effects reducing *Fpr1* and *Fpr2* mRNA levels (Figure 3B-C). These data highlight the involvement of GPR43 in neutrophil recruitment during colon inflammatory condition and confirm that FBA was able to reduce PMN infiltration compared to DSS group.

FBA modulates pro-and anti-inflammatory mediators in colon and serum. Protein expression of inducible nitric oxide synthase (iNOS), which is responsible for NO production, and mRNA levels of *Ccl2* gene, which encode monocyte chemoatractant protein (MCP)-1, were greater in the colon of DSS challenged mice rather than those of control group (Figure 4A

and 4B). Similarly, *Tnf* and *Il6* mRNA levels were greater in DSS group than in CON (Figure 4C-D). Conversely, the transcriptional levels of anti-inflammatory cytokines *Il10* and *Tgfb* resulted impaired by DSS (Figure 4E-F). FBA treatments, similarly reduced proinflammatory iNOS and *Ccl2*, *Tnf* and *Il6* and at least in part restored *Il10* and *Tgfb* transcripts in colon tissue compared to DSS group. Moreover, we also analysed the TNF-α levels in serum (n=5, each group) to confirm the systemic anti-inflammatory properties of FBA and butyrate (CON=1,67±0,34 pg/ml and DSS=28,74±4,57, P<0.001; P-FBA=15,22±3,85 and T-FBA=12,89±2,76, P<0.05 *Vs* DSS, respectively).

FBA preserves MCT-1 transporter and epigenetically counteracts inflammation induced by DSS. DSS-challenged mice showed lower mRNA levels of the monocarboxylic acid carrier, the solute transporter family 16 *Slc16a1*, than the CON group (figure 5A). This reduction was preserved only by therapeutic treatments with FBA or butyrate. To determine the mechanisms involved in butyrate effect, the modulation of several transcription factors and histone acetylation were analyzed. DSS challenge significantly reduced the PPAR-γ (*Pparg)* mRNA (Figure 5B) and increased nuclear NF-κB p50 protein (Figure 5D) compared to control group. These effects were counteracted by preventive and therapeutic treatments with FBA. We also assessed the FBA capability to normalize the transcriptional levels of a specific member of class IIA HDAC involved in the pathogenesis of colitis in mice (de Zoeten et al., 2010), the HDAC9, which resulted impaired in DSS animals. As shown in figure 5C, FBA, such as sodium butyrate, counteracted the DSS-induced up-regulation of *Hdac9* mRNA levels. However, FBA when used as therapeutic scheme was able to significantly increase histone H3 acetylation confirming HDAC inhibition (Figure 5E). Interestingly, FBA and butyrate did not modify histone 3 (H3) acetylation when used as preventive treatment.

Effects of FBA on intestinal barrier integrity and tight-junction expression. To evaluate barrier integrity, we determined the distribution of two tight junction proteins (TJs), occludin and ZO-1, in distal colon. Staining for occludin (Figure 6A) and ZO-1 (Figure 6C) in colonic mucosa of DSS-fed mice displayed less intensity than CON group, as confirmed even by the integrated pixel intensity analysis for occludin and ZO-1 (Figure 6B and D, respectively). However, compared to DSS group, P-FBA and T-FBA significantly restored the distribution of occludin and ZO-1, through colonic mucosa with a continuous staining pattern. These effects were highlighted by the evaluation of mRNA transcriptional levels for both proteins in colon sections (see Supplemental figure 3). Furthermore, both FBA-based treatments more than P-B and T-B, were able to preserve not only the TJs distribution but even the architecture of colons in DSS-fed mice similarly to that of control mice.

Discussion

In this study, we demonstrated that FBA, a butyrate-releasing derivative, protects mice from DSS-induced colon injury, by reducing inflammation and restoring epithelial barrier integrity. The effects of SCFA, in particular butyrate, in intestinal diseases and their role on colonic functions are known (Canani et al., 2011; Hamer et al., 2008; Hamer et al., 2009). However, despite the wide spectrum of possible indications, the major limits of butyrate in clinical practice are its unpleasant taste and odour, when orally administered, or discomfort, by rectal preparations. Even if dietary fiber intake, leading to SCFA production, has shown benefits in IBD (Hanai et al., 2004; Wedlake et al., 2014), other data had demonstrated an inverse association between intake of fruits and vegetables and risk of IBD (Amre et al., 2007) and more recently dietary intake and risk of developing IBD have been reviewed (Ananthakrishnan et al., 2013; Hou et al., 2011). Very recently the rational identification of diet-derived postbiotics in restoring intestinal microbiota composition and function has been reviewed (Klemashevich et al., 2014). For all these reasons the use of postbiotics, such as butyrate, may be potential alternative to the use of live probiotic organisms or dietary fiber intake as prebiotics. In fact, the beneficial effects of sodium butyrate in different models of DSS-induced colitis in mice have been already reported after oral or topical administration (Mishiro et al., 2013; Vieira et al., 2012). Recently, we investigated the role of butyrate and FBA in pain behaviour, identifying different and converging non-genomic and genomic mechanisms of action, which cooperate in nociception maintenance (Russo et al., 2016). Notably, we found a major activity of both compounds on inflammatory visceral pain probably due to butyrate physiological role in gut and to the possible elevated number of its transporters (i.e. MCT1) involved in its absorption.

Here, we tested oral FBA efficacy in DSS colitis which leads to a significant loss of body weight, associated to diarrhea, rectal bleeding and colon shortening (Yan et al., 2009). These macroscopic and pathologic changes were counteracted by FBA, similarly to sodium butyrate, especially when used as therapeutic protocol.

The role of immune cells infiltration in the inflammatory response during the development of colitis was already assessed, as well as the efficacy of butyrate supplementation in limiting myeloid and lymphoid cells recruitment into the colonic mucosa (Tsou et al., 2007). According with these findings, FBA reduced DSS-induced PMN infiltration in colonic mucosa, decreasing MPO activity and *Ly6g* mRNA levels. Ly-6G, reacting only in neutrophils, is a very useful marker to detect specifically cells of the neutrophil lineage (Tsou et al., 2007). Dense neutrophil infiltration and crypt abscess formation are pathological characteristics in the inflamed mucosa of UC patients (Raab et al., 1993). In fact, in Japan, granulocyte adsorption apheresis therapy had been reported to show a remarkable therapeutic effect in active UC patients (Shimoyama et al., 2001). Moreover, the faecal neutrophil-derived biomarkers, calprotectin and lactoferrin, represent an ideal non-invasive test for detecting intestinal inflammation (Sipponen, 2013). These findings strengthen the pivotal role of neutrophils in the pathogenesis of UC. Moreover, trans-epithelial migration of PMNs from the microcirculation to the mucosa results in impaired barrier function and tissue destruction (Nusrat et al., 1997).

Among SCFAs, butyrate can influence chemotaxis of immune cells through GPR43 receptor but this effect depends on the type of immune cells and butyrate concentration (Maslowski et al., 2009; Sina et al., 2009). Here, *Ffar2* mRNA expression was increased by FBA alongside of the reduction of *Ly6g* mRNA transcripts mediated by both FBA-based treatments. These data confirm the involvement of GPR43 in neutrophil recruitment during inflammation and, at the same time, display FBA ability in reducing PMN infiltration. Therefore, we

hypothesize that, when neutrophil recruitment is reduced by FBA treatments, GPR43 could be expressed on other cell populations, e.g. intestinal enteroendocrine L and epithelial cells, which are involved in intestinal barrier integrity.

We also assessed if protective effect of FBA was associated with modulation of pro-resolving factors, such as Annexin A1 and its receptors, Fpr1 and Fpr2. Annexin A1, a member of the superfamily of annexins, is a downstream mediator of glucocorticoids action (Gerke et al., 2005). In resting conditions, neutrophils, monocytes and macrophages constitutively contain high levels of Annexin A1 in their cytoplasm (Mulla et al., 2005; Perretti et al., 2000), that are promptly secreted following cell activation (Perretti et al., 1996). Increased expression and secretion of Annexin A1 has been reported to occur in inflamed mucosal tissues in rodents and humans (Vergnolle et al., 1995; Vergnolle et al., 2004). In particular, increased Anx-A1 was observed in the intestinal epithelium and infiltrating leukocytes in the mucosa of UC patients compared with normal intestinal mucosa (Leoni et al., 2013). Anx-A1 has also been reported to inhibit neutrophil influx and promote neutrophil apoptosis at the site of resolving inflammation (Perretti et al., 1996). In our experiments, a co-localisation between Ly-6G and Anx-A1 positive cells was obtained: FBA reduced Anx-A1 levels as well as Ly-$6G^+$ cells. Moreover, both schemes of treatment with FBA reduced the transcriptional levels of *Fpr1* and *Fpr2*. Therefore, in these settings Anx-A1 and its receptors seem to be a marker of neutrophil infiltration.

Our data clearly demonstrate that the preventive and therapeutic treatments with FBA corrected the imbalance between pro- and anti-inflammatory mediators reducing iNOS protein expression and *Ccl2*, *Tnf*, and *Il6* transcripts in colon tissue; at the same time both FBA protocols recovered, at least in part, mRNA levels of *Tgfb* and *Il10*. Consistently, previous data showed the reduction of several pro-inflammatory mediators in colon *lamina propria* macrophages by butyrate (Chang et al., 2014).

Butyrate regulates epigenetically gene expression by inhibiting HDAC, specifically class IIA and I (Steliou et al., 2012), and its anti-inflammatory effects are related to this mechanism in many cell types (Chang et al., 2014). In particular class IIA HDAC has been reported to suppress regulatory T cells (Tregs) expansion (Smith et al., 2013) and the inhibition of HDAC9 increases Treg function, reducing colitis in mice (Glauben et al., 2006). Due to HDAC inhibition, butyrate can avoid NF-κB activation in human colonic epithelial cells (CECs) (Segain et al., 2000). NF-κB regulates many cellular genes involved in early immune inflammatory response frequently dysregulated in IBDs (Schwab et al., 2007). Here, FBA reproduced the same effect of butyrate limiting the *Hdac9* transcript up-regulation induced by DSS challenge. Accordingly, FBA, especially when used as therapeutic treatment, inhibits NF-κB activation and promotes histone H3 acetylation.

Moreover, we demonstrate *in vivo* model that FBA restored *Pparg* transcription in colonic mucosa, confirming previous *in vitro* data displaying PPARγ involvement in butyrate anti-inflammatory activity (Schwab et al., 2006). Genetic ablation of PPARγ resulted in increased susceptibility to experimental colitis in mice (Dubuquoy et al., 2006) and PPARγ protein expression is 60% lower in the inflamed colonic mucosa of UC patients than in controls (Dubuquoy et al., 2003). Previous data have shown that PPARγ can inhibit NF-κB activation and cytokine expression in monocytes and CECs (Desreumaux et al., 2001).

Interestingly, we also observed a strong reduction of MCT1 transporter in colonic mucosa of DSS mice, highlighting an impairment of butyrate uptake. MCT1 plays an important role in the absorption of butyrate by the colonocytes (Cuff et al., 2002) and previous data report that butyrate stimulates MCT1 promoter activity in Caco-2, IEC-6 and in rat intestinal mucosa (Borthakur et al., 2012; Borthakur et al., 2008). Both therapeutic FBA and butyrate preserved *Slc16a1* down-regulation, normalizing its transcriptional levels. These results may have translational potential, since down-regulation of MCT1 in IBD patients could result in

butyrate uptake deficiency (Thibault et al., 2010). We hypothesize that in our experimental conditions the stronger effect of FBA could be related not only to MCT1 induction by butyrate released from FBA, but also to the capability of undissociated FBA to interact and be carried by several members of SLCs, such as those of phenyl-alanine.

TJ alteration in UC results in impaired barrier function, which may lead to increased uptake of luminal antigens and/or adjuvant that overcome the net suppressive tone of the mucosal immune system. SCFAs modulate key epithelial cell functions that help to maintain intestinal epithelial barrier integrity preventing injury (Peng et al., 2009). Analysis of the distribution and intensity of occludin and ZO-1 staining and mRNA confirmed the beneficial effect elucidated by both butyrate-based compounds. Gut permeability integrity is essential to limit bacterial translocation and preserves mucosal immune homeostasis. In fact, damage- and pathogen-associated molecular patterns penetration determines a strong recruitment of immune cells in impaired mucosa causing subsequent inflammation.

In conclusions, FBA treatments confirm and improve the beneficial effect of butyrate at intestinal level, counteracting colon inflammation, neutrophil recruitment and alteration of intestinal permeability in DSS-induced colitis model. Indeed, our data indicate the potential clinical utility of FBA as preventive or therapeutic strategy for UC, as an optimization of a direct “postbiotic” approach, diversifying this treatment from prebiotic (i.e. fibers) or probiotic (i.e. bacteria) one. Since this synthetic derivative of butyrate does not have the characteristic odour of rancid cheese, it may represent a viable alternative to butyrate, favouring a better oral compliance and a greater effectiveness.

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Conflicts of interest

All authors have not conflicts of interest.

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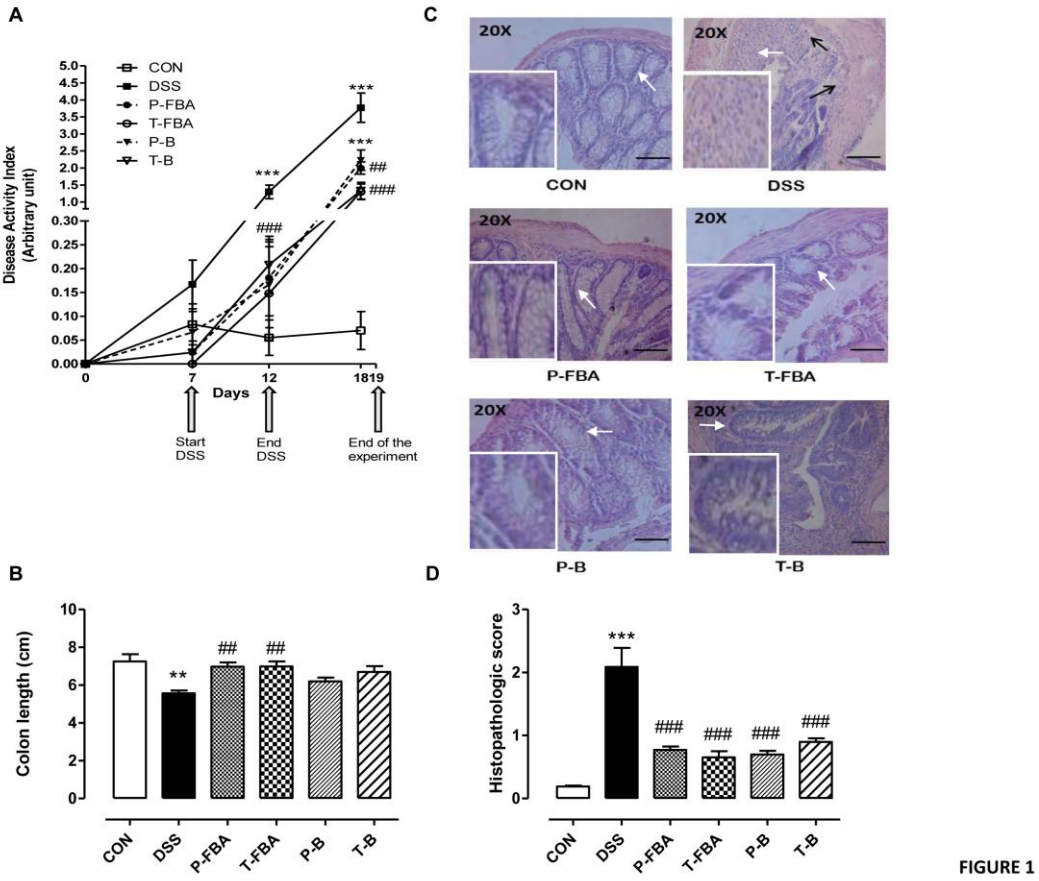

Figure 1

Effects of FBA and butyrate on DSS-induced colitis. (A) Disease activity index (DAI) on day 0, 7, 12 and 18. (B) Colon length expressed in cm. (C) Distal colons were stained with H&E. Scale bar:100 µm. White arrows indicate areas shown in the inset squares (objective 40X). Black arrows indicate infiltrated cells in the *submucosa*. (D) Histopathological scores were determined in a blinded fashion. Colons were excised at day 19. Data are means ± SEMs, *n*=8. ** $P<0.01$ and *** $P<0.001$ *Vs* CON; ## $P<0.01$ and ### $P<0.001$ *Vs* DSS.

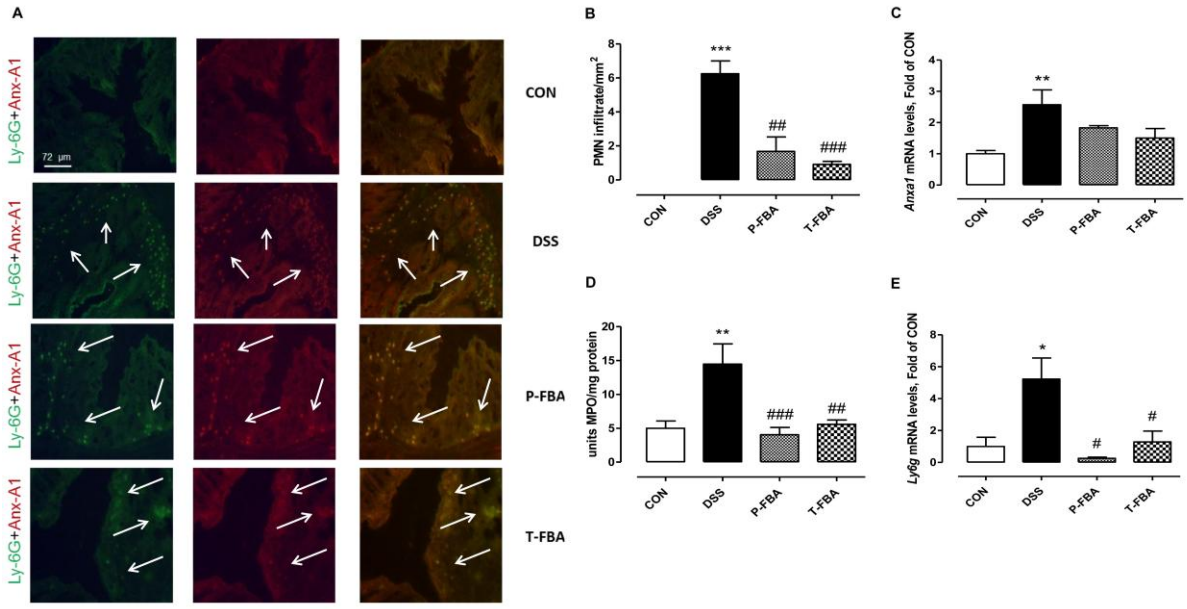

FIGURE 2

Figure 2

FBA reduces PMN infiltration in colonic mucosa. (A) Double-staining immunofluorescence detection of Anx-A1 and Ly-6G (Ly-6G+Anx-A1 yellow staining) in DSS compared to control and FBA groups. White arrows indicate PMN infiltrate. (B) PMN infiltration score was obtained by counting Ly-6G$^+$ cells in four random mucosal and submucosal views of three different sections from the descendent colon and was expressed as number of cells·mm^{-2}. (C) Real-time PCR of *Anxa1* is shown. (D) MPO activity measurement and *Ly6g* mRNA levels (E) are also reported. Real-Time data are presented as means ± SEMs, n=8. * P<0.05, ** P<0.01 and *** P<0.001 *Vs* CON; # P<0.05, ## P<0.01 and ### P<0.001 *Vs* DSS. Immunofluorescence stainings are representative of 3 slides for each group, magnification 200X.

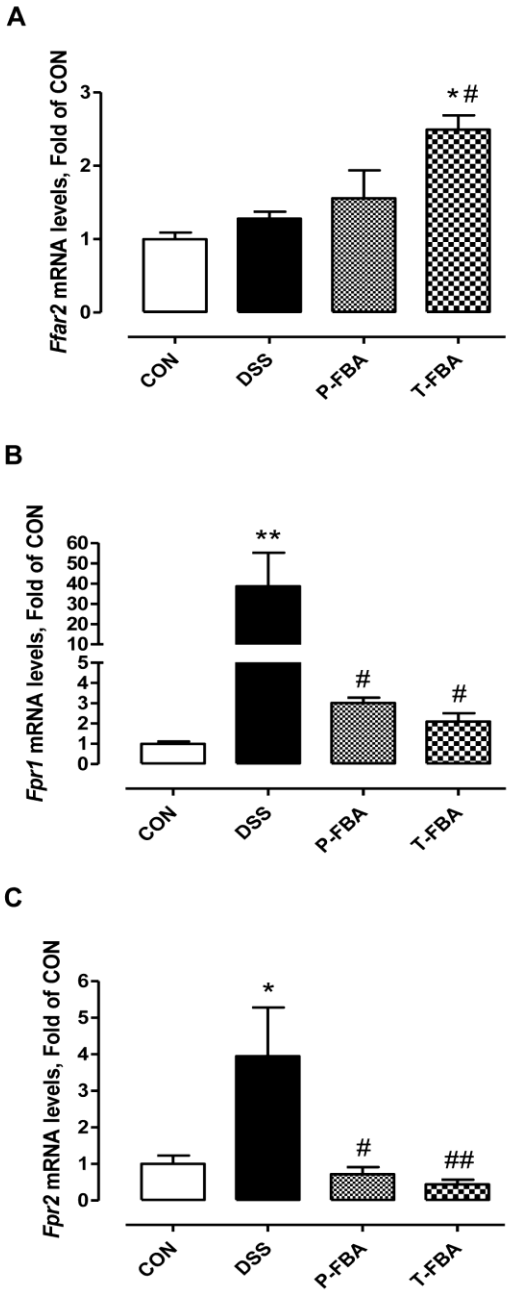

FIGURE 3

Figure 3

Effect of FBA on GPR43 expression and neutrophil markers in colonic mucosa. Transcriptional levels of *Ffar2* (A), *Fpr1* (B) and *Fpr2* (C) were also evaluated following treatment with FBA (preventive and therapeutic). Real-Time PCR data are presented as means ± SEMs, n=8. * P<0.05 and ** P<0.01 *Vs* CON; # P<0.05, ## P<0.01 *Vs* DSS.

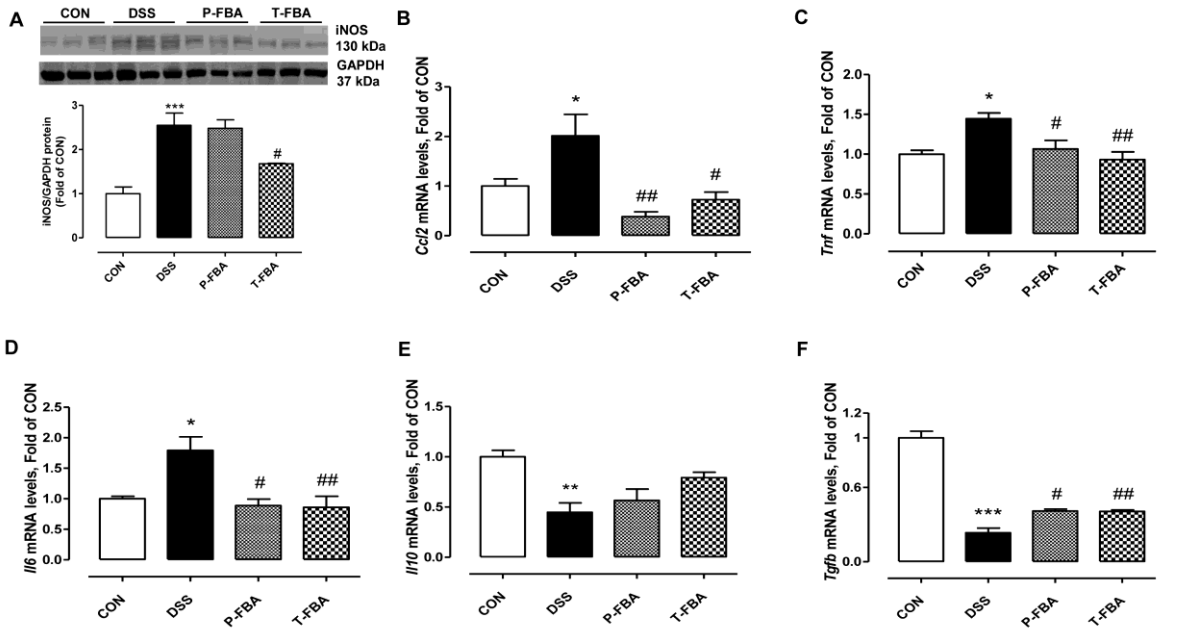

FIGURE 4

Figure 4

FBA reduces pro-inflammatory mediators and increases anti-inflammatory markers in colon tissue. (A) Western blot showing iNOS protein expression. GAPDH blot was used as equal loading control. mRNA transcriptional levels of *Ccl2* (B), *Tnf* (C) and *Il6* (D) are also shown. Real-time PCR of *Il10* (E) and *Tgfb* (F) were performed in colon from CON and DSS mice treated or not with FBA. Data are presented as means ± SEMs, *n*=8. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ *Vs* CON; # $P<0.05$ and ## $P<0.01$ *Vs* DSS.

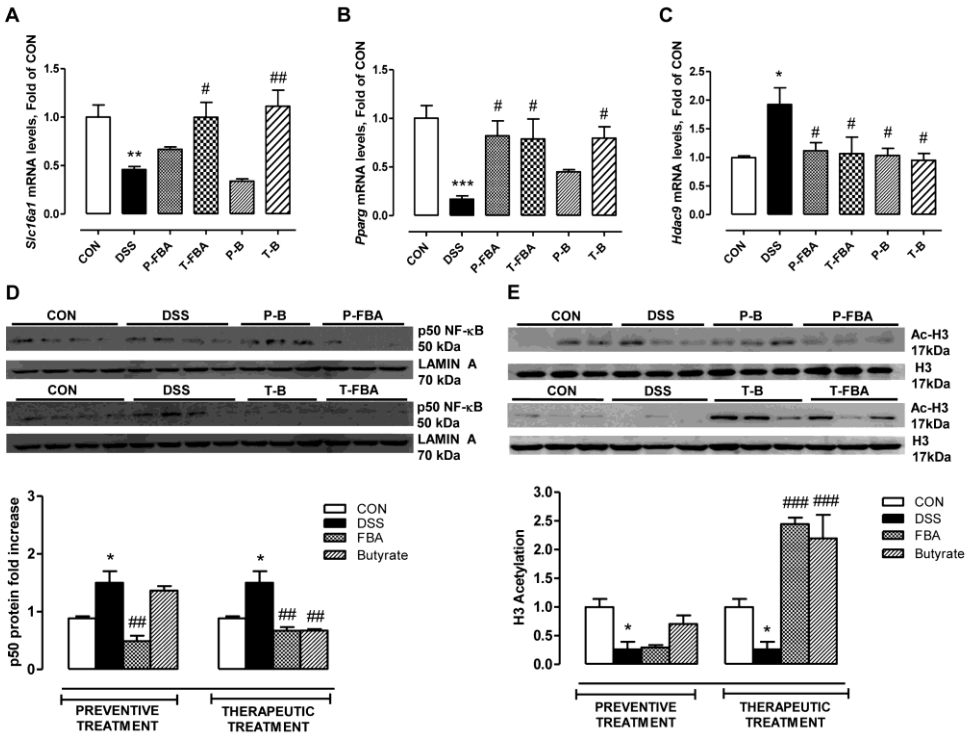

FIGURE 5

Figure 5

Mechanisms of anti-inflammatory action mediated by FBA and sodium butyrate in DSS-induced colon damage. (A) mRNA expression of *Slc16a1*, (B) *Pparg* and (C) *Hdac9* are shown. Real-Time PCR data are presented as means ± SEMs, *n*=8. (D) Western blot showing p50 NF-κB expression in nuclear extract is also reported. Both butyrate-based compounds reduced *Hdac9* transcriptional levels (C) and induced histone H3 acetylation (E). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ *Vs* CON; # $P<0.05$, ## $P<0.01$ and ### $P<0.001$ *Vs* DSS.

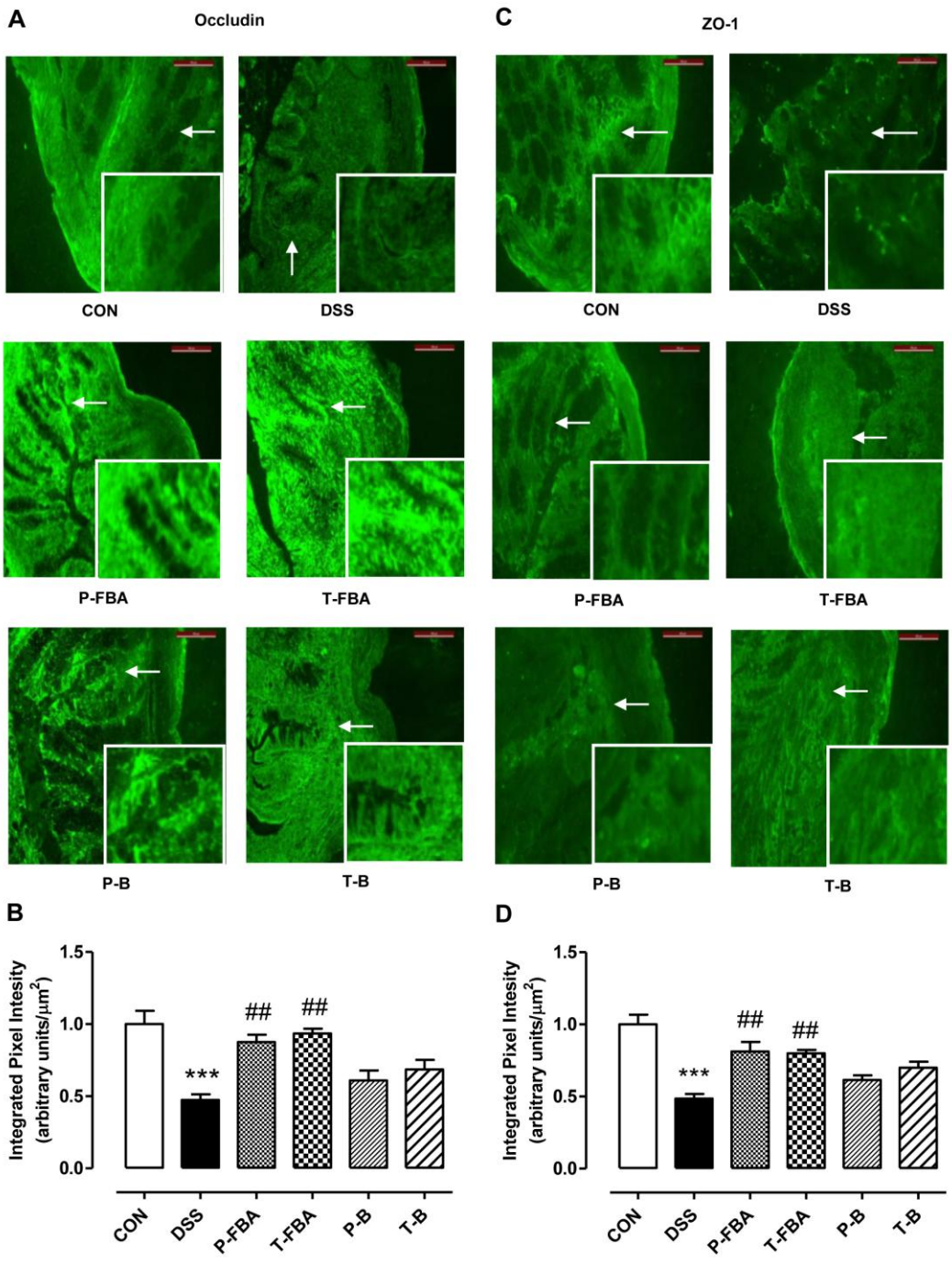

FIGURE 6

Figure 6

FBA-based compounds restore TJ-barrier function improving intestinal permeability. Immunofluorescence staining for occludin (A) and ZO-1 (C) in colons of DSS-fed mice treated or not with FBA or butyrate before or after DSS consumption is shown. Scale bar: 100 μm. White arrows indicate areas shown in the inset squares (objective 40X). Quantification of occludin (B) and ZO-1 (D) expression is reported as integrated pixel intensity/area. Data are presented as means ± SEMs, n=4. *** P<0.001 *Vs* CON; ## P<0.01 *Vs* DSS.