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The association of minocycline and the probiotic *Escherichia coli* Nissle 1917 results in an additive beneficial effect in a DSS model of reactivated colitis in mice

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ABSTRACT

Antibiotics have been empirically used for human inflammatory bowel disease, being limited to short periods. Probiotics are able to attenuate intestinal inflammation due to its immunomodulatory properties, being considered as safe when chronically administered. The aim was to test the association of minocycline, a tetracycline with immunomodulatory properties, and the probiotic *Escherichia coli* Nissle 1917 (EcN) in a mouse model of reactivated colitis. For this purpose, female C57BL/6J mice were assigned to different groups: non-colitic and dextran sodium sulfate (DSS)-control groups (without treatment), minocycline (50 mg/kg/day; p.o.), EcN (5×10^8 CFU/day; p.o.), and minocycline plus EcN treated groups. Colitis was induced by adding DSS in the drinking water (3%) for 5 days; 2 weeks later, colitis was reactivated by subsequent exposure to DSS. The inflammatory status was evaluated daily by a disease activity index (DAI); colonic damage was assessed histologically and biochemically by evaluating mRNA relative expression of different mediators by qPCR. Finally, a microbiological analysis of the colonic contents was performed. Minocycline and EcN exerted intestinal anti-inflammatory effect and attenuated the reactivation of the colitis, as shown by the reduced DAI values, being these effects greater when combining both treatments. This was evidenced histologically and biochemically, by reduced expression of TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS and MMP-9, together with increased MUC-3 and ZO-1 expression. Finally, the altered microbiota composition of colitic mice was partially restored after the different treatments. In conclusion, EcN supplementation to minocycline treatment improves the recovery of the intestinal damage and prevents the reactivation of experimental colitis.

1. Introduction

The term inflammatory bowel disease (IBD) mainly includes Crohn's disease (CD) and ulcerative colitis (UC). These complex conditions are considered as multifactorial and, although their etiology is not fully understood, it has been proposed that concurrence of genetic predisposition and an aberrant intestinal immune response to environmental factors triggers the disease [1]. Among the latter, intestinal microbiota seems to play a key role, and different studies have suggested that these bacteria may represent the stimulus for the chronic inflammation that occurs in IBD [2]. The pathogenic mechanisms proposed to explain this include persistent infections with a specific pathogen and increased exposure to normal luminal flora that lead to a dysbiosis [3]. In fact, it has been reported that there is a marked reduction in the diversity of the microbiota, both in CD and UC, when compared with healthy subjects [4–7]. As a result, an imbalance in normal microbial population takes place, with an overabundance of potentially pathogenic bacteria and/or a loss of potentially protective bacteria [8,9]. Despite the relevance of the gut microbiota in IBD, therapy has been mainly focused on suppressing the immune system rather than on restoring the composition of the altered gut microbiota, a strategy that may be achieved through the use of antibiotics or the administration of probiotics or prebiotics [3].

Antibiotics have been long used in the treatment of human IBD, but the results obtained have been conflicting up to date. Since no

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Table 1
Primer sequences used in real-time PCR assays in colonic tissue.

Gene	Sequence (5′–3′)	Annealing temperature (°C)
TNFα	F: AACTAGTGGTGCCAGCCGAT R: CTTCACAGAGCAATGACTCC	56
IL-1β	F: TGATGAGAATGACCTGTTCT R: CTTCTTCAAAGATGAAGGAA	55
IL-2	F: CTTCAAGCTCCACTTCAAGCT R: CCATCTCCTCAGAAAGTCCACC	60
MIP-2	F: CAGTGAGCTGCGCTGTCCAATG R: CAGTTAGCCTTGCCTTTGTTCAG	62
MCP-1	F: AGCCAACTCTCACTGAAG R: TCTCCAGCCTACTCATTG	60
ICAM-1	F: GAGGAGGTGAATGTATAAGTTATG R: GGATGTGGAGGAGCAGAG	60
iNOS	F: GGCAGAATGAGAAGCTGAGG R: GAAGGCGTAGCTGAACAAGG	55
MMP-9	F: TGGGGGGCAACTCGGC R: GGAATGATCTAAGCCCAG	60
MUC-3	F: CGTGGTCAACTGCGAGAATGG R: CGGCTCTATCTCTACGCTCTCC	62
ZO-1	F: GGGGCCTACACTGATCAAGA R: TGGAGATGAGGCTTCTGCTT	56
GAPDH	F: CATTGACCTCAACTACATGG R: GTGAGCTTCCCGTTCAGC	55

pathogen has been specifically targeted, broad-spectrum antibiotics have been the most frequently studied, mainly metronidazole and ciprofloxacin [10]. In a recent systematic review and meta-analysis about antibiotic therapy in IBD, it is concluded that some antibiotics, alone or in combination, may induce remission in active CD and UC [11]. It is interesting to note that the beneficial effect exerted by the antibiotics in the treatment of these conditions has been mainly attributed to their antimicrobial properties [12]. However, different studies have reported the ability of many of them to modulate both the innate and the adaptative immune responses by acting directly on different inflammatory cells [13,14]. In fact, the combination of both antimicrobial and immunomodulatory properties can be of great interest in the treatment of IBD, and can support the use of some antibiotics for this purpose. In particular, minocycline has been recently reported to exert intestinal anti-inflammatory effects in experimental models of rodent colitis due to its ability to combine immunomodulatory and antimicrobial properties [15,16]. Unfortunately, several studies have reported that discontinuation of antibiotic therapy results in a high relapse rate, suggesting a need for long-term therapy and then increasing the risk of drug side effects [10].

The use of probiotics in IBD therapy is even more controversial. Despite the large number of probiotics that have shown beneficial effects in experimental models of intestinal inflammation [17], the studies describing their efficacy in human IBD, mainly ulcerative colitis and pouchitis, are less abundant. The strongest evidence comes from clinical trials conducted with *Escherichia coli* Nissle 1917 or with the probiotic mixture VSL#3 [18,19], revealing their greater usefulness in maintaining the disease in remission and preventing the relapses rather than in inducing remission, when studied in more severe active forms of IBD [20]. Different mechanisms have been proposed to participate in their beneficial effects, including the suppression of the growth of enteric pathogenic bacteria and their epithelial binding and subsequent invasion, the immunoregulatory activity, either by inducing protective cytokines (IL-10 and transforming growth factor-β) or by inhibiting pro-inflammatory cytokines (TNFα) in the intestinal mucosa, as well as the improvement of the intestinal barrier function by decreasing mucosal permeability [21,22]. However, it is important to note that not all probiotic bacteria have similar therapeutic effects; each may have individual mechanisms of action, and the host condition may determine which probiotic species and even strains may be optimal [23]. Furthermore, although most of them are considered safe in humans, their administration in some circumstances may be associated with certain risks [24].

For this reason, it is interesting to develop combined approaches that would restore the local ecological conditions in the gut lumen, thus correcting dysbiosis, and reinstate the altered immune response that takes place in the inflamed intestine in the long term. Although the combination of antibiotics and probiotics has been proposed to play a role in the treatment of IBD, with the rationale of opening a microbial niche with the antibiotics that the probiotics can then occupy [3,25], there are only few studies supporting this strategy [26,27]. The aim of the present study was to characterize the beneficial effects derived from the association of the antibiotic minocycline and the probiotic *E. coli* Nissle 1917 in an experimental model of reactivated colitis in mice. For this purpose, a curative treatment protocol was followed: once dextran sodium sulfate (DSS) experimental colitis had been established, minocycline was initially administered for 7 days; then, the antibiotic treatment was substituted by the probiotic administration, which continued until the end of the experiment in order to maintain the remission. Fourteen days after the beginning of the treatment, mice were subjected to a second cycle of DSS intake for 5 days, thus promoting a relapse in the colonic inflammatory process, which would allow evaluating the impact of the association in preventing the reactivation, in comparison with each individual treatment. The results revealed that the combination of the antibiotic and the probiotic improved the colonic inflammatory status in mice, thus supporting the potential use of this association in controlling intestinal inflammation.

2. Material and methods

All studies were carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health. All chemicals, including the antibiotic minocycline, were obtained from Sigma Chemical (Madrid, Spain), unless otherwise stated. *E. coli* strain Nissle 1917 (O6:K5:H1) was provided by Ardeypharm GmbH (Herdecke, Germany).

Table 2
Primer sequences used for microbiological analysis in real-time PCR assays in the colonic contents.

Target bacterial group	Sequence (5′–3′)	Annealing temperature (°C)
Bacteroides group	g-Bfra-F: ATAGCCTTTCGAAAGRAAGAT g-Bfra-R: CCAGTATCAACTGCAATTTTA	50
Clostridium cluster XIVa–XIVb	g-Ccoc-F: AAATGACGGTACCTGACTAA g-Ccoc-R: CTTTGAGTTTCATTCTTGCGAA	50
Bifidobacterium group	g-Bifid-F: CTCCTGGAAACGGGTGG g-Bifid-R: GGTGTTCTTCCCGATATCTACA	50
Lactobacillus group	Lab 159: GGAAACAG(A%G)TGCTAATACCG Lab 677: CACCGCTACACATGGAG	61

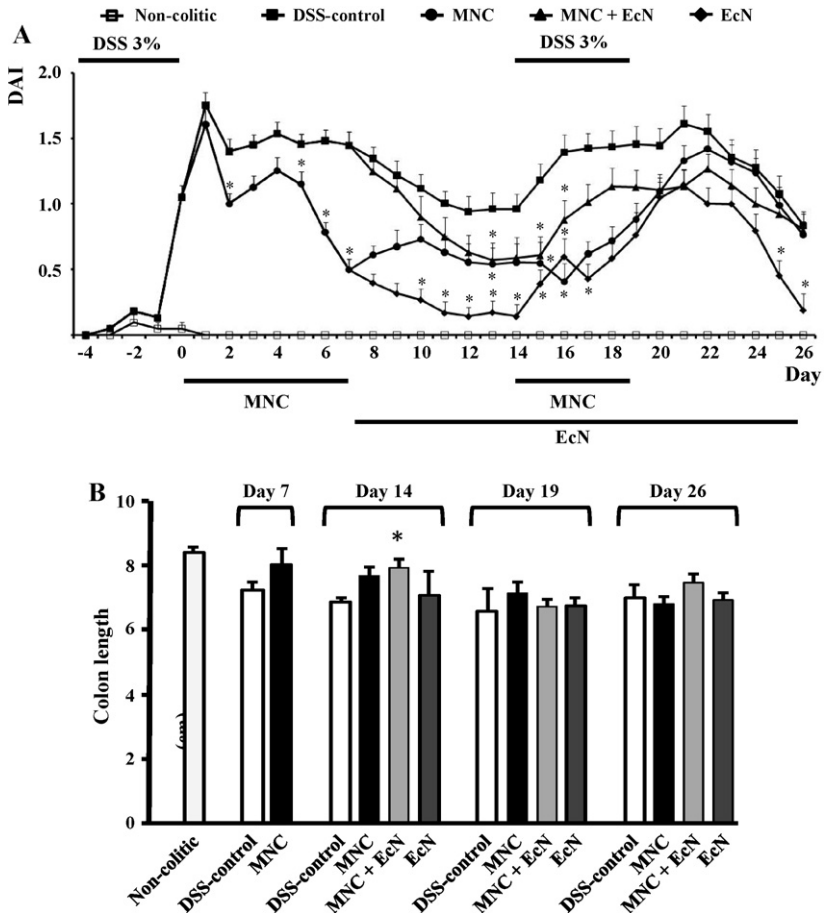

Fig. 1. (A) Effects of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) on disease activity index (DAI) values in DSS mice colitis over the 31-day experimental period, based on the criteria proposed previously [29]. $^{*}P < 0.05$ *vs.* DSS control group. (B) Effects of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) on colonic length of DSS colitic mice at the different time points. $^{*}P < 0.05$ *vs.* DSS control group; all colitic groups significantly differ from healthy group, except MNC at day 7 and MNC + EcN at day 14.

2.1. Dextran sodium sulfate (DSS) model of reactivated mouse colitis.

Female C57BL/6J mice (7–9 weeks old; approximately 20 g) obtained from Janvier (St Berthevin Cedex, France) were randomly assigned to different groups: non-colitic ($n = 10$) and DSS colitic groups ($n = 140$). The colitis was induced by adding DSS (3%, w/v) (36–50 kDa, MP Biomedicals, Ontario, USA) in the drinking water for a period of 5 days [28]. Then, colitic mice were divided in two groups of 60 animals each: control mice, which were given distilled water (200 μL), and minocycline-treated group, which received orally 30 mg/kg/day of minocycline dissolved in of 200 μL of distilled water for 7 days. At this time point, half of the mice from each group (control and minocycline-treated), received the probiotic *E. coli* Nissle 1917 at doses of 5×10^8 colony forming units (CFU) per mouse (suspended in 200 μL of distilled water), until the end of the experiment. Fourteen days after, all colitic mice received a new cycle of DSS for other 5 days. Concurrently with DSS administration, all the mice that had received minocycline previously were orally, and daily, treated again with the same doses of antibiotic. Mice from the different groups ($n = 10$) were sacrificed 7, 14, 19 and 26 days after the removal of the first cycle of DSS.

Animal body weight, the presence of gross blood in the feces and stool consistency were evaluated daily for each mouse by an observer unaware of the treatment. These parameters were assigned a score according to the criteria proposed previously [29] and they were used to calculate an average daily disease activity index (DAI). Once the animals were sacrificed, the colon was removed, the contents were collected aseptically and its length was measured under a constant load (2 g). Representative whole gut specimens (0.5 cm length) were taken from the distal inflamed region and fixed in 4% buffered formaldehyde for the histological studies; equivalent colonic segments were also obtained from the non-colitic group. The remaining colonic tissue was subsequently sectioned in different longitudinal fragments for RNA isolation.

2.2. Histological studies

Cross-sections were selected and embedded in paraffin. Full-thickness sections of 5 μm were obtained at different levels and stained with hematoxylin and eosin. The histological damage was evaluated by a pathologist observer, who was blinded to the experimental groups, according to the criteria previously described [30].

2.3. Analysis of gene expression in colonic samples by RT-qPCR

Total RNA from colonic samples was isolated using RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer and 2 μg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK).

Real-time quantitative PCR amplification and detection was performed on optical-grade 96-well plates in a 7500 RT-PCR System (PE Applied Biosystems, CA, USA). Each reaction was composed of 25 μL of FastStart Universal SYBR Green Master (ROX)

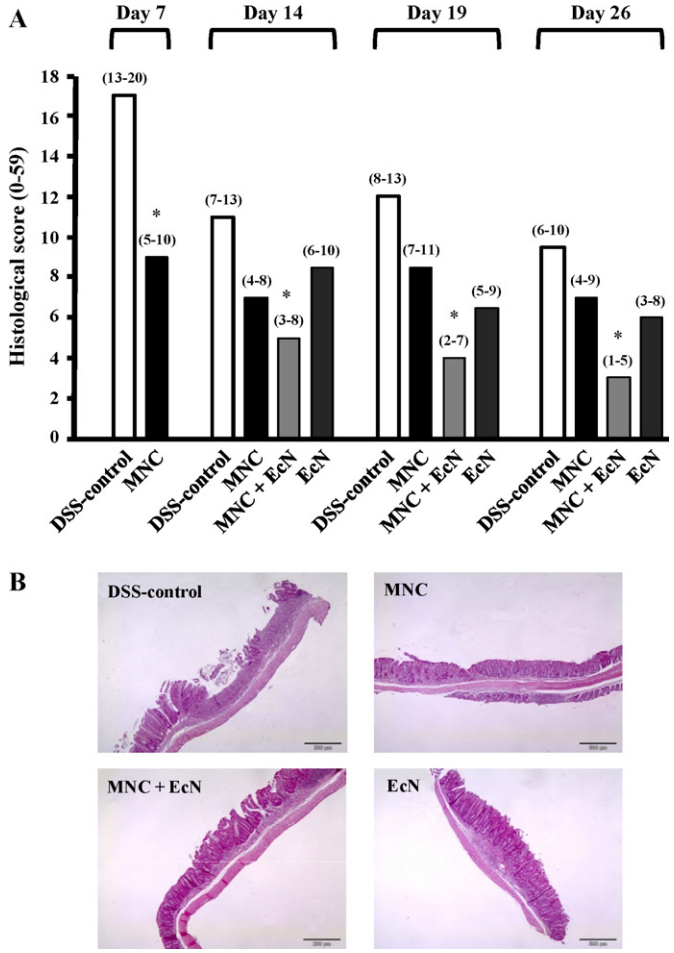

Fig. 2. Histological analysis of the colonic segments from DSS colitic mice treated with minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) at the different time points. (A) Microscopic score assigned according the criteria previously described [30]; data are expressed as median (range); $^{*}P < 0.05$ *vs.* DSS control group. (B) Representative histological sections of colonic segments stained with hematoxylin and eosin, showing the intestine anti-inflammatory effect of the treatments at day 26.

Mix (Roche Applied Science, Indianapolis, IN), each amplification primer at a concentration of 0.3 μmol/L, 25 ng of cDNA from the RT reaction and PCR-grade water up to a final volume of 50 μL.

The thermal cycling program consisted of an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at T_a (see below). Fluorescence was measured at the end of the annealing period of each cycle to monitor the progress of amplification, and dissociation curves were added to confirm the specificity of the amplification signal in each case. To normalize mRNA expression, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. For each sample, both the housekeeping and target genes were amplified in triplicate and the mean was used for further calculations. The mRNA relative quantitation was done using the $\Delta\Delta C_t$ method. The specific primers used are indicated in Table 1.

2.4. Microbial analysis of the colonic contents

For DNA extraction, samples from colonic contents were diluted 1:10 (w/v) in PBS. DNA was extracted from 2 mL of the dilution using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 20 μL of buffer AE (provided in the kit), and the purified DNA extracts were stored at −20 °C. qRTi-PCR was used to characterize the bacterial DNA present in colonic content samples as reported previously [31]. For this purpose, a series of genus-specific primer pairs were used (Table 2). PCR amplification and detection was performed as described above. In this case, each reaction mixture (50 μL) was composed of 25 μL of FastStart Universal SYBR Green Master (ROX) Mix (Roche Applied Science, Indianapolis, IN), 0.5 μL of each specific primer at a concentration of 30 μM) and 4 μL of template DNA. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from pure cultures with a bacterial population ranging from 2 to 9 $\log_{10}$ colony forming units (CFUs), as determined by plate counts. One strain belonging to each of the bacterial genera or groups targeted in this study was used to construct the standard curve. More specifically, the strains from which the DNA was extracted were the following: *Bifidobacterium longum* CECT 4551, *Clostridium coccoides* DSMZ 935, *Bacteroides fragilis* DSMZ 2151, *Lactobacillus salivarius* CECT 2197. All of them were obtained from the Spanish Collection of Type Cultures (CECT) or the German Collection of Microorganisms and Cell Cultures (DSMZ).

2.5. Statistics

All results are expressed as the mean ± SEM. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. Differences between proportions were analyzed with the chi-squared test. Non-parametric data (DAI values and histological score) were analyzed using the Mann–Whitney *U*-test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Maryland), with statistical significance set at $P < 0.05$.

3. Results

3.1. Evaluation of the colonic inflammatory status at day 7 after DSS removal

The administration of 3% (w/v) DSS dissolved in the drinking water for 5 days to mice resulted in a progressive increase in DAI values, due to the body weight loss and the excretion of diarrheic/bleeding feces. Oral minocycline treatment promoted the recovery of DSS colitic mice, as evidenced by the significant decrease observed in the DAI during the 7 days following DSS administration in comparison with untreated colitic mice (Fig. 1), mainly associated with an improvement in the weight loss, rather than in the amelioration of feces consistency. Macroscopically, the inflammatory process in the untreated control group, but not in the minocycline-treated, was related to a significant shortening of the colonic length in comparison with healthy mice (Fig. 1). Histologically, 7 days after DSS removal, the colonic specimens from control colitic mice were characterized by mucosa epithelial ulceration, that typically affected more than 75% of the surface, and marked crypt hyperplasia with goblet cell depletion. A chronic inflammatory cell infiltration into the lamina propria was also observed, and edema was evidenced between the mucosa and muscularis layers of the intestine. The microscopic score assigned, expressed as median (range) was 17 (13–20). Minocycline-treated colitic mice showed a significant improvement of the altered colonic histology associated with the inflammatory process, the mucosal epithelium appeared restored, and there was a lower inflammatory infiltrate of mononuclear cells in the lamina propria and slight edema in the submucosa. This resulted in a significant reduction in the microscopic score compared with the untreated control group, showing a value of 9 (5–10) ($P < 0.05$ *vs.* colitic control) (Fig. 2). The qPCR analysis of the mRNA expression of different inflammatory markers in the colonic segments also corroborated the intestinal anti-inflammatory effect exerted by minocycline at this time point. The colonic inflammation induced by DSS was characterized by an increased expression of the pro-inflammatory cytokines TNFα,

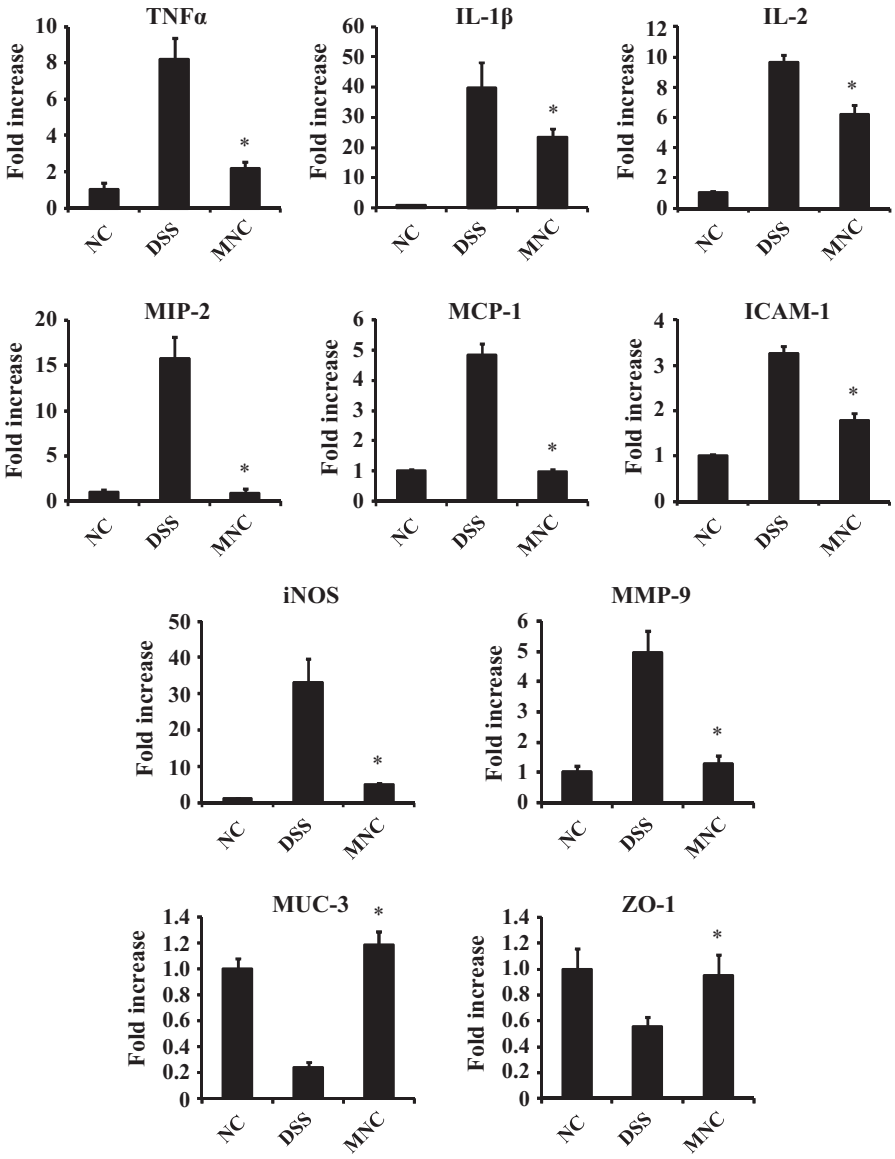

Fig. 3. Biochemical evaluation of the effects of minocycline (MNC) after 7 days of treatment; mRNA expression of TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS, MMP-9, MUC-3 and ZO-1 was quantified by real-time PCR, and fold increases are expressed as means ± SEM; $^{*}P < 0.05$ *vs.* DSS control group.

IL-1β and IL-2, the chemokines MIP-2 and MCP-1, the adhesion molecule ICAM-1 and the enzymes iNOS and MMP-9, as well as by decreased expression of some biochemical markers of the epithelial integrity, including MUC-3 and zona occludens-1 (ZO-1) (Fig. 3). Mice treated with minocycline showed a significant restoration of the expression of the different markers when compared with the untreated colitic group (Fig. 3). In addition, the microbiota composition of the colonic contents was modified as a result of the inflammatory process. The ratio between non-pathogenic (lactobacilli and bifidobacteria) and potentially pathogenic (bacteroides and clostridia) bacteria was reduced in the DSS-control animals when compared with non-colitic mice. The beneficial effect observed in minocycline-treated colitic mice was associated with a significant increase in this ratio in comparison with untreated control mice (Fig. 4).

3.2. Evaluation of the colonic inflammatory status at day 14 after DSS removal

Seven days after DSS removal, the administration of minocycline was interrupted and half of the mice from both colitic groups (treated or not with the antibiotic) received a daily dose of the probiotic *E. coli* Nissle 1917 until the end of the experiment. DAI time-course evaluation during the following 7 days revealed that probiotic administration improved the recovery of the colitic mice when compared with DAI values showed by those mice without probiotic treatment, that remained constantly high (Fig. 1). During this period, in addition to a reduction in the body weight loss, probiotic supplementation to minocycline treatment did have a positive impact on feces consistency. When the colonic length was measured, only the group that received the antibiotic followed by the probiotic showed no statistical differences with healthy mice as well as a significant increase in comparison with untreated control mice (Fig. 1). The histological study confirmed the beneficial effect of the probiotic in colitic mice 14 days after DSS removal. Although in the control colitic mice the colonic damage was partially recovered, these intestine segments still showed ulceration of the mucosa affecting 50% of the surface. The inflammatory infiltrate was slight to moderate, mainly composed by mononuclear cells, and there were a moderate crypt hyperplasia and goblet cell depletion. The microscopic score value assigned to these mice was 11 (7–13). *E. coli* Nissle 1917 administration

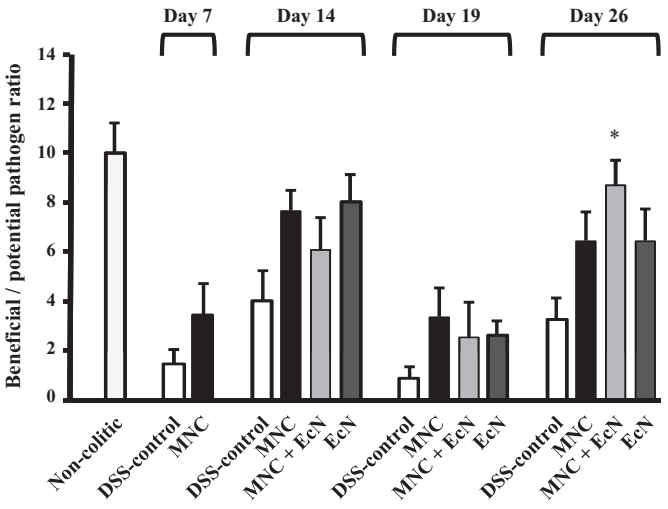

Fig. 4. Effects of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) on the bacterial profile of the colonic contents from DSS colitic mice. Data are expressed as means ± SEM of the ratio between non-pathogenic (lactobacilli and bifidobacteria) and potentially pathogenic (bacteroides and clostridia). $^{*}P < 0.05$ *vs.* DSS control group.

improved the recovery of the colonic tissue in all colitic mice, treated or not with minocycline. The mucosal layer appeared almost completely preserved, with goblet cells full of mucin content and only a slight crypt hyperplasia. It is remarkable that those mice previously treated with the antibiotic showed a lower microscopic score (5 (3–8)) than those without minocycline treatment (8.5 (6–10)) ($P < 0.05$). Of note, the group of colitic mice that only received minocycline also showed a lower microscopic score value (7 (4–8)) than the control mice ($P < 0.05$) (Fig. 2). No statistical differences were observed among the different treatments at this time point. When the biochemical markers were analyzed, the expression of IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS and MMP-9 still remained increased in control colitic mice when compared with healthy mice, whereas MUC-3 and ZO-1 expressions persisted reduced (Fig. 5). In this regard, the association of minocycline and *E. coli* Nissle 1917 was the most effective of the treatments assayed, since the reduction in the expression of IL-1β, IL-2, MIP-2, MCP-1, ICAM-1 and iNOS was greater than in the groups of mice that received a single treatment. Similarly, the expression of ZO-1 was significantly increased when compared with the other colitic mice, without showing differences with the non-colitic group. However, no significant modifications were observed among the three treated groups when MMP-9 was considered (Fig. 5). When the colonic microbiota was analyzed, the beneficial/potential pathogen bacteria ratio was still significantly reduced in control colitic mice in comparison with the non-colitic group. All treated groups revealed an increase in this ratio; and, although the values were not statistically different from the untreated colitic control group, no significant differences were either observed when compared with healthy mice (Fig. 4).

3.3. Evaluation of the colonic inflammatory status after colitis reactivation

The exacerbation of the intestine inflammatory process after the second cycle of DSS was evidenced by a progressive increase in the DAI values in all groups, although it was more moderate than that obtained during the first colitis onset. The mice previously treated with minocycline again received the antibiotic during the second exposure to DSS. All treated mice showed lower DAI values when compared with untreated colitic mice, being these differences more evident in those treated with the antibiotic than in the mice that only received the probiotic (Fig. 1). When the mice were sacrificed at the end of this period, the histological evaluation showed no significant differences between those mice which received either minocycline or the probiotic alone when compared with control colitic group. However, the combined treatment improved colonic histology as evidenced by the lower histological damage score obtained (Fig. 2). As expected, the colitis reactivation was associated with an increased expression of all the pro-inflammatory makers assayed, together with a decreased expression of MUC-3 and ZO-1 (Fig. 6). The combination of minocycline and the probiotic resulted to be once more the most effective treatment, since it decreased all the pro-inflammatory mediators studied, whereas each individual treatment was only able to significantly modify some of them. Regarding the expression of the mediators involved in the intestine barrier integrity, the concurrent administration of the antibiotic and the probiotic significantly increased colonic ZO-1 expression in comparison with untreated colitic mice. However, none of the treatments significantly modified colonic MUC-3 expression (Fig. 6). Furthermore, the reactivation of the colonic inflammatory process was associated with an alteration in the microbiota composition. However, the administration of the antibiotic, the probiotic or their combination partially restored the non-pathogenic/potential pathogenic ratio (Fig. 4).

After these 5 days, minocycline treatment was stopped, and the rest of the mice were sacrificed 7 days later, i.e. 31 days after starting the experiments, being the colonic inflammatory status evaluated once more. During this period, the mice that received the combination of treatments throughout the study recovered almost completely. Their DAI evolution showed significantly lower values than the other colitic mice, as a result of the amelioration of both body weight loss and feces consistency, and no significant differences were seen in comparison with the healthy mice at the end of the study (Fig. 1). Microscopically, these animals showed the lowest histological score (Fig. 2) and the qPCR analysis revealed that the administration of minocycline and *E. coli* Nissle 1917 significantly decreased the expression of the pro-inflammatory mediators studied (TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1 MMP-9 and iNOS), while increasing MUC-3 and ZO-1 expressions (Fig. 7). Finally, the ratio between beneficial and potential pathogen bacteria, that remained reduced in the control colitic group in comparison with healthy mice, was increased in all the treated groups, but even more in the group that received the combination of both treatments (Fig. 4).

4. Discussion

Although the etiology of IBD is not completely elucidated, recent studies have attributed a key role to the intestine microbiota in its pathogenesis. However, no specific microorganism has been clearly involved as a causative agent of this pathology; most probably, this intestinal condition may be due to an aberrant immunological reaction against luminal contents in a susceptible host [2,3] and/or due to an altered luminal microbiota composition leading to dysbiosis [7,9]. Therefore, the development of therapeutic strategies that combine an immunomodulatory activity and the ability to restore the luminal microbial balance in the intestine could be very interesting in the management of IBD. This could be the case of a therapy based on antibiotics and probiotics.

In a previous study, we reported that the antibiotic minocycline exerted intestinal anti-inflammatory effect in two experimental models of rodent colitis, the TNBS model in rats and the DSS model in mice, through the modulation of several immune and microbiological parameters [16]. The present study confirms these findings, since minocycline was able to promote the recovery of DSS-induced colitis in mice when it was administered for 1 week after colonic

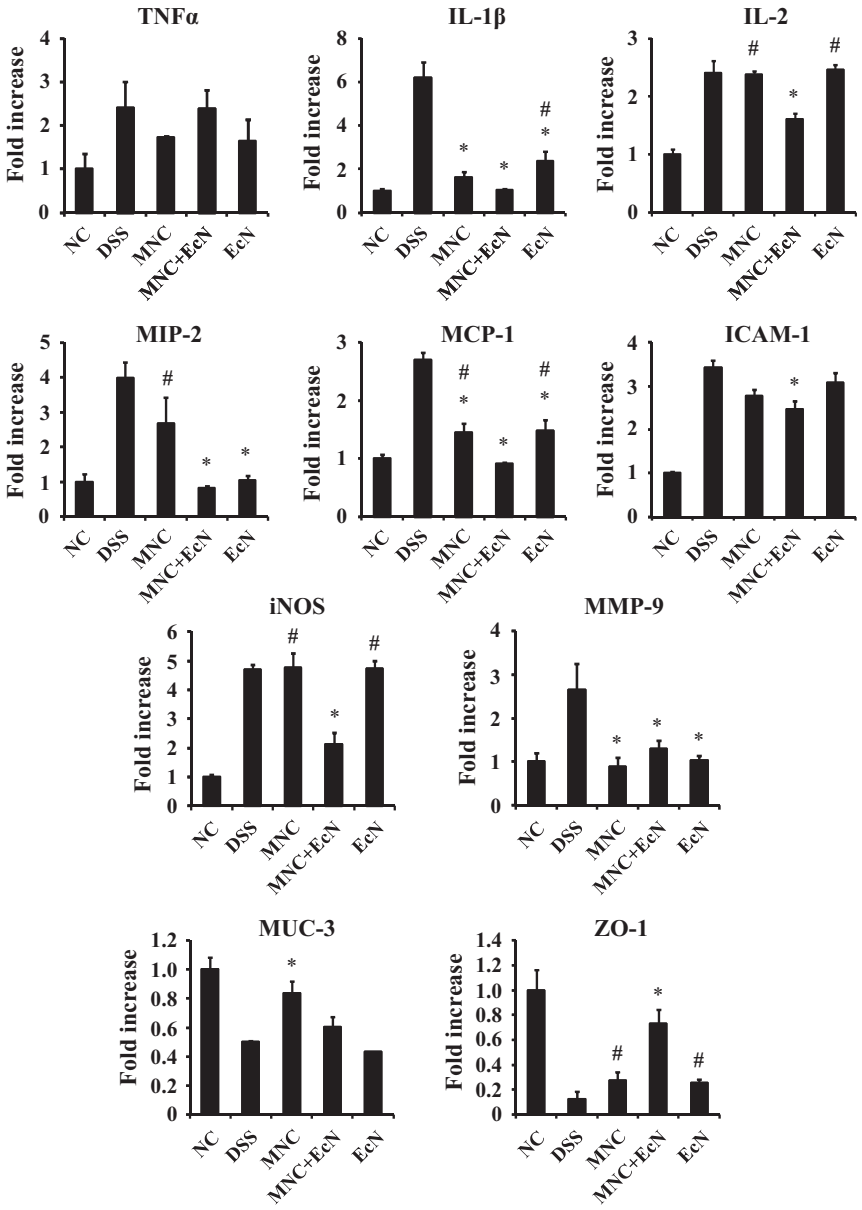

Fig. 5. Biochemical evaluation of the effects of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) at day 14; mRNA expression of TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS, MMP-9, MUC-3 and ZO-1 was quantified by real-time PCR, and fold increases are expressed as means ± SEM; $^{*}P < 0.05$ *vs.* DSS control group, $^{\#}P < 0.05$ *vs.* MNC + EcN treated group.

damage induction. This beneficial effect was associated with an amelioration of the altered immune response, as evidenced by a decrease in the expression of the pro-inflammatory cytokines TNFα, IL-1β and IL-2, the chemokines MIP-2 and MCP-1, the adhesion molecule ICAM-1 and the enzymes iNOS and MMP-9, being all of them key players in the pathogenesis of IBD [2,3]. In addition, the treatment with this antibiotic improved the defensive mechanisms of the intestine epithelial barrier, whose architecture appeared restored in the histological studies. Biochemically, minocycline counteracted the reduced expression of MUC-3, one of the primary constituents of the mucus layer in the colon [32], and of ZO-1, a transmembrane protein that maintains tight junctions integrity [33], similarly to that previously described in other models of experimental colitis [16,34]. Finally, the administration of minocycline was also able to restore the balance in the intestinal microbiota, which was altered in the DSS-induced inflammatory process, in accordance to that reported in the TNBS model of rat colitis [16].

However, long-term use of antibiotics can be associated with an increased risk of drug side effects; therefore, the chronic administration that could be required in IBD therapy is limited [10]. For this reason, in the present study, the administration of minocycline to colitic mice was stopped after 1 week of treatment. The DAI evolution during the following 7 days showed constant values for these mice, being lower than those of the untreated colitic group. Nevertheless, the absence of an additional recovery in these mice after the interruption of the treatment contrasts with that achieved in the same model of colitis when the antibiotic treatment was maintained longer [16]. For this reason, we evaluated if the subsequent administration of the probiotic *E. coli* Nissle 1917 could have a positive impact in these treated mice. In fact, the probiotic treatment caused a significant improvement of DAI values, regardless of whether the mice were previously treated with the antibiotic or not. These results support previous studies that reported the beneficial effects of this probiotic in intestinal inflammation, both in experimental models and in human IBD [18,35], ascribed to its ability to interfere with the intestine microbiota and to modulate the intestine immune response. The evaluation of the colonic inflammatory status 1 week after

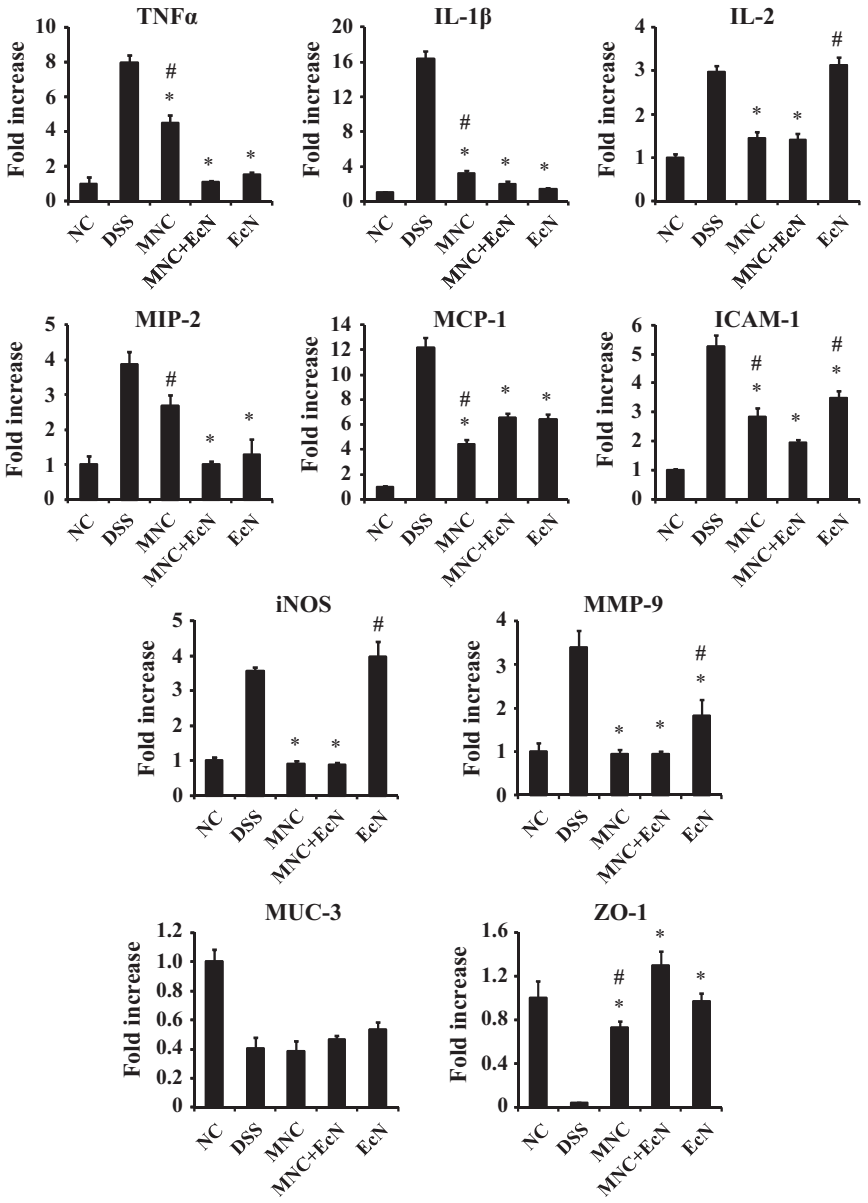

Fig. 6. Biochemical evaluation of the effects of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) after colitis reactivation (day 19); mRNA expression of TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS, MMP-9, MUC-3 and ZO-1 was quantified by real-time PCR, and fold increases are expressed as means ± SEM; $^{*}P < 0.05$ *vs.* DSS control group, $^{\#}P < 0.05$ *vs.* MNC + EcN treated group.

confirmed this beneficial effect. The histological damage score in those mice treated with the probiotic alone was similar to that obtained in the group previously treated only with minocycline. However, the group of mice that received the combined therapy, i.e. first the antibiotic followed by the probiotic, showed a greater improvement in the colonic histology when compared with the other experimental groups, revealing the additional beneficial effect of the association, which was confirmed in the biochemical analysis. The immunomodulatory properties reported for minocycline [16,36] and *E. coli* Nissle 1917 [35,37] could clearly contribute to reduce the expression of some of the pro-inflammatory markers. However, these effects were increased when the antibiotic treatment was followed by the administration of the probiotic, since all the mediators assayed were clearly improved.

As a result, the vicious cycle generated by the different mediators involved in IBD pathogenesis, responsible for maintaining the chronic inflammatory response, can be blocked, thus facilitating the recovery of the inflamed colonic tissue. The decreased levels of the pro-inflammatory cytokines TNF-α, IL-2 and IL-1β can lead to a downregulation of the expression of the chemokines MIP-2 and MCP-1, as well as of the adhesion molecule ICAM-1, which cause a reduced inflammatory cell infiltrate in the colonic tissue. As a consequence, the expression of enzymes like iNOS and MMP-9 is reduced, since they are mainly synthesized by inflammatory cells, particularly T cells, macrophages and polymorphonuclear leucocytes [38,39]. In sum, all these mechanisms help to prevent the deleterious effect that these activated cells may exert in inflammatory conditions [2].

Furthermore, the combination of both treatments also restored intestine integrity increasing the expression of colonic MUC-3 and ZO-1, as previously reported for the antibiotic [16] and the probiotic [40] separately. The altered epithelial barrier function can be considered as one of the key pathogenic mechanisms in intestinal inflammation since it may facilitate the access of antigens from the intestinal lumen, thus promoting the exacerbated immune response that occurs in this condition [41,42].

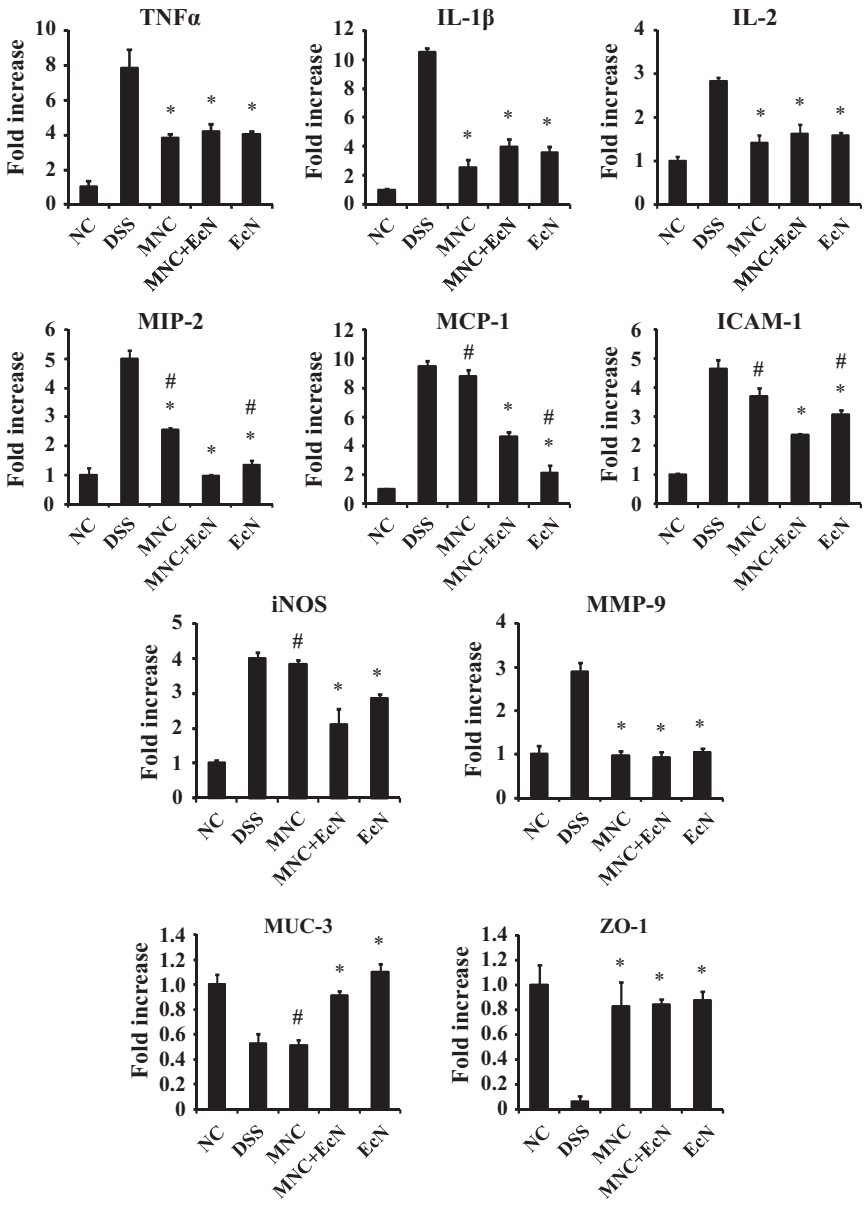

Fig. 7. Biochemical evaluation of the effects of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) at the end of the study (day 26); mRNA expression of TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS, MMP-9, MUC-3 and ZO-1 was quantified by real-time PCR, and fold increases are expressed as means ± SEM; $^{*}P < 0.05$ *vs.* DSS control group, $^{\#}P < 0.05$ *vs.* MNC + EcN treated group.

In order to study the impact of the combined therapy in the prevention of the relapses that usually takes place in human IBD, colitic mice were submitted to a second cycle of DSS. This resulted in a worsening of the intestine inflammatory process with increased DAI values, as a result of the body weight loss and the presence of diarrhea in these mice. All the treatments assayed attenuated the aggravation of the inflammatory status and lately promoted the recovery of the mice, but the group that received both the antibiotic and the probiotic displayed a lower colonic damage, as evidenced by DAI evolution and histologically, appearing at day 26 almost fully recovered. This was also associated with a significant down-regulation of the expression of all the biochemical pro-inflammatory markers evaluated, and again, the treatment was able to preserve the intestine integrity, which was affected in the reactivation of the colitis. It is interesting to note that in previous assays we have tested the sensitivity of EcN to minocycline, revealing that the probiotic is sensitive to the antibiotic from concentrations of 2 μM (unpublished results) although this fact has no detrimental impact on the probiotic activity.

Finally, when the microbiota was evaluated throughout the experiment, all treatments were able to restore the ratio between beneficial and potential pathogen bacteria, which was modified due to the colitic process, in a similar way. Although this activity may have a role in the greater effect observed with the combined therapy, the absence of clear differences among the treated groups may point out that their ability to regulate the altered immune response and to preserve intestine integrity could justify the greater anti-inflammatory effect displayed with the association of both treatments.

In conclusion, the supplementation of minocycline treatment with the probiotic *E. coli* Nissle 1917 improves the recovery of the intestinal damage and prevents the reactivation of experimental colitis, supporting the potential use of this new therapeutic strategy in the treatment of human IBD.

Conflict of interest

The authors declare no conflict of interest.

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