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Highlights

- Nonsteroidal anti-inflammatory drugs (NSAIDs) can damage the intestinal tract
- The use of prebiotics and probiotics can protect against NSAID-induced enteropathy
- Lactoferrin and *Bifidobacterium longum* counteracted diclofenac-induced enteropathy
- The underlying mechanisms include anti-inflammatory and antioxidant actions
- These effects are likely to depend on the modulation of TLR-2/-4/NF-kB pathways

Protective effects of the combination *Bifidobacterium longum* plus lactoferrin against NSAID-induced enteropathy

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Abbreviations

NSAIDs, nonsteroidal anti-inflammatory drugs; GI, gastrointestinal; MPO, myeloperoxidase; MDA, malondialdehyde; TLR, Toll-like receptors; NF-kB, nuclear factor-kB; MyD88, myeloid-differentiation primary response gene 88; DIC, diclofenac; LAC, lactoferrin; BIF, *Bifidobacterium longum* BB536, LPS, lipopolysaccharide.

Abstract

Objectives: Non-steroidal anti-inflammatory drugs can exert detrimental effects in the lower digestive tract. This study examined the protective effects of a combination of the probiotic *Bifidobacterium longum* BB536 (*Bifidobacterium*) with the prebiotic lactoferrin in a rat model of diclofenac-induced enteropathy.

Methods: Enteropathy was induced in 40-week-old male rats by intragastric diclofenac (4 mg/kg BID, 14 days). Lactoferrin (100 mg/kg BID), *Bifidobacterium* ($2.5 \cdot 10^6$ CFU/rat BID) or their combination were administered 1 hour before diclofenac. At the end of treatments, the ileum was processed for the evaluation of histological damage, myeloperoxidase (MPO) and malondialdehyde (MDA) levels, as well as the expression of toll-like receptors 2 and 4 (TLR-2/-4) and the activation of downstream signaling molecules (MyD88 and NF-kB p65). Blood hemoglobin and fecal calprotectin were also assessed.

Results: Diclofenac induced intestinal damage, along with increments of MPO and MDA, overexpression of TLR-2, TLR-4, MyD88 and NF-kB p65, increase in fecal calprotectin and decrease in blood hemoglobin levels. Lactoferrin or *Bifidobacterium* alone prevented diclofenac-induced enteric damage, and the changes in blood hemoglobin, MPO, MDA, fecal calprotectin and NF-kB p65. *Bifidobacterium*, but not lactoferrin, decreased TLR-4 expression, while none of them affected MyD88 overexpression. TLR-2 expression was slightly enhanced by all treatments. The combined administration of lactoferrin and *Bifidobacterium* reduced further the intestinal damage, and restored MPO and blood hemoglobin levels.

Conclusions: Diclofenac induced ileal mucosal lesions by activation of inflammatory and pro-oxidant mechanisms. These detrimental actions were prevented by the combination of lactoferrin with *Bifidobacterium* likely through the modulation of TLR-2/-4/NF-kB pro-inflammatory pathways.

Keywords: Nonsteroidal anti-inflammatory drugs; intestinal damage; lactoferrin; *Bifidobacterium longum*; probiotics; prebiotics

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are very effective medications, but their use is associated with a broad spectrum of adverse reactions, including upper gastrointestinal (GI) adverse effects, such as dyspepsia, heartburn and abdominal discomfort, as well as more serious events, such as peptic ulcer with life-threatening complications of bleeding and perforation [1,2]. Over the past decade, it has been increasingly acknowledged that NSAIDs can trigger adverse effects also in the lower digestive tract [3,4].

The pathophysiology of NSAID-induced small bowel damage is not completely understood. However, enteric bacteria and bile acids are currently regarded as the most prominent pathogenic factors [5]. A large body of experimental evidence suggests that NSAID-induced enteropathy results from direct effects, characterized by the accumulation of usually acidic NSAIDs into mucosal cells via damage to the membrane brush border and disruption of mitochondrial oxidative phosphorylation, with consequent ATP deficiency [6]. These events would lead to increased mucosal permeability [7], with facilitation of tissue entry and detrimental actions of luminal factors, such as dietary macromolecules, bile acids, components of pancreatic juice and bacteria, which activate the inflammatory cascade [6]. Amongst the luminal aggressors, enteric bacteria act as the most prominent neutrophil chemoattractants. In line with this concept, several studies have shown that antimicrobial agents can attenuate NSAID-enteropathy, thus supporting further the pathogenic role of enteric bacteria [8]. However, the enteroprotective use of antimicrobial drugs is associated with local/systemic adverse effects, possible increase in bacterial resistance, and remains to be conclusively validated in the clinical setting.

Recently, several lines of evidence have suggested that the use of probiotics and/or prebiotics could represent a valuable strategy for the management of various bowel inflammatory conditions, including NSAID-induced enteropathy, without the occurrence of significant adverse reactions [9]. For instance, *Bifidobacterium longum* BB536 was found to exert protective effects against experimental colitis in rats [10]. In addition, *Bifidobacterium longum* CECT7347 was shown to protect against experimental small bowel inflammation induced by gliadin in rat [11], while *Bifidobacterium infantis* attenuated tissue injury in rat experimental necrotizing enterocolitis [12]. Prebiotics, such as lactoferrin, can exert protective effects in experimental models of intestinal inflammation. In particular, lactoferrin induced entero-protective effects against small bowel injury in a rat model of lipopolysaccharide (LPS)-induced endotoxemia [13], and in a model of colitis induced by dextran sulphate sodium [14]. Moreover, lactoferrin counteracted intestinal bleeding and the elevation of intestinal myeloperoxidase (MPO) levels associated with NSAID-induced experimental enteropathy [15].

Based on current background, the present study was designed to assess the ability of the combination of *Bifidobacterium longum* BB536 (*Bifidobacterium)* with lactoferrin in preventing the intestinal injury associated with NSAID therapy in a rat model of diclofenac-induced enteropathy. The main mechanisms underlying their entero-protective actions have been examined as well.

Materials and Methods

Animals

Albino male Sprague Dawley rats, 500 to 600 g body weight (40 weeks old), were used throughout the study. The animals were fed standard laboratory chow and tap water ad libitum, and were not subjected to experimental procedures for at least 1 week after their delivery to the laboratory. They were housed in solid-bottomed cages, equipped wire-mesh bottom inserts to prevent coprophagy, and located in temperature-controlled rooms (22-24°C and 50-60% humidity) under a 12-h light cycle (06:00-18:00 hours). Their care and handling were in accordance with the provisions of the European Community Council Directive 210/63/UE, recognized and adopted by the Italian Government. The experiments were approved by the Ethical Committee for Animal Experimentation of the University of Pisa and by the Italian Ministry of Health (authorization n. 465/2016).

Experimental design

PRELIMINARY DOSE-FINDING STUDY

A preliminary study was performed in order to identify the effective doses of *Bifidobacterium* and lactoferrin able to exert protective effects against diclofenac-induced small intestinal injury. Diclofenac-induced enteropathy was elicited according to the methodology developed by Fornai et al. [16]. Briefly, non-fasted rats were treated twice daily by intragastric route for 14 days with diclofenac (4 mg/kg) suspended in 1% methylcellulose (0.3 ml/rat). Rats were euthanized 24 hours after the last diclofenac administration. Subgroups of animals (n=6 per group) receiving diclofenac were treated with *Bifidobacterium* ($2.5 \cdot 10^5$, $2.5 \cdot 10^6$ or $2.5 \cdot 10^7$ CFU/rat BID) or lactoferrin (50, 100 or 200 mg/kg BID) 1 hour before DIC for 14 days. The doses of *Bifidobacterium* and lactoferrin were selected in the range of doses found to be effective against experimental colitis in rat [10,14]. At the end of treatments, the

ileum was removed and processed for: 1) histological assessment of mucosal damage; 2) evaluation of tissue MPO levels, as an index of polymorphonuclear cell infiltration; 3) tissue malondialdehyde (MDA) concentration, as an index of tissue oxidative stress. The experimental procedures are described in detail below.

MAIN STUDY

Diclofenac-induced enteropathy was made up as described above. Based on the results obtained in the preliminary dose-finding study, we selected the dose of 100 mg/kg BID for lactoferrin, and $2.5x10^6$ CFU/rat BID for *Bifidobacterium* for subsequent evaluations. The choice of these doses was based mainly on the results obtained from the microscopic score of bowel lesions, regarded as the most relevant outcome of treatment efficacy. Indeed, the intermediate, but not the lowest, dose of both lactoferrin and *Bifidobacterium* was able to prevent the occurrence of type 3 lesions, while such values did not differ significantly from those obtained with the highest doses. Likewise, the intermediate, but not the lowest, dose of lactoferrin was able to counteract the increase in MDA and MPO levels in a statistically significant fashion, while such effects did not differ significantly from those obtained in animals treated with the highest dose. With regard for the effects exerted by *Bifidobacterium* on MPO and MDA, we did not detect any significant difference among the three tested doses. Therefore, we selected the intermediate one, representing the least dose able to prevent the occurrence of type 3 lesions. *Bifidobacterium* and lactoferrin were administered, either alone or in combination, 1 hour before diclofenac for 14 days. Twenty-four hours after the last dose of diclofenac, non-fasted rats were anesthetized with chloral hydrate. Blood samples were collected by cardiac puncture from each animal for hemoglobin measurement. Fecal pellets were collected directly from the sigmoid colon and stored at -80°C for calprotectin measurement. The whole gastrointestinal tract was excised and examined macroscopically.

Samples of ileum were snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis of the following parameters; 1) Assay of tissue MPO and MDA levels (ELISA and colorimetric assays); 2) Expression of toll-like receptors-2/-4 (TLR-2/-4) in the mucosa (Western blot); 3) Activation of molecular pathways downstream to TLR receptors: nuclear factor kB (NF-kB) and myeloid-differentiation primary response-gene 88 (MyD88) in the mucosa (Western blot). Other portions of ileal tissue, collected as described below, were fixed in 10% formalin for subsequent evaluation of microscopic damage.

The experimental groups were arranged as follows:

- Group 1: animals treated with vehicle (normal controls, n=10)
- Group 2: animals treated with diclofenac 4 mg/kg BID (n=10)
- Group 3: animals treated with diclofenac and *Bifidobacterium* $2.5 \cdot 10^6$ CFU/rat BID (n=10)
- Group 4: animals treated with diclofenac and lactoferrin 100 mg/kg BID (n=10)
- Group 5: animals treated with diclofenac, *Bifidobacterium* $2.5 \cdot 10^6$ CFU/rat BID and lactoferrin 100 mg/kg BID (n=10)

Assessment of blood haemoglobin concentration

Blood haemoglobin concentration was assumed as an index of digestive bleeding. The analysis was performed on blood samples collected as reported above, by means of Quantichrom Hemoglobin assay kit (Bioassay Systems, Hayward, CA, USA) and expressed as g/dL.

Microscopic assessment of intestinal damage

The histological evaluation of intestinal injury in the ileum was carried out as previously described [16], detailed in supplementary material.

Evaluation of tissue myeloperoxidase and malondialdehyde levels

MPO was assumed as a quantitative index to estimate the degree of mucosal infiltration by polymorphonuclear cells, and thereby the severity of enteropathy elicited by diclofenac, while MDA concentration in intestinal tissues was determined to obtain quantitative estimates of membrane lipid peroxidation. MPO and MDA levels in the ileum were assessed according to previously adopted methods [16], described in supplementary material.

Western blot analysis of TLR-2, TLR-4, NF-kB p65 subunit and MyD88 expression

Western blot assays were performed as previously described by Colucci et al. [17]. Specimens of mucosa were excised from ileum, weighed and homogenized in lysis buffer using a polytron homogenizer. Mucosal homogenates were spun by centrifugation at 15,000 g for 15 minutes at 4°C, and the resulting supernatants were then separated from pellets and stored at −80°C for subsequent quantification of TLR-2, TLR-4, MyD88 and p65 subunit. The detailed procedure as well as primary and secondary antibodies employed are described in Supplementary material.

Assay of fecal calprotectin

Calprotectin, a calcium binding protein of neutrophil granulocytes that correlates well with neutrophil infiltration of the intestinal mucosa was measured in fecal pellets, as previously described by Colucci et al. [17] (see Supplementary material).

Statistical analysis

Results are presented as mean ± standard error of mean (S.E.M.). The statistical significance of data was evaluated by one way analysis of variance (ANOVA) followed by *post hoc*

analysis by Student–Newman–Keuls test, and p values less than 0.05 were considered significant. All statistical calculations were performed using GraphPad Prism™ 3.0 software (GraphPad, San Diego, CA, USA). Unless otherwise specified in the figure legends, the differences between groups were not statistically significant.

Results

Preliminary dose-finding study

Microscopic damage

In the ileum from control animals, microscopic analysis did not reveal any type of lesion. Treatment with diclofenac elicited the development of type 1, 2 and 3 lesions in the ileum (Table 1). The concomitant administration of lactoferrin 50 mg/kg BID partly prevented the development of type 1 and 3 lesions, while unaffecting type 2 ones (Table 1). Doses of lactoferrin of 100 and 200 mg/kg BID significantly decreased the occurrence of type 1 and 2 lesions, and prevented completely the development of type 3 lesions. Similar results were obtained with *Bifidobacterium*, as the middle and highest doses were the most effective in counteracting the development of small intestinal lesions (Table 1). In control animals, treatment with lactoferrin or *Bifidobacterium* did not exert any significant effect on the microscopic appearance of ileal tissue (not shown).

Tissue myeloperoxidase levels

MPO concentration in the ileum from control animals was 6.00 ng/mg of tissue (Fig. 1A). In rats treated with diclofenac, MPO levels were significantly increased (34.40 ng/mg of tissue). In the ileum from diclofenac-treated animals, the co-administration of lactoferrin 50 mg/kg BID reduced MPO levels, without reaching the level of significance. By contrast, treatment with lactoferrin at doses of 100 and 200 mg/kg BID counteracted significantly the diclofenac-

induced increment of MPO levels (Fig. 1A). Likewise, in animals treated with diclofenac, the concomitant administration of *Bifidobacterium* significantly blunted the increase in MPO levels, at all tested doses (Fig. 1A). Administration of lactoferrin or *Bifidobacterium* to control animals did not modify tissue MPO concentration (not shown).

Tissue malondialdehyde levels

In control animals, MDA levels in the ileum accounted for 24.50 ng/mg tissue (Fig. 1B). Diclofenac elicited a significant increase in MDA levels (41.8 ng/mg tissue). Under these conditions, co-treatment with lactoferrin 50 mg/kg BID did not modify MDA levels, while the doses of 100 and 200 mg/kg BID significantly counteracted the diclofenac-induced MDA increment (Fig. 1B). The concomitant administration of *Bifidobacterium* to rats with diclofenac-induced enteropathy counteracted significantly the increase in MDA levels, at all tested doses (Fig. 1B). MDA levels remained unchanged in control animals treated with lactoferrin or *Bifidobacterium* (not shown).

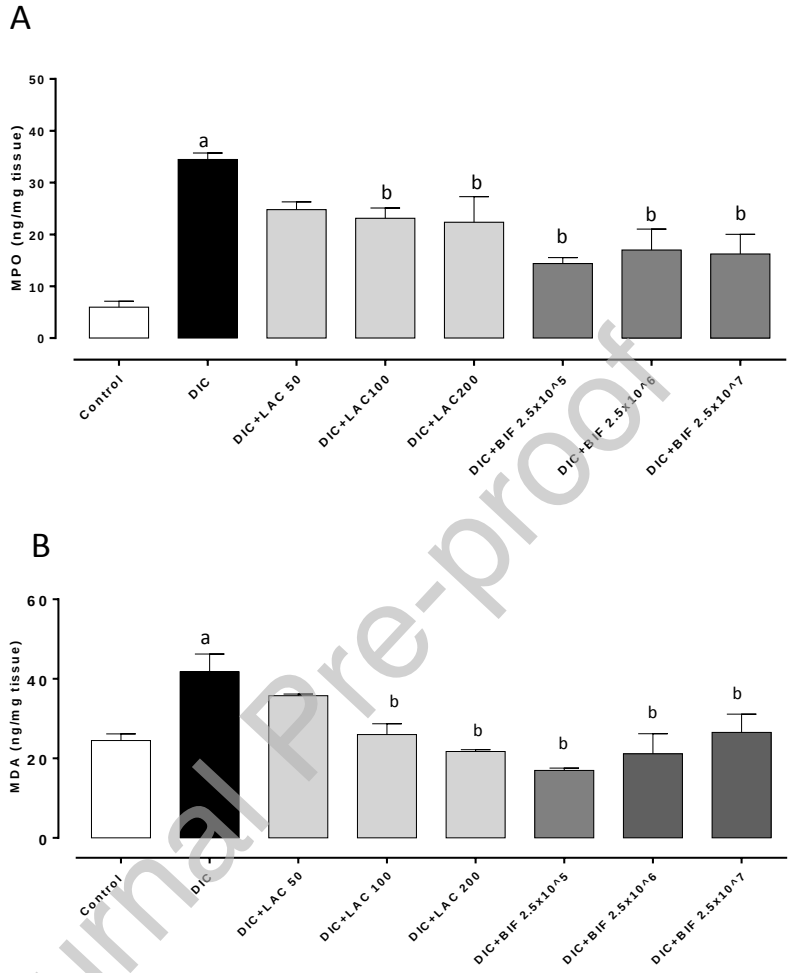

Fig. 1. Tissue levels of myeloperoxidase (MPO) **(A)** or malondialdehyde (MDA) **(B)** in the ileum from rats treated with vehicle (control) or diclofenac (DIC, 4 mg/kg BID), either alone or in concomitance with lactoferrin (LAC, 50, 100 or 200 mg/kg BID) or *Bifidobacterium longum* BB536 (BIF, $2.5 \cdot 10^5$, $2.5 \cdot 10^6$ or $2.5 \cdot 10^7$ CFU/rat BID) for 14 days. Each column represents the mean±S.E.M. from 6 animals. [a]P<0.05; significant difference *versus* Control; [b]P<0.05, significant difference vs diclofenac alone ~~[b]P<0.05, significant difference vs DIC+LAC 50; [c]P<0.05, significant difference vs DIC+BIF 2.5*10[5]~~

Main study

Microscopic damage

As expected, control animals did not display any type of lesions in the ileum, while diclofenac elicited various degrees of type 1, 2 and 3 lesions (Fig. 2). Lactoferrin or *Bifidobacterium* administration reduced significantly both type 1 and 2 lesions, while prevented completely the occurrence of type 3 lesions (Fig. 2). Administration of lactoferrin plus *Bifidobacterium* to animals with diclofenac-induced enteropathy resulted in a further significant decrease of type 1 and 2 lesions (Fig. 2).

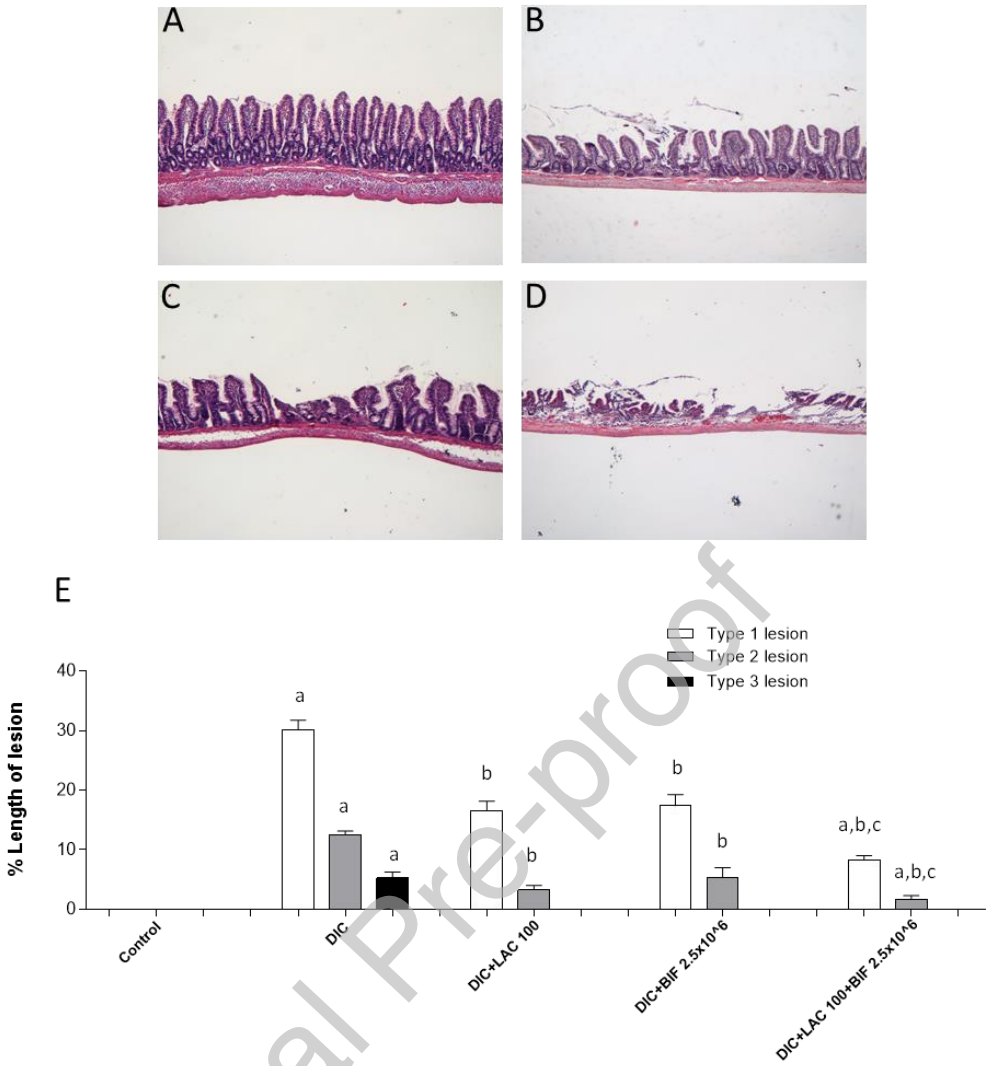

Fig. 2. Histological analysis of mucosal damage in the ileum from rats treated with vehicle (control) or diclofenac (DIC, 4 mg/kg BID), either alone or in concomitance with lactoferrin (LAC, 100 mg/kg BID), *Bifidobacterium longum* BB536 (BIF, $2.5 \cdot 10^6$ CFU/rat BID) or LAC+BIF for 14 days. . Representative pictures showing the microscopic appearance of ileal mucosa from control animals (A), as well as type 1 (B), type 2 (C) or type 3 (D) lesions, observed in animals treated with DIC. Effects of treatments on type 1, type 2 or type 3 lesions (E). Each column represents the mean±S.E.M. from 10 animals. $^{a}P<0.05$; significant difference *versus* Control; $^{b}P<0.05$, significant difference vs diclofenac alone; $^{c}P<0.05$; significant difference *versus* DIC+LAC 100 and DIC+BIF $2.5 \cdot 10^6$

Blood hemoglobin levels

In animals with diclofenac-induced small intestinal damage, the mean concentration of hemoglobin was significantly reduced, as compared with the value recorded in controls (9.97 g/dL vs 16.17 g/dL) (Fig. 3). The administration of lactoferrin to rats with diclofenac-induced enteropathy promoted a significant increase in hemoglobin concentration. Treatment with *Bifidobacterium* elicited also a significant increment of hemoglobin levels (Fig. 3). The administration of lactoferrin plus *Bifidobacterium* was associated with a further increase in blood hemoglobin levels, although being not statistically significant as compared to the administration of lactoferrin or *Bifidobacterium* alone (Fig. 3).

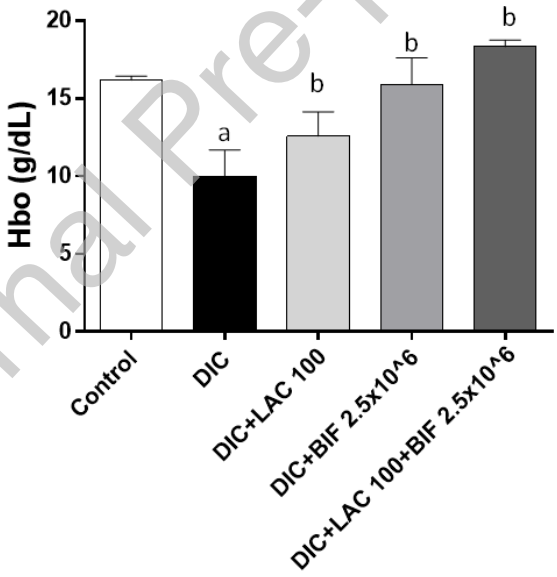

Fig. 3. Effects of vehicle (control) or diclofenac (DIC, 4 mg/kg BID), either alone or in combination with lactoferrin (LAC, 100 mg/kg BID), *Bifidobacterium longum* BB536 (BIF, $2.5 \cdot 10^6$ CFU/rat BID) or LAC+BIF for 14 days on blood hemoglobin levels. Each column represents the mean±S.E.M. from 10 animals. $^{a}P<0.05$; significant difference vs Control; $^{b}P<0.05$, significant difference vs diclofenac alone.

Myeloperoxidase levels in the ileum

Control rats displayed a mean MPO concentration in the ileum of 6.55 ng/mg of tissue. In animals treated with diclofenac, MPO levels in the ileum were significantly elevated, as compared with control animals (21.18 ng/mg of tissue) (Fig. 4A). Treatment of animals with diclofenac-induced enteropathy with lactoferrin or *Bifidobacterium* resulted in a significant decrease in MPO levels, with a similar magnitude (Fig. 4A). In rats treated with lactoferrin plus *Bifidobacterium*, MPO concentration in the ileum was reduced further, although not statistically significant as compared with the effects observed with single treatments (Fig. 4A).

Malondialdehyde levels in the ileum

MDA concentrations in the ileum from control rats accounted for 20.64 nmol/mg of tissue. Treatment with diclofenac was associated with a significant increase in MDA levels (41.78 nmol/mg of tissue) (Fig. 4B). In rats with enteropathy induced by diclofenac, treatment with lactoferrin, *Bifidobacterium* or their combination significantly reduced the MDA concentrations in the ileum, with similar effects (Fig. 4B).

Fecal calprotectin levels

In feces collected from control animals, mean calprotectin concentrations were 1.68 ng/mg of feces, while treatment with diclofenac resulted in a significant increase in fecal calprotectin (3.82 ng/mg of feces) (Fig. 4C). The administration of lactoferrin, *Bifidobacterium* or their combination exerted similar effects in counteracting the diclofenac-induced increase in fecal calprotectin levels (Fig. 4C).

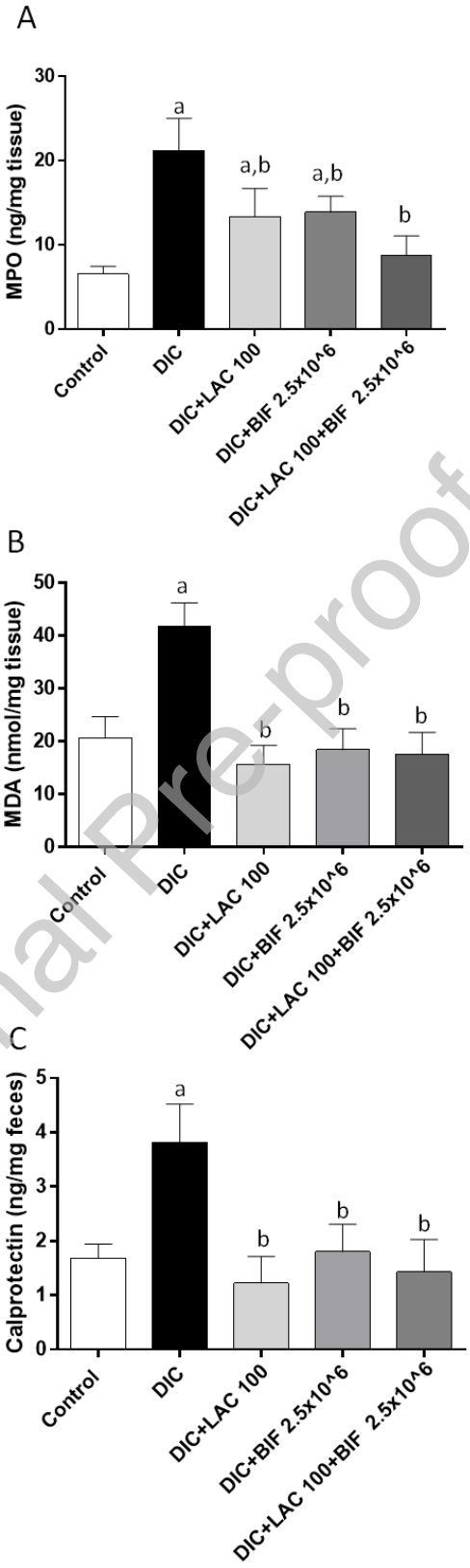

Fig. 4. Effects of vehicle (control) or diclofenac (DIC, 4 mg/kg BID), either alone or in concomitance with lactoferrin (LAC, 100 mg/kg BID), *Bifidobacterium longum* BB536 (BIF, $2.5 \cdot 10^6$ CFU/rat BID) or LAC+BIF for 14 days on tissue myeloperoxidase (MPO) **(A)**, malondialdehyde (MDA) **(B)** or fecal calprotectin **(C)**. Each column represents the mean±S.E.M. from 10 animals. [a]P<0.05; significant difference *versus* Control; [b]P<0.05, significant difference vs diclofenac alone.

Expression of TLR-2, TLR-4, p65 and MyD88

In the ileum from animals treated with diclofenac, TLR-4 expression was increased, as compared with controls (Fig. 5A). The administration of lactoferrin caused a slight, not significant, decrease in TLR-4 expression, while *Bifidobacterium* or lactoferrin plus *Bifidobacterium* significantly reduced TLR-4 protein levels (Fig. 5A).

The expression of TLR-2 receptors was enhanced in the ileum from diclofenac-treated rats, as compared with controls (Fig. 5B). The administration of lactoferrin, *Bifidobacterium* or their combination resulted in a further increment of TLR-2 expression, which, however, did not reach the level of statistical significance (Fig. 5B).

In rats treated with diclofenac, the expression of MyD88 in the ileum was increased, as compared with control animals (Fig. 5C). Under these conditions, co-administration of lactoferrin, *Bifidobacterium* or lactoferrin plus *Bifidobacterium* did not modify the pattern of MyD88 protein expression (Fig. 5C).

In ileal tissues isolated from rats with diclofenac-induced enteropathy, the expression of p65 was enhanced (Fig. 5D). Treatment with lactoferrin, *Bifidobacterium* or lactoferrin plus *Bifidobacterium* counteracted such an increase (Fig. 5D).

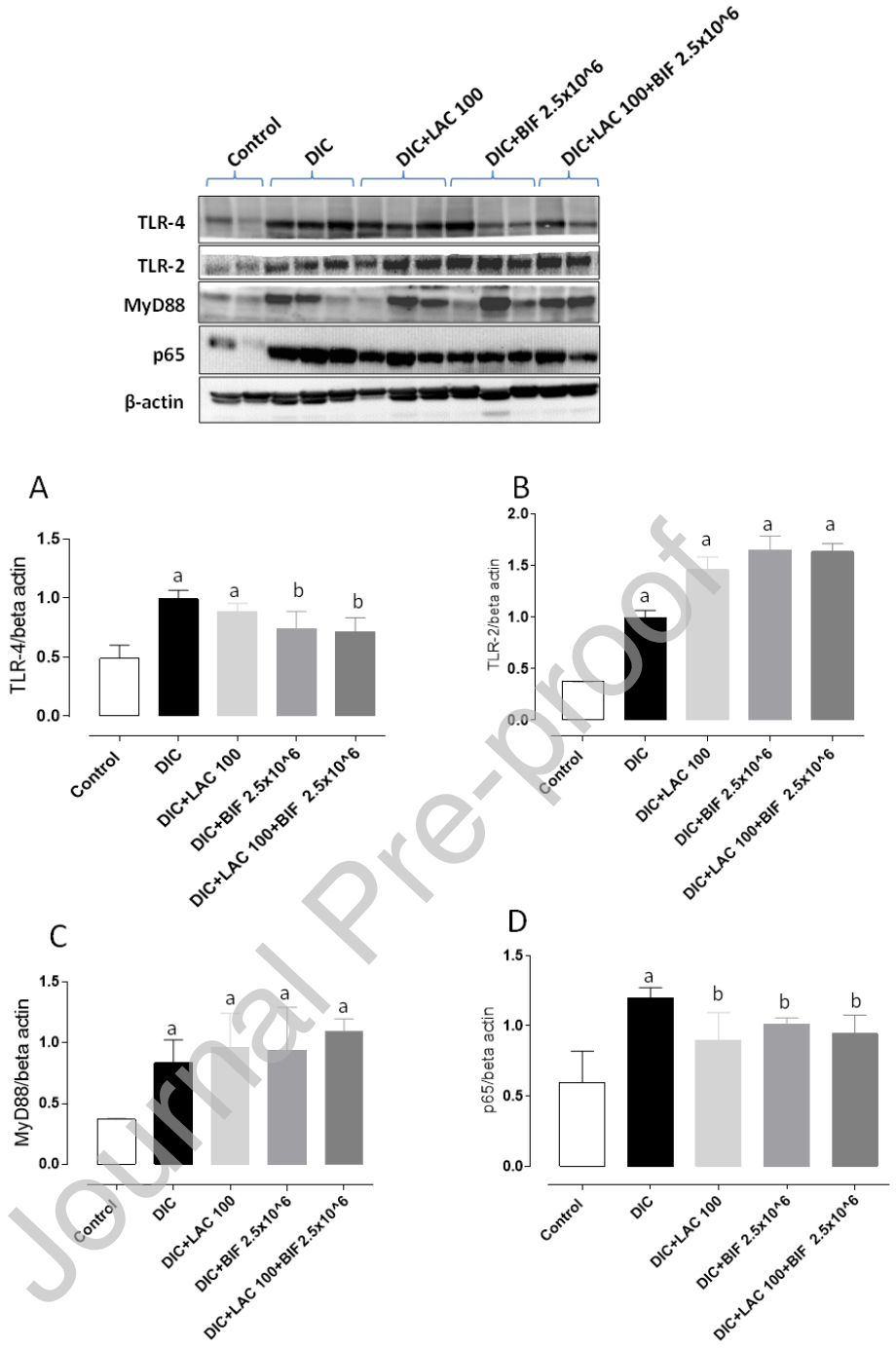

Fig. 5. Western blot analysis of toll-like receptor-4 (TLR-4) **(A)**, toll-like receptor-2 (TLR-2) **(B)**, myeloid-differentiation primary response gene 88 (MyD88) **(C)** and activated nuclear factor-kB (p65) **(D)**, in the ileum of rats treated with vehicle (control) or diclofenac (DIC, 4 mg/kg BID), either alone or in combination with lactoferrin (LAC, 100 mg/kg BID), *Bifidobacterium longum* BB536 (BIF, $2.5 \cdot 10^6$ CFU/rat BID) or LAC+BIF for 14 days. Each

column represents the mean±S.E.M. from 10 animals. [a]P<0.05; significant difference *versus* Control; [b]P<0.05, significant difference vs diclofenac alone.

Discussion

The use of NSAIDs is associated with the development of enteric damage and occult bleeding, the so-called enteropathy [2], which occur even more frequently than upper digestive injury [3]. In the present study, consistently with current evidence [17,18,19], we observed that such enteric damaging effects are characterized by the development of tissue inflammation, oxidative stress, and intestinal bleeding. Despite the above knowledge, at present, in the clinical setting no effective and specific therapeutic interventions are available as protective strategies for prevention of NSAID-induced enteropathy. However, a growing body of evidence supports the contention that the use of probiotics and prebiotics could represent a valuable tool for the protection of the intestinal tract against the detrimental effects associated with chronic NSAID therapy [15,20,21,22]. In this respect, our aim was to investigate the putative enteroprotective effects of lactoferrin and *Bifidobacterium* in a validated experimental model of diclofenac-induced enteropathy.

In our experiments, separate administrations of lactoferrin or *Bifidobacterium* to rats with diclofenac-induced enteropathy resulted in a significant decrease in small bowel damage, with a complete disappearance of the most severe type 3 lesions and a significant decrease in both type 1 and 2 lesions. Of note, the combined administration of lactoferrin plus *Bifidobacterium* was found to be more effective in reducing the intestinal damage as compared with each single treatment, thus suggesting that this combination could represent a suitable therapeutic tool for the protection of digestive mucosa against NSAID-induced enteropathy. With regard for the other examined parameters, no significant differences were detected when comparing separate treatments with lactoferrin and *Bifidobacterium* with their combined administration.

However, trends in changes in plasma hemoglobin levels and MPO, as well as in reduction of the expression of TLR-4 and p65, were observed for the combined treatment. Therefore, it is likely that the overall sum of such trends concurred to produce a significantly better efficacy against the histologic damage upon administration of lactoferrin plus *Bifidobacterium*, which represents the most significant and clinically relevant outcome. Of note, the effects exerted by the present treatments on the majority of such examined parameters correlated with their ability to reduce the extent and severity of intestinal damage, thus highlighting the relevance of such effects. Among them, fecal calprotectin is of particular interest, since it has been shown to undergo a significant increase both in experimental models of enteropathy and in patients treated with NSAIDs. In particular, in a clinical trial on healthy volunteers, treatment with indomethacin resulted in a significant increase in mean fecal calprotectin levels, to a similar extent to that observed in our experimental model (2-3 fold increase) [23]. Moreover, the administration of a probiotic mixture was able to prevent completely such increments of fecal calprotectin, thus indicating a clinical reliability of such index. Taken together, these observations suggest that the effects of treatment with LAC and BIF, as observed in our model, can be considered as clinically meaningful.

Intestinal bleeding is one of the main complication and life-threatening event associated with NSAID-induced intestinal injury [6]. In our experimental model, diclofenac elicited a significant decrease in blood hemoglobin levels, which indirectly reflects the occurrence of enteric bleeding [18]. Under these conditions, lactoferrin and *Bifidobacterium* blunted significantly the hemoglobin decrease. In keeping with our findings, previous reports indicate that lactoferrin counteracted intestinal bleeding in different experimental models, including NSAID/enteropathy [14,15]. Data concerning the specific effects of *Bifidobacterium* on gut bleeding in NSAID-induced enteropathy are currently lacking. However, in accordance with the present data on tissue damage, the combination of lactoferrin plus *Bifidobacterium* was

more effective than separate treatments in reducing digestive bleeding, thus corroborating further the suitability of this combined intervention as valuable tool for the protection of intestinal mucosa against NSAID-induced damage.

MPO concentration in intestinal tissues represents a reliable marker reflecting the inflammatory infiltration of polymorphonuclear cells [24]. In line with previous observations [17,18], repeated administrations of diclofenac resulted in a significant increment of ileal MPO concentrations. Under these conditions, treatment with lactoferrin or *Bifidobacterium* counteracted significantly such an increment, while ileal MPO levels were normalized when these agents were administered in combination. Of note, these results are in keeping with the patterns of protective activity of lactoferrin and *Bifidobacterium* against intestinal damage and bleeding, and therefore they suggest the involvement of anti-inflammatory mechanisms.

Oxidative stress is a condition commonly associated with intestinal damage, particularly in the presence of NSAID-induced enteropathy [16,18]. Herein, we confirmed that treatment with diclofenac resulted in a significant elevation of MDA concentration in the ileum. In this setting, both lactoferrin and *Bifidobacterium*, either alone or in combination, prevented completely the increment of MDA induced by diclofenac. Of interest, the antioxidant properties of lactoferrin have been documented in different experimental conditions [25]. In particular, lactoferrin can directly scavenge reactive oxygen species (ROS) and reduce neutrophil oxidative bursts [26,27]. In addition, previous studies provided evidence that some probiotics belonging to the *Bifidobacterium* genus are characterized by the ability of counteracting the occurrence of oxidative stress at intestinal level [28,29]. Thus, based on our findings and current knowledge, it is conceivable that the activation of antioxidant mechanisms can take a significant part in the protective effects exerted by lactoferrin and *Bifidobacterium* against the intestinal injury induced by diclofenac.

Fecal calprotectin is a calcium binding protein, which correlates well with the infiltration of neutrophils in the intestinal mucosa, and thereby representing a reliable marker of bowel inflammatory response [30]. In previous studies, treatment with diclofenac has been associated with a significant increment of fecal calprotectin both in experimental models [17] and human subjects [31]. In keeping with these findings, in the present study rats with diclofenac-induced enteropathy displayed elevated levels of fecal calprotectin. Under these conditions, lactoferrin, *Bifidobacterium* or their combination prevented completely this calprotectin elevation, thus corroborating further our hypothesis that the beneficial effects exerted by both these agents are related to the activation of anti-inflammatory mechanisms.

In accordance with our previous findings [17], we observed an enhanced expression of both TLR-4 and TLR-2 in the ileum from rats treated with diclofenac. These data are consistent with the results of Watanabe et al. [32], who reported an up-regulation of TLR-4 in an acute model of NSAID-enteropathy, thus suggesting the involvement of Gram-negative bacteria in the pathogenesis of mucosal inflammation and intestinal lesions. Our findings, showing an enhanced expression of TLR-2, suggest a possible role also for Gram-positive microorganisms. Indeed, TLR-4 has been found to act as a receptor for LPS (a major component of the outer membrane of Gram-negative bacteria), while TLR-2 binds preferentially peptidoglycan and lipoteichoic acid (two major cell wall components of Gram-positive bacteria) [33]. Interestingly, in our experiments treatment with lactoferrin did not modify the increased expression of TLR-4, while *Bifidobacterium* reduced significantly the TLR-4 levels in rats with enteropathy, either alone or in combination with lactoferrin. It is therefore conceivable that the downregulation of TLR-4 is driven mainly by *Bifidobacterium*. In support of this conclusion, it has been shown that *B. longum* counteracts the upregulation of TLR-4 expression in rat intestinal epithelial cells stimulated with enteropathogenic *Escherichia coli* endotoxin [34]. Since there is evidence that TLRs mediate pro-inflammatory

signaling in the intestinal mucosa, it is likely that the present downregulation of TLR-4 promoted by *Bifidobacterium* accounts for its anti-inflammatory action and protective effect against diclofenac-induced enteropathy.

Another interesting observation made in our study was that both lactoferrin and *Bifidobacterium*, either alone or in combination, increased further the expression of TLR-2 as compared with rats treated with diclofenac alone, even though such an increment did not reach the level of statistical significance. TLR-2 are thought to mediate the anti-inflammatory actions of some *Bifidobacterium* strains, including the strain BB536 tested in the present study. Indeed, Tomosada et al. [35] observed that BB536 was able to counteract the inflammatory response of intestinal epithelial cells to stimulation with the enterotoxigenic *Escherichia coli* pathogen-associated molecular patterns, and that such an effect was mediated by the activation of TLR-2. Of interest, TLR-2 stimulation with lipoarabinomannan or lipoteichoic acid has been found to counteract indomethacin-induced small intestinal damage through a decrease in TLR-4 expression [36]. Therefore, it appears that the anti-inflammatory effects exerted by *Bifidobacterium* in our study could be the consequence of TLR-2 activation leading to a suppression of TLR-4 signaling. Data concerning a putative involvement of TLR-2 in the anti-inflammatory effects of lactoferrin are currently lacking, and therefore the possible role of TLR-2 in the protective effects exerted by this prebiotic remains to be clarified.

The stimulation of TLRs is known to activate MyD88-dependent NF-kB signaling, which plays a critical role in immune/inflammatory responses [33]. In our experiments, this pathway was found to be activated in rats with diclofenac-induced enteropathy. However, neither lactoferrin nor *Bifidobacterium* were able to modify this increased pattern of MyD88 expression. Nevertheless, both treatments were able to counteract the activation of the pro-inflammatory NF-kB signalling induced by diclofenac. Similar observations have been made

previously in different models of inflammation. Indeed, Li et al. [37] found that lactoferrin counteracted the pro-inflammatory NF-kB activation in a model of LPS-induced endometritis in mice. Furthermore, Guo et al. [38] found that *Bifidobacterium infantis* was able to counteract NF-kB activation in Caco-2 cells stimulated with IL-1β. Thus, it is likely that, in our experiments, lactoferrin and *Bifidobacterium* were able to downregulate NF-kB signaling without modifying the expression pattern of upstream MyD88, which was enhanced by diclofenac and was likely to serve both the anti-inflammatory and pro-inflammatory actions mediated by TLR-2 and TLR-4, respectively.

Another point, deserving attention, is the putative effect of lactoferrin and *Bifidobacterium* on gut microbiota composition. In our preliminary experiments, the total load of enteric bacteria was not affected by diclofenac (see Supplementary material), either alone or in combination with lactoferrin and *Bifidobacterium*. Moreover, we have previously observed that diclofenac did not affect significantly the relative abundance of Actinobacteria, which include the genus *Bifidobacterium* [17]. Taken together, these findings support the view that putative changes in the relative abundance of *Bifidobacteria* would be unlikely to take a relevant part in the entero-protective effects of lactoferrin and *Bifidobacterium*, which appear to be driven mostly on anti-inflammatory actions exerted at the level of intestinal mucosa.

Conclusions

Based on the present findings, it can be concluded that the protective effects exerted by lactoferrin and *Bifidobacterium* against diclofenac-induced enteropathy could be supported by antioxidant and anti-inflammatory effects. These appear to be mediated, at least in part, by the modulation of TLR-2/-4/NF-kB pathways. Overall, we have provided original evidence that the combination of lactoferrin and *Bifidobacterium* may represent a suitable therapeutic intervention for the prevention of NSAID-induced small bowel damage. Accordingly, these

observations encourage clinical investigations designed to explore the value of the lactoferrin/ *Bifidobacterium* combination in the management of the risk of NSAID-induced enteropathy.

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Authorship

MF, LA, CB, RC: conception and design of the study; CP, LB, ET, DG, LR, GN, EP: generation, collection, assembly, analysis and/or interpretation of data; MF, CB, EG: drafting or revision of the manuscript; CB, RC, LA, EG: approval of the final version of the manuscript.

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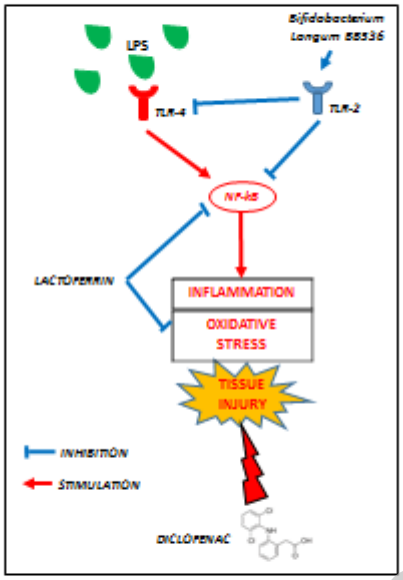
Table 1. Microscopic analysis of mucosal damage in the ileum from rats treated with vehicle (control) or diclofenac (DIC, 4 mg/kg BID), either alone or in concomitance with lactoferrin (LAC, 50, 100 or 200 mg/kg BID) or *Bifidobacterium longum* BB536 (BIF, $2.5 \cdot 10^5$, $2.5 \cdot 10^6$ or $2.5 \cdot 10^7$ CFU/rat BID) for 14 days.

	Control	DIC	DIC+LAC 50	DIC+LAC 100	DIC+LAC 200	DIC+BIF $2,5x10^5$	DIC+BIF $2,5x10^6$	DIC+BIF $2,5x10^7$
Type 1 lesion	0	29,25±1,07[a]	18,25±1,66[a,b]	16,81±1,14[a,b]	13,89±0,80[a,b]	18,20±1,42[a,b]	15,90±2,35[a,b]	13,61±0,64[a,b]
Type 2 lesion	0	13,39±1,39[a]	8,67±0,67[a]	2,98±0,53[a,b,c]	3,44±0,19[a,b,c]	9,09±0,55[a]	4,77±0,19[a,b,d]	4,66±0,34[a,b,d]
Type 3 lesion	0	4,91±0,47[a]	2,18±0,39[a,b]	0[c]	0[c]	1,57±0,64[a,b]	0[d]	0[d]

Each number represents the mean±S.E.M. from 6 animals. [a]P<0.05; significant difference versus Control; [b]P<0.05, significant difference vs diclofenac alone; [c]P<0.05, significant difference vs DIC+LAC 50; [d]P<0.05, significant difference vs DIC+BIF $2.5x10^5$

Graphical abstract



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: