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RESEARCH ARTICLE

Use of *Saccharomyces boulardii* CNCM I-745 as therapeutic strategy for prevention of nonsteroidal anti-inflammatory drug-induced intestinal injury

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Abstract

Background and Purpose: Nonsteroidal anti-inflammatory drugs (NSAIDs) can be associated with severe adverse digestive effects. This study examined the protective effects of the probiotic *Saccharomyces boulardii* CNCM I-745 in a rat model of diclofenac-induced enteropathy.

Experimental Approach: Enteropathy was induced in 40-week-old male rats by intragastric diclofenac (4 $mg \cdot kg^{-1}$ BID for 14 days). *S. boulardii* CNCM I-745 (3 $g \cdot kg^{-1}$ BID by oral gavage) was administered starting 14 days before (preventive protocol) or along with (curative protocol) diclofenac administration. Ileal damage, inflammation, barrier integrity, gut microbiota composition and toll-like receptors (TLRs)–nuclear factor κB (NF-κB) pathway were evaluated.

Key Results: Diclofenac elicited intestinal damage, along with increments of myeloperoxidase, malondialdehyde, tumour necrosis factor and interleukin-1β, overexpression of TLR2/4, myeloid differentiation primary response 88 (Myd88) and NF-κB p65, increased faecal calprotectin and butyrate levels, and decreased blood haemoglobin levels, occludin and butyrate transporter monocarboxylate transporter 1 (MCT1) expression. In addition, diclofenac provoked a shift of bacterial taxa in both faecal and ileal samples. Treatment with *S. boulardii* CNCM I-745, in both preventive and curative protocols, counteracted the majority of these deleterious changes. Only preventive administration of the probiotic counteracted NSAID-induced decreased expression of MCT1 and increase in faecal butyrate levels. Occludin expression, after probiotic treatment, did not significantly change.

Abbreviations: Myd88, myeloid differentiation primary response 88; NSAIDs, nonsteroidal anti-inflammatory drugs.

Vanessa D'Antongiovanni, Luca Antonioli and Laura Benvenuti contributed equally to the manuscript.

Conclusions and Implications: Treatment with *S. boulardii* CNCM I-745 prevents diclofenac-induced enteropathy through anti-inflammatory and antioxidant activities. Such effects are likely to be related to increased tissue butyrate bioavailability, through an improvement of butyrate uptake by the enteric mucosa.

KEYWORDS
enteropathy, intestinal damage, microbiota, nonsteroidal anti-inflammatory drugs, probiotics, *Saccharomyces boulardii*

1 | INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most consumed drugs in the world owing to their beneficial actions as analgesic, anti-inflammatory and antipyretic agents (Scarpignato et al., 2015). However, it is well established that the chronic administration of these drugs is associated with a broad spectrum of adverse reactions, including those in the upper gastrointestinal (GI) tract (Pereira-Leite et al., 2017). Over the last years, it has been increasingly acknowledged that NSAIDs can also trigger the development of adverse events in the lower digestive tract (mostly, but not limited to the small bowel) in a large proportion of patients (Bjarnason & Rainsford, 2021; Colucci et al., 2018; Fornai et al., 2014; Lanas et al., 2009; Pereira-Leite et al., 2017; Scarpignato & Bjarnason, 2019; Watanabe et al., 2020). The most common side effects complained by patients, include dyspepsia, heartburn and/or abdominal discomfort, intestinal perforation and ulcers (Lanas et al., 2009; Svistunov et al., 2018).

In the initial stage, NSAID-induced enteropathy depends on direct effects on intestinal mucosa. This is due to the accumulation of NSAIDs within epithelial cells via a damage to the brush border of cell membrane and disruption of the mitochondrial processes of oxidative phosphorylation, with consequent **ATP** deficiency, activation of apoptotic cell death and alterations of tight junction protein expression (Bjarnason et al., 2018; Wallace, 2013). These events would then lead to an increased mucosal permeability, with facilitation of the entry of injurious luminal factors (i.e. dietary macromolecules, bile acids and enteric bacteria), which trigger an immune/inflammatory response. In parallel, it has been reported that NSAID treatment can also modify the composition of intestinal microbiota (condition known as dysbiosis), by affecting bacterial metabolism/growth and/or inducing microbial cell death (Wang et al., 2021). In particular, preclinical studies observed a significant increase in the abundance of Gram-negative bacteria following NSAID treatment and such increases are positively correlated with the severity of NSAID-induced intestinal damage (Wang et al., 2021), suggesting the relevant role of intestinal dysbiosis in the pathophysiology of NSAID-induced enteropathy. In line with this concept, several studies have shown that antimicrobials (**rifaximin**, **tetracycline**, **kanamycin**, **metronidazole** or **neomycin** plus bacitracin) can attenuate or prevent NSAID-induced enteropathy, thus corroborating the pathogenic role of enteric bacteria (Colucci et al., 2018; Fornai et al., 2016; Lanas & Scarpignato, 2006). Indeed, it is well known that the increase of bacterial endotoxin, as a result of the increased Gram-negative bacteria composition, significantly contributes to ulceration in the intestine associated with NSAID therapy (Liang et al., 2015; Watanabe et al., 2020). Consequently, cyclic antibiotic administration, getting rid of enteric pathogenic bacteria, would protect the intestine from the damaging effect of anti-inflammatory compounds. In this respect, clinical studies showed that metronidazole (an antimicrobial targeting most Gram-negative and Gram-positive anaerobic bacteria) was able to reduce inflammation and blood loss in patients taking NSAIDs, thus suggesting a therapeutic potential of antimicrobials (Bjarnason et al., 1992; Leite et al., 2001). However, in

What is already known

- The use of NSAIDs is associated with a broad spectrum of gastrointestinal adverse reactions.
- Currently, no effective and specific therapeutic interventions are available for prevention of NSAID-induced enteropathy.

What does this study add

- Treatment with *S. boulardii* CNCM I-745 prevents diclofenac-induced enteropathy through anti-inflammatory and antioxidant activities.
- *S. boulardii* CNCM I-745 is able to exert beneficial effects on tissue butyrate bioavailability.

What is the clinical significance

- *S. boulardii* CNCM I-745 administration may represent a strategy for the prevention of NSAID-induced enteropathy.
- *S. boulardii* CNCM I-745 administration should be started before any scheduled therapy with NSAIDs.

the clinical setting, the use of antibiotics is associated with a significant risk of adverse effects and increase in bacterial resistance.

Recently, several lines of evidence have suggested that appropriate manipulations of the enteric microbiome through probiotics could represent a useful strategy for the management of bowel inflammatory conditions, including NSAID-induced enteropathy, without the occurrence of significant adverse reactions (Montalto et al., 2010; Satoh & Takeuchi, 2012). For instance, *Bifidobacterium* and *Lactobacillus* strains have been proven to protect against NSAID-induced intestinal damage (X. Chen et al., 2022; Endo et al., 2011; Engel et al., 2022; Fornai et al., 2020; Gotteland et al., 2001; Montalto et al., 2010; Mortensen et al., 2019; Ocón et al., 2013; Suzuki et al., 2017; Yoshihara et al., 2020). The mechanisms underlying the enteroprotective effects of some probiotics remain poorly understood. However, it is conceivable that such strains could exert strengthening effects on the intestinal epithelial barrier (IEB) and counteract the development of inflammatory reactions within the enteric mucosa. In parallel, non-bacterial probiotics, including *Saccharomyces boulardii* (*S. boulardii*), have also been shown to exert enteroprotective actions by exerting anti-inflammatory effects and strengthening the intestinal mucosal barrier, as well as counteracting intestinal dysbiosis under different experimental conditions (X. Chen et al., 2013; Pothoulakis, 2009; Roy Sarkar et al., 2021; Soyturk et al., 2012). Indeed, *S. boulardii*, besides reducing the intestinal mucosal expression of pro-inflammatory cytokines, is able to compete with pathogens that adhere to host cells, thus favouring their elimination (Mumy et al., 2008; Pais et al., 2020). Of note, the interaction with enteropathogenic microorganisms and the beneficial effects on the intestinal mucosa represent the potential mechanisms underlying the enteroprotective effects of the probiotic yeast, although further studies are needed.

Based on the above background and current knowledge, the present study was designed to assess the ability of *S. boulardii* CNCM I-745, strain produced by Biocodex, in preventing intestinal damage and enteric inflammation associated with NSAID administration in a rat model of **diclofenac**-induced enteropathy. In addition, the main mechanisms underlying their enteroprotective actions have also been examined.

2 | METHODS

2.1 | Animals

Albino male Wistar rats (40 weeks old) were used throughout the study. The animals were fed with standard laboratory chow and tap water *ad libitum*, and were not subjected to experimental procedures for 2 weeks after their delivery to the laboratory. They were housed individually in solid-bottomed cages, equipped with wire-mesh bottom inserts to prevent coprophagy and located in temperature-controlled rooms, 22–24°C, under a 12-h light cycle. The cages were washed two/three times a week. All the procedures involving animals were carried out following the guidelines of the European Community Council Directive 86-609 and in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki, EU Directive 2010/63/EU for animal experiments). The experiments have been approved by the Ethical Committee for Animal Experimentation of the University of Pisa and by the Italian Ministry of Health (Authorization No. 547/2020-PR). All efforts to reduce and minimize the number of animals and their suffering were carried out. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). A randomization of animals between groups was carried out in order to generate groups of equal size. The design in this study complies with the recommendations on experimental design in pharmacology (Curtis et al., 2022).

2.2 | Experimental design

Diclofenac-induced enteropathy has been performed according to the methodology developed by Fornai et al. (2020). Briefly, non-fasted rats were administered by intragastric route for 14 days with diclofenac 4 $mg \cdot kg^{-1}$ BID suspended in 1% methylcellulose (0.3 ml per rat). Groups of animals receiving diclofenac or vehicle were treated with *S. boulardii* CNCM I-745 (Biocodex, France) 1 h before diclofenac administration (3 $g \cdot kg^{-1}$ BID suspended in drinking water and administered by gavage). The dose of diclofenac and *S. boulardii* was selected according to previous studies (Briand et al., 2019; Fornai et al., 2020; Girard et al., 2014). Animals were randomly divided into 6 groups of 10–15 rats per group, following the experimental design reported in Figure 1. An a priori power analysis was carried out to identify the necessary number of animals; blood haemoglobin was considered as the reference parameter. In addition, a different number of animals for each group of treatment were employed to compensate the different mortality rates affecting the diclofenac-treated groups, as observed also in our previous studies (Colucci et al., 2018; Fornai et al., 2014).

At Days −14, 0 and +14, rats were placed individually in clean cages and faeces from each animal were collected with clean tweezers immediately after the pellet extrusion.

Twenty-four hours after the last diclofenac or vehicle administration, animals were euthanized with CO_2 and subsequent cervical dislocation, blood samples were collected and employed for the evaluation of haemoglobin content and the small intestine was removed. Samples of ileum were snap frozen in liquid nitrogen and stored at −80°C for subsequent evaluations. Other portions of ileal tissue, collected as described below, were fixed in 10% formalin for subsequent evaluation of microscopic damage.

2.3 | Histological assessment of small bowel injury

The histological evaluation of intestinal injury was carried out as previously described (Fornai et al., 2020; Pellegrini et al., 2018). Upon removal, the whole intestinal tract was opened along the antimesenteric border and cleaned of faecal content. In particular, in order to rule out any bias, ileal samples were taken in accordance to the following procedure: (1) The full length of small intestine was measured, (2) 1 cm of tissue proximal to the ileocecal valve was discarded and

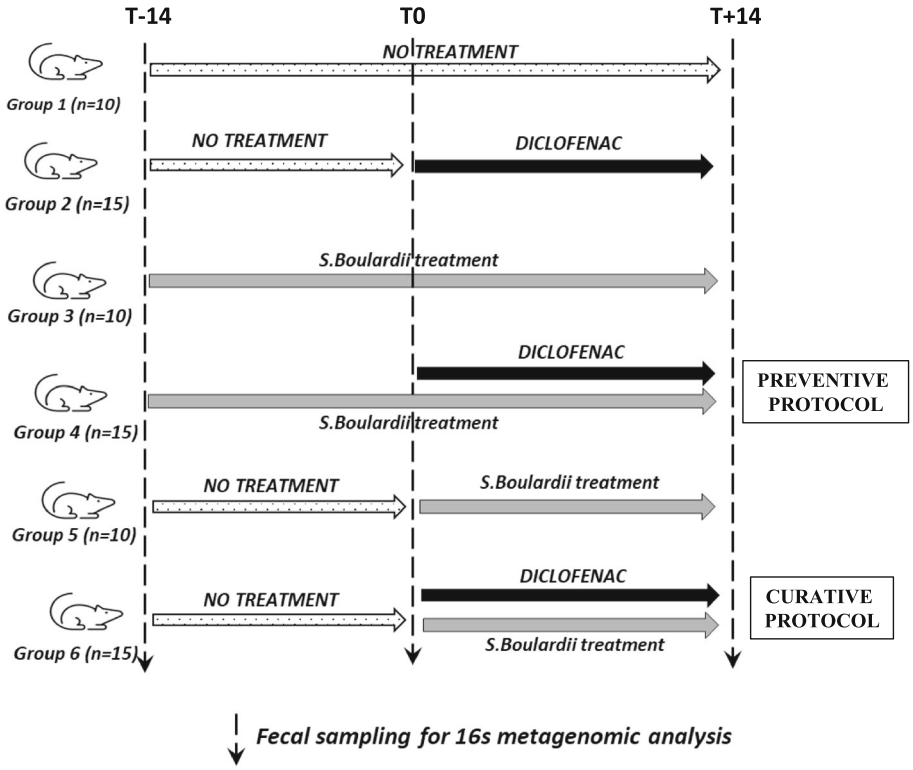

FIGURE 1 Schematic representation of the experimental design. Non-fasted rats were administered by intragastric route for 14 days with diclofenac (4 mg·kg^{-1} BID). Groups of animals receiving diclofenac or vehicle were treated with *Saccharomyces boulardii* CNCM I-745 (3 g·kg^{-1} BID by oral gavage) 14 days before (preventive protocol, i.e. 28 days of treatment) or in concomitance (curative protocol, i.e. 14 days of treatment) with diclofenac administration. Faeces were collected before treatment (T_{-14}) and before the first administration of diclofenac (T_0). At the end of treatment (T_{+14}), blood samples and faeces were collected from all animals for the evaluation of haemoglobin content and metagenomic analysis, respectively. Samples of ileum were snap frozen in liquid nitrogen and stored at −80°C for subsequent evaluations.

two specimens of 1.5–2 cm of ileum were taken at this edge, as well as 5 cm away from the first ileum sample and (3), specimens were fixed in 10% formalin for 24 h, embedded into paraffin blocks and cut into consecutive serial 3-μm sections for staining with haematoxylin and eosin. Histological damage was assessed by two observers blind to the treatment, according to the score criteria reported in Table 1. The intestinal damage was expressed as percentage of the total length of lesions over the total length of the histological section.

The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

TABLE 1 Microscopic criteria for quantitative estimation of the intestinal injury elicited by diclofenac.

Type 1 injury	Damage confined to the tunica mucosa De-epithelization Significant morphologic alterations of villi
Type 2 injury	Inflammatory infiltration in the submucosa, with thickening of the tunica muscularis or serosa The morphologic framework of tunica mucosa is preserved
Type 3 injury	Damage involves the full thickness of intestinal wall The morphologic patterns of tunicae are lost Inflammatory reaction widely extended to the tunica serosa with a significant increase in thickness

2.4 | 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 earlier by means of QuantiChrom Hemoglobin Assay Kit (Bioassay Systems, Hayward, CA, USA) and expressed as grams per decilitre.

2.5 | Evaluation of tissue myeloperoxidase (MPO) and malondialdehyde (MDA) levels

Myeloperoxidase 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. MDA concentration in intestinal tissues was determined to obtain quantitative estimates of membrane lipid peroxidation (Antonioli et al., 2021; Fornai et al., 2020). MPO and MDA levels in the ileum were assessed according to previously adopted methods (Antonioli et al., 2021; Fornai et al., 2020), described in the supporting information.

For MPO evaluation, specimens of ileum (30 mg) were homogenized on ice with a polytron homogenizer (QIAGEN, Milan, Italy) in 0.6 ml of ice-cold lysis buffer (200 mM of NaCl, 5 mM of ethylenediaminetetraacetic acid [EDTA], 10 mM of Tris, 10% glycerine, 1 mM of phenylmethylsulfonyl fluoride [PMSF], 1 μg·ml^{-1} of leupeptin and 28 μg·ml^{-1} of aprotinin) (pH 7.4). The homogenate was centrifuged two times at 4°C for 15 min at 1500 g. The supernatant was diluted 1:5 and used for determination of myeloperoxidase concentration by means of enzyme-linked immunosorbent assay (ELISA) (Hycult Biotech, Uden, Netherlands). All samples were stored at −80°C and

assayed within 2 days after collection. The results were expressed as nanograms of MPO per milligram of intestinal tissue.

For MDA analysis, intestinal tissue was excised, weighed, minced by forceps, homogenized in 2 ml of cold buffer (20 mM of Tris–HCl, pH 7.4) using a polytron homogenizer (QIAGEN) and centrifuged at 1500 g for 10 min at 4°C. Aliquots of supernatants were then used for subsequent assay procedures. Tissue MDA concentrations were estimated using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA). The results were expressed as nanomoles of MDA per milligram of intestinal tissue.

2.6 | Evaluation of tissue tumour necrosis factor (TNF) and interleukin (IL)-1β levels

TNF and **IL-1β** levels in the ileum were measured by ELISA kits (Abcam), as previously described (D'Antongiovanni et al., 2021; Pellegrini et al., 2021, 2022). For this purpose, tissue samples from ileum, stored previously at −80°C, were weighed, thawed, homogenized in 0.4 ml of phosphate-buffered saline (PBS), pH 7.2/20 mg of tissue at 4°C and centrifuged at 10,000 g for 5 min. Aliquots (100 µl) of supernatants were then used for assay. Tissue TNF and IL-1β levels are expressed as picograms per gram of tissue and picograms per milligram of proteins, respectively.

2.7 | Assay of faecal calprotectin

Calprotectin, a calcium binding protein of neutrophil granulocytes that correlates well with neutrophil infiltration of the intestinal mucosa, was measured in faecal pellets, as previously described (Colucci et al., 2018). In particular, freeze-dried faecal pellets were reconstituted in 1 ml of PBS, along with 50 µl 1% ($wt \cdot vol^{-1}$) **ascorbic acid**. Samples were then homogenized for 10 min (4°C). Homogenates were diluted with 2 ml of lysis buffer (0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.02% sodium azide, 5 mM of disodium EDTA and 1× Halt protease/phosphatase inhibitor cocktail [Thermo Fisher Scientific Inc, Waltham, MA, USA] in PBS). Homogenates were further homogenized for 30 s and centrifuged (5800 g, 10 min, 4°C), and supernatants were snap frozen in liquid nitrogen and stored at −80°C. Faecal calprotectin levels were determined using rat calprotectin ELISA kit and analysed following the manufacturer's instructions (DBA, Milan, Italy).

2.8 | Protein extraction and Western blot analysis

The ileum was collected from rats and flushed of faecal content with ice-cold PBS, as described previously (D'Antongiovanni et al., 2022; Pellegrini et al., 2020). Tissues were minced and homogenized using a Potter-Elvehjem Grinder homogenizer. Samples were then sonicated and boiled for 5 min at 95°C. Proteins were quantified with the Bradford assay. Total lysates were run on a 4%–20% Criterion™ TGX™ Precast Midi Protein Gel (Bio-Rad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride (PVDF) membranes (Trans-Blot Turbo™ PVDF Transfer packs, Bio-Rad). Membranes were blocked with 3% bovine serum albumin (BSA) diluted in Tris-buffered saline (TBS; 20 mM of Tris–HCl, pH 7.5 and 150 mM of NaCl) with 0.1% Tween 20. Primary antibodies against β-actin (Abcam, Cat# ab8227, RRID:AB_2305186), monocarboxylate transporter 1 (MCT1; Thermo Fisher Scientific, Cat# PA5-76687, RRID:AB_2720414), myeloid differentiation primary response 88 (MyD88; Santa Cruz Biotechnology, Cat# sc-136970, RRID:AB_2146721), nuclear factor κB (NF-κB; Santa Cruz Biotechnology, Cat# sc-136970, RRID:AB_2146721), occludin (Abcam, Cat# ab216327, RRID:AB_2737295), **toll-like receptor 2 (TLR2**; Abcam, Cat# ab213676, RRID:AB_2892080) and **toll-like receptor 4 (TLR4**; Abcam, Cat# ab22048, RRID:AB_446735) were used. Secondary antibodies were obtained from Abcam (goat anti-mouse, Cat# ab97040, RRID:AB_10698223 and goat anti-rabbit, Cat# ab6721, RRID:AB_955447). Protein bands were detected with enhanced chemiluminescence (ECL) reagents (Clarity Western ECL Blotting Substrate, Bio-Rad). Densitometry was performed by iBright Analysis Software.

2.9 | Metagenomic analysis of bacterial populations in faeces and ileal tissues

Microbial genomic DNAs extracted from faeces and ileal tissues were subjected to 16S rRNA gene sequencing. Metagenomic analyses and data processing were performed by Novogene (Beijing, China). In particular, genomic DNA was extracted from faeces and ileal tissues by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. V3–V4 regions of the 16S rRNA gene were amplified using specific primer pairs. PCR products were run on a 2% agarose gel electrophoresis and purified with the Qiagen Gel Extraction Kit (Qiagen). Libraries were then generated using NEBNext Ultra DNA Library Prep Kit for Illumina. Library was sequenced using the HiSeq Illumina platform. Raw data were filtered using the QIIME quality control process (Version 1.7.0, http://qiime.org/index.html). Operational taxonomic units (OTUs) were clustered with a ≥97% similarity cutoff by the Uparse software (Uparse, Version 7.0.1001, http://drive5.com/uparse/). Phylogenetic relations between OTUs were assessed with the MUSCLE software (Version 3.8.31, http://www.drive5.com/muscle/). Alpha- and beta-diversity analyses were performed using the QIIME (Version 1.7.0) and R (Version 2.15.3) software.

2.10 | Evaluation of faecal levels of short-chain fatty acids (SCFAs)

Faces were collected and kept at −80°C until further processing. Up to 100 mg of frozen material was used to prepare faecal samples. Feaces were processed with 700 µl of 1-butanol (ACROS Organics, Fisher Scientific) followed by 1 min of gentle vortexing and then kept at room temperature (RT) for 50 min. After centrifugation at 300 g for

10 min, the supernatant was transferred to another glass tube containing 1 ml of boron trifluoride–1-butanol solution (~10% in 1-butanol). Tubes were vortexed and kept at RT for 30 min; 2.5 ml of hexane, 1 ml of distilled water and 0.2 ml of saturated sodium chloride solution were then added to the formulations and samples were vortexed and kept at RT for 50 min. An aliquot of the supernatant, hexane containing the butylated SCFAs, was diluted 1:4 with methanol.

The identification and quantification were carried out by liquid chromatography-tandem mass spectrometry (LC-MS/MS). An Agilent 1100 high-performance liquid chromatography (HPLC) system (Agilent, Palo Alto, CA, USA) was coupled to a mass spectrometry Agilent Technologies QQQ triple quadrupole 6420 equipped with an electrospray ionization (ESI) source, using positive mode (ESI+). A C-18 Zorbax Eclipse Plus column (Agilent) with 5 µm of particle size and 50 × 4.6 mm was used with a mobile phase of CH_3OH/H_2O/CHOOH (70/30/0.1, v/v/v) at a flow rate of 0.5 ml·min^{-1}. N_2 was used as a nebulizing gas with a pressure of 50 psig, drying gas temperature of 300°C, flow of 11 L·min^{-1} and 4000 V of capillary voltage. For each standard, the precursor ion $[M + H]^+$ was determined during a full scan in MS and subsequently, the obtained product ion (PI) was monitored for each transition in multiple reaction monitoring (MRM) mode in MS/MS. Parameters of source, such as cone voltage or fragmentor (CV) and collision energy (CE), have been optimized for each MRM transition. Butyl propionate and butyl butyrate transitions were [131 → 75] and [145 → 89], respectively.

The MassHunter workstation acquisition software was used to collect data, which was then analysed for qualitative and quantitative analyses. Butyric and propionic acid (Supelco) and butyl butyrate and butyl propionate, boron trifluoride–1-butanol solution (~10% in 1-butanol), formic acid, n-hexane, water, methanol and LiChrosolv gradient grade for liquid chromatography.

2.11 | Statistical analysis

The statistical analysis of data complies with the requirements of good laboratory practices (GLPs), according to the guidelines of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022). The statistical analysis was undertaken only for studies where each group size was at least n = 5, where n = number of independent values. In particular, the results are presented as mean ± standard error of the mean (SEM). Values less than Q1 − 1.5 * IQR or greater than Q3 + 1.5 * IQR were identified as outliers and excluded from the analysis. The statistical significance of data was evaluated by two-way or one-way analysis of variance (ANOVA). Post hoc tests, specifically Tukey's or Dunnett's test, were conducted only if F in anova achieved $P < 0.05$. Resulting P values < 0.05 were considered significant. Calculated full P values are shown in results section for analysis of faecal and ileal bacterial populations. All statistical procedures were performed by two different operators, blinded to the treatment, using GraphPad Prism 7.0 software (GraphPad Prism, San Diego, CA, USA). Both Shannon index and Chao1 index values were compared with one-way ANOVA followed by post hoc Tukey's test for the different experimental groups. Differences in specific taxa abundance in the different treatments were evaluated with Student's unpaired *t*-tests.

2.12 | Materials

Diclofenac, methylcellulose, formalin, EDTA, Tris, glycerine, PMSF, leupeptin, aprotinin, ascorbic acid, butyric acid, propionic acid, butyl butyrate, butyl propionate, boron trifluoride–1-butanol solution, formic acid, hexane, methanol, BSA and sodium chloride were obtained from Sigma Ltd (St. Louis, MO, USA). *S. boulardii* CNCM I-745 was provided by Biocodex (Gentilly, France). Details of other materials and suppliers are provided in the specific sections.

2.13 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Fabbro, et al., 2021a, b; Alexander, Kelly, et al., 2021).

3 | RESULTS

3.1 | Histological assessment of small bowel injury

In the ileum from control animals, as well as from those treated with *S. boulardii* CNCM I-745 alone over 14 or 28 days, microscopic analysis did not reveal any type of lesion. The administration of diclofenac was associated with the development of type 1–3 lesions in the ileum (Figure 2a–e). Under these conditions, both the preventive and the curative administration of *S. boulardii* CNCM I-745 resulted in a significant decrease of type 1–3 lesions (Figure 2a–e).

3.2 | Blood haemoglobin levels

In animals with diclofenac-induced small intestinal damage, the concentration of haemoglobin was significantly reduced, as compared with the value recorded in controls (13.7 ± 0.44 vs. 17.06 ± 0.17 g·dl^{-1}) (Figure 2f). In rats treated with *S. boulardii* CNCM I-745 for 14 days followed by treatment with *S. boulardii* CNCM I-745 plus diclofenac for 14 days, the haemoglobin levels were significantly increased compared with animals treated with diclofenac alone. Likewise, animals treated with diclofenac plus *S. boulardii* CNCM I-745 for 14 days displayed a significant increase in blood haemoglobin concentration (Figure 2f). No significant changes were detected in animals treated with *S. boulardii* CNCM I-745 alone for 28 or 14 days compared to controls.

3.3 | Myeloperoxidase (MPO) levels in the ileum

In animals treated with diclofenac, myeloperoxidase levels in the ileum were significantly enhanced, as compared with control animals (5.52 ± 0.78 vs. 2.24 ± 0.58 ng·mg^{-1} of tissue; Figure 3a). Both the preventive and the curative administration of *S. boulardii* CNCM I-745 were associated with a significant decrease in myeloperoxidase levels compared with animals treated with diclofenac alone. In rats treated with *S. boulardii* CNCM I-745 alone for 14 or 28 days no significant changes in myeloperoxidase levels were detected compared to control (Figure 3a).

3.4 | Evaluation of tissue TNF and IL-1β levels

Treatment with diclofenac significantly increased TNF levels compared to controls (25.55 ± 2.47 vs. 13.74 ± 2.81 pg·mg^{-1} of tissue; Figure 3b). Rats treated with diclofenac plus *S. boulardii* CNCM I-745 for 14 days or *S. boulardii* CNCM I-745 for 14 days followed by treatment with *S. boulardii* CNCM I-745 plus diclofenac for 14 days did not show any significant change in ileal TNF levels, as compared with animals treated with diclofenac alone (Figure 3b). No significant changes in TNF levels were also detected in animals treated with *S. boulardii* CNCM I-745 alone for 14 or 28 days compared to control animals (Figure 3b).

IL-1β levels in diclofenac-treated rats were significantly raised compared to control animals (11.34 ± 2.44 vs. 1.18 ± 0.37 pg·mg^{-1} of tissue; Figure 3c). Treatment with *S. boulardii* CNCM I-745 for 14 days followed by treatment with *S. boulardii* CNCM I-745 plus diclofenac for 14 days caused a significant reduction in IL-1β levels compared to diclofenac-treated animals. On the other hand, no significant changes were detected in animals treated with *S. boulardii* CNCM I-745 plus diclofenac for 14 days, as well as *S. boulardii* CNCM I-745 alone for 14 or 28 days (Figure 3c).

3.5 | Malondialdehyde (MDA) levels in the ileum

In animals treated with diclofenac, ileal levels of malondialdehyde were significantly elevated, compared to control animals (4.83 ± 0.50 vs. 2.15 ± 0.22 μmol·mg^{-1} of tissue) (Figure 3d). Rats treated with diclofenac plus *S. boulardii* CNCM I-745 for 14 days or *S. boulardii* CNCM I-745 for 14 days followed by treatment with *S. boulardii* CNCM I-745 plus diclofenac for 14 days showed a significant reduction in malondialdehyde levels, compared to animals treated with diclofenac alone. No significant changes of malondialdehyde were detected in animals treated with *S. boulardii* CNCM I-745 alone for 14 or 28 days compared to controls (Figure 3d).

3.6 | Faecal calprotectin levels

At T_{-14} and T_0, no significant changes in faecal calprotectin levels in all treatment groups were detected (not shown). At T_{+14}, diclofenac-treated rats showed a significant increase in faecal calprotectin compared with the respective control values (Figure 3e). Treatment with diclofenac plus *S. boulardii* CNCM I-745 for 14 days or *S. boulardii* CNCM I-745 for 14 days followed by treatment with *S. boulardii* CNCM I-745 plus diclofenac for 14 days determined a significant decrease of calprotectin values compared with rats treated with diclofenac alone. No significant changes of calprotectin were detected in animals treated with *S. boulardii* CNCM I-745 alone for 14 or 28 days (Figure 3e).

3.7 | Expression of TLR2 and TLR4 in the intestinal mucosa

Western blot analysis showed that the expression of TLR2 and TLR4 was significantly increased in the ileum from rats treated with diclofenac in comparison with control animals (Figure 4a,b). Treatment with *S. boulardii* CNCM I-745 for 14 days followed by treatment with *S. boulardii* CNCM I-745 plus diclofenac for 14 days was associated with a significant decrease in the expression levels of both TLR2 and TLR4, as compared with rats treated with diclofenac alone (Figure 4a,b). Treatment with *S. boulardii* CNCM I-745 plus diclofenac for 14 days did not exert any effect on TLR2 expression, whereas it significantly counteracted the increase in ileal TLR4 expression associated with diclofenac administration (Figure 4a,b). In rats treated with *S. boulardii* CNCM I-745 alone for 28 or 14 days, no significant changes in TLR2 and TLR4 expression were detected compared to controls (Figure 4a,b).

3.8 | Expression of MyD88 and NF-κB in the intestinal mucosa

In animals with enteropathy induced by diclofenac, the expression of MyD88 and NF-κB was increased compared to control group (Figure 4c,d). Treatment with *S. boulardii* CNCM I-745 for 14 days followed by treatment with *S. boulardii* CNCM I-745 plus diclofenac for 14 days was associated with a significant decrease in MyD88 and NF-κB expression compared to animals treated with diclofenac alone (Figure 4c,d). Likewise, rats treated with *S. boulardii* CNCM I-745 plus diclofenac for 14 days, both parameters were significantly reduced (Figure 4c,d). In rats treated with *S. boulardii* CNCM I-745 alone for 28 or 14 days, no significant changes in MyD88 and NF-κB expression were detected compared to controls (Figure 4c,d).

3.9 | Analysis of faecal bacterial populations

Rarefaction curves confirmed an adequate depth and quality of sequencing (Figure S1A). Chao1 indexes of alpha diversity differed only when rats were treated with the curative protocol compared with those treated with only diclofenac ($P = 0.0099$; Figure S1C). No

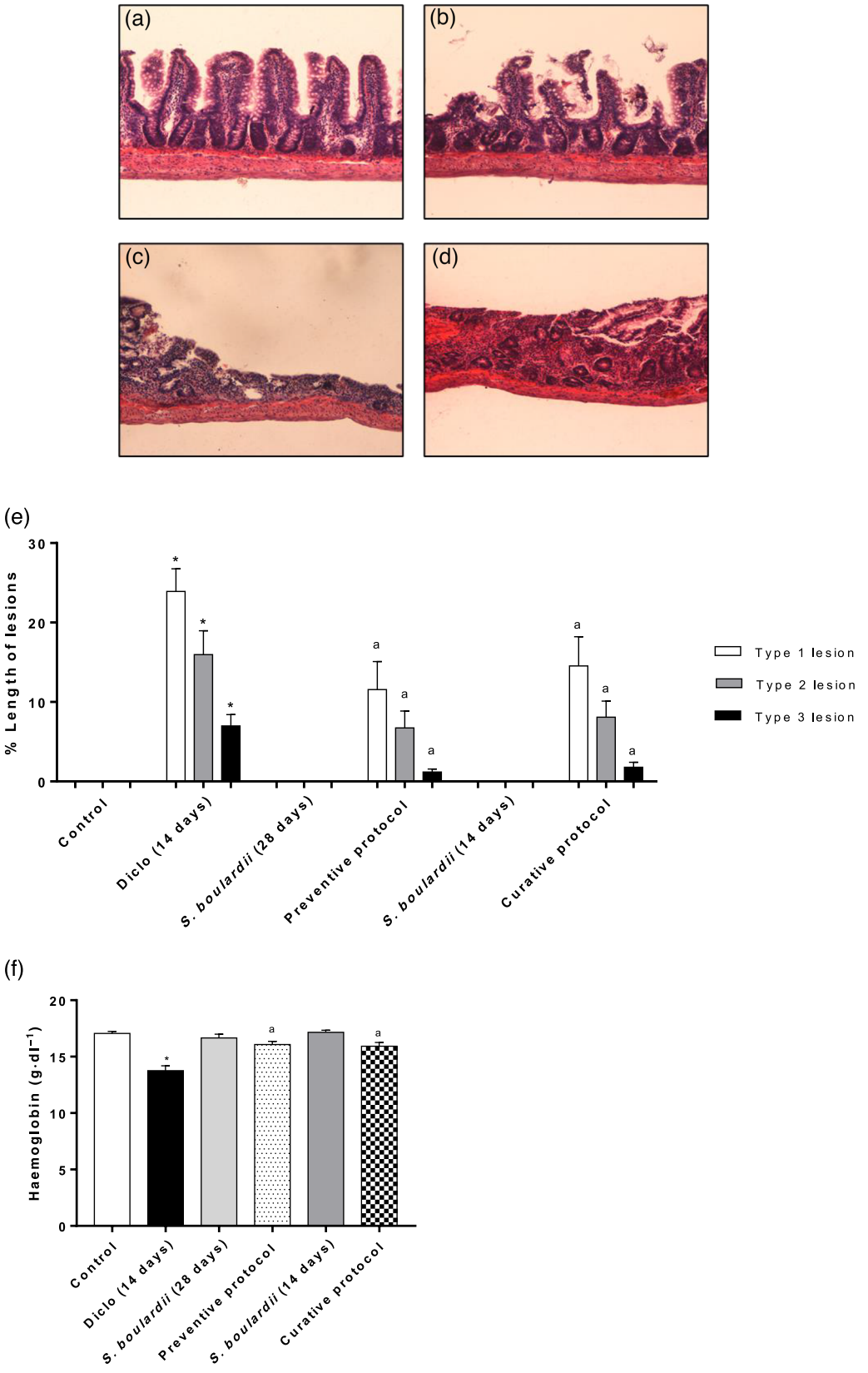

FIGURE 2 Legend on next page.

FIGURE 2 Histological analysis of intestinal mucosal damage. Representative images showing the microscopic appearance of ileal mucosa from control animals (a) (scale bar = 100 μm), as well as type 1 (b) (scale bar = 100 μm), type 2 (c) (scale bar = 100 μm) or type 3 (d) (scale bar = 100 μm) lesions defined according to criteria presented in Table 1, observed in animals treated with diclofenac (Diclo). (e) Effects of vehicle (control), Diclo (4 mg·kg^{-1} BID) for 14 days, *Saccharomyces boulardii* CNCM I-745 for 28 days, *S. boulardii* CNCM I-745 for 14 days followed by *S. boulardii* CNCM I-745 plus Diclo for 14 days, *S. boulardii* CNCM I-745 for 14 days and *S. boulardii* CNCM I-745 plus Diclo for 14 days on type 1–3 lesions. (f) Blood haemoglobin levels. Effects of vehicle (control), Diclo (4 mg·kg^{-1} BID) for 14 days, *S. boulardii* CNCM I-745 for 28 days, *S. boulardii* CNCM I-745 for 14 days followed by *S. boulardii* CNCM I-745 plus Diclo for 14 days, *S. boulardii* CNCM I-745 for 14 days and *S. boulardii* CNCM I-745 plus Diclo for 14 days on blood haemoglobin levels. Each column represents the mean ± SEM from 9–15 animals. $^{*}P < 0.05$, significant difference versus control; $^{a}P < 0.05$, significant difference versus Diclo alone.

significant differences were demonstrated by Shannon (Figure S1E). Conversely from principal coordinate analysis (PCoA) (Figure S2C), non-metric multidimensional scaling (NMDS) highlighted that the diclofenac + *S. boulardii* (14 days) group did not cluster with the others (Figure S2A).

The control group showed *Lactobacillus reuteri*, *Ruminococcus bromii* and *Ruminococcus flavefaciens* as the main bacterial species in faecal samples. When rats were treated with diclofenac, a higher prevalence of *Escherichia coli* and *Enterococcus faecalis* was revealed, while *Lachnospiraceae* showed a significant reduction as compared with the control group ($P = 0.0050$; Figure 5b,e). The administration of *S. boulardii* for 14 associated with a higher abundance of *Pediococcus* ($P = 0.0152$), especially of *Pediococcus pentosaceus*, than in control, while the administration of the preventive protocol caused an increase in the abundance of *Pediococcus* ($P = 0.0048$) and *Lachnospiraceae* ($P = 0.0039$), as well as decreasing levels of *Enterococcus* ($P = 0.0273$) and *Clostridiales* ($P = 0.0162$), when compared with rats treated with diclofenac alone. Interestingly, the probiotic administration through these two protocols re-established *L. reuteri* as the most abundant species. The curative protocol with *S. boulardii* CNCM I-745 in concomitance with diclofenac showed a comparable trend for *Enterococcus* ($P = 0.0158$) and *Clostridiales* ($P = 0.0057$), as compared with diclofenac-treated rats. Moreover, also, *Firmicutes* ($P = 0.0389$; Figure 5a), *Lactobacillus* ($P = 0.0005$) and *Jeotgalicoccus* ($P = 0.0018$) were reduced, while *Bacteroidetes* ($P = 0.0443$) and *Pediococcus* ($P = 0.0127$) increased (Figures 5b,e and S3).

3.10 | Analysis of ileal bacterial populations

As reported for faeces, the evaluation of rarefaction curves from ileal tissues showed an appropriate depth of sequencing (Figure S1B). Shannon indexes were not significantly different between treatments (Figure S1F) unlike Chao1 values, which were higher in rats treated with *S. boulardii* for 14 days compared with control ($P = 0.0002$) and with the curative protocol compared with diclofenac alone ($P = 0.0267$; Figure S1D). Non-metric multidimensional scaling (NMDS) showed that rats treated with *S. boulardii* with both preventive and curative protocols were not clustering with the other treatments, different from PCoA (Figure S2B,D).

In all the tested groups, *Lactobacillus murinus*, *L. reuteri* and *Lactobacillus johnsonii* were identified as the main species in the rat ileum, with *E. coli* was found to be prevalent only in diclofenac-treated animals. Treatment with diclofenac provoked an increase in *Enterococcus* ($P = 0.0483$), *Corynebacterium* ($P = 0.0137$) and *Staphylococcus* ($P = 0.0396$) compared with controls (Figure 5d,f). The administration of *S. boulardii* for 14 days determined increasing levels of *Proteobacteria* ($P = 0.0374$; Figure 5c), *Ruminococcus* ($P = 0.0336$) and *Roseburia* ($P = 0.0142$), as well as decreasing abundances of *Staphylococcus* ($P = 0.0025$). Rats treated with the preventive protocol in comparison with diclofenac alone displayed an increase of *Rothia* ($P = 0.0345$) and *Corynebacterium* ($P = 0.0456$), as well as a reduction in *Enterococcus* ($P = 0.0071$), *Ruminococcus* ($P = 0.0022$) and *Staphylococcus* ($P = 0.0148$). The curative protocol instead, demonstrated a statistically significant increase in *Actinobacteria* ($P = 0.0357$), *Bacteroidetes* ($P = 0.0093$; Figure 5c), *Bifidobacterium* ($P = 0.0460$), *Ruminococcus* ($P = 0.0327$), *Corynebacterium* ($P = 0.0490$) and *Roseburia* ($P = 0.0444$), but a reduction in *Firmicutes* ($P = 0.0034$) and *Enterococcus* ($P = 0.0111$; Figures 5d,f and S3).

3.11 | Evaluation of faecal levels of short-chain fatty acids (SCFAs)

At T_{-14} and T_0, no significant changes were detected in faecal butyrate and propionate levels in all the treatment groups (not shown). In rats with enteropathy induced by diclofenac, faecal butyrate levels at T_{+14} were significantly increased, as compared with the control group (Figure 6a,b). Treatment with *S. boulardii* CNCM I-745 in preventive, but not in curative, protocol significantly reduced the faecal butyrate concentration, as compared with animals treated with diclofenac alone (Figure 6a,b). Of note, the range of faecal butyrate concentrations reported in our study is in line with the literature (Choi et al., 2021).

No significant changes were observed on faecal propionate concentrations (Figure 6a,b).

3.12 | Expression of intestinal butyrate transporter MCT1 in the intestinal mucosa

In animals with enteropathy induced by diclofenac, the expression of MCT1 transporter was significantly reduced, as compared with the control group (Figure 6c). Treatment with *S. boulardii* CNCM I-745 for 14 days followed by treatment with *S. boulardii* CNCM I-745 plus diclofenac for 14 days was associated with a significant increase in

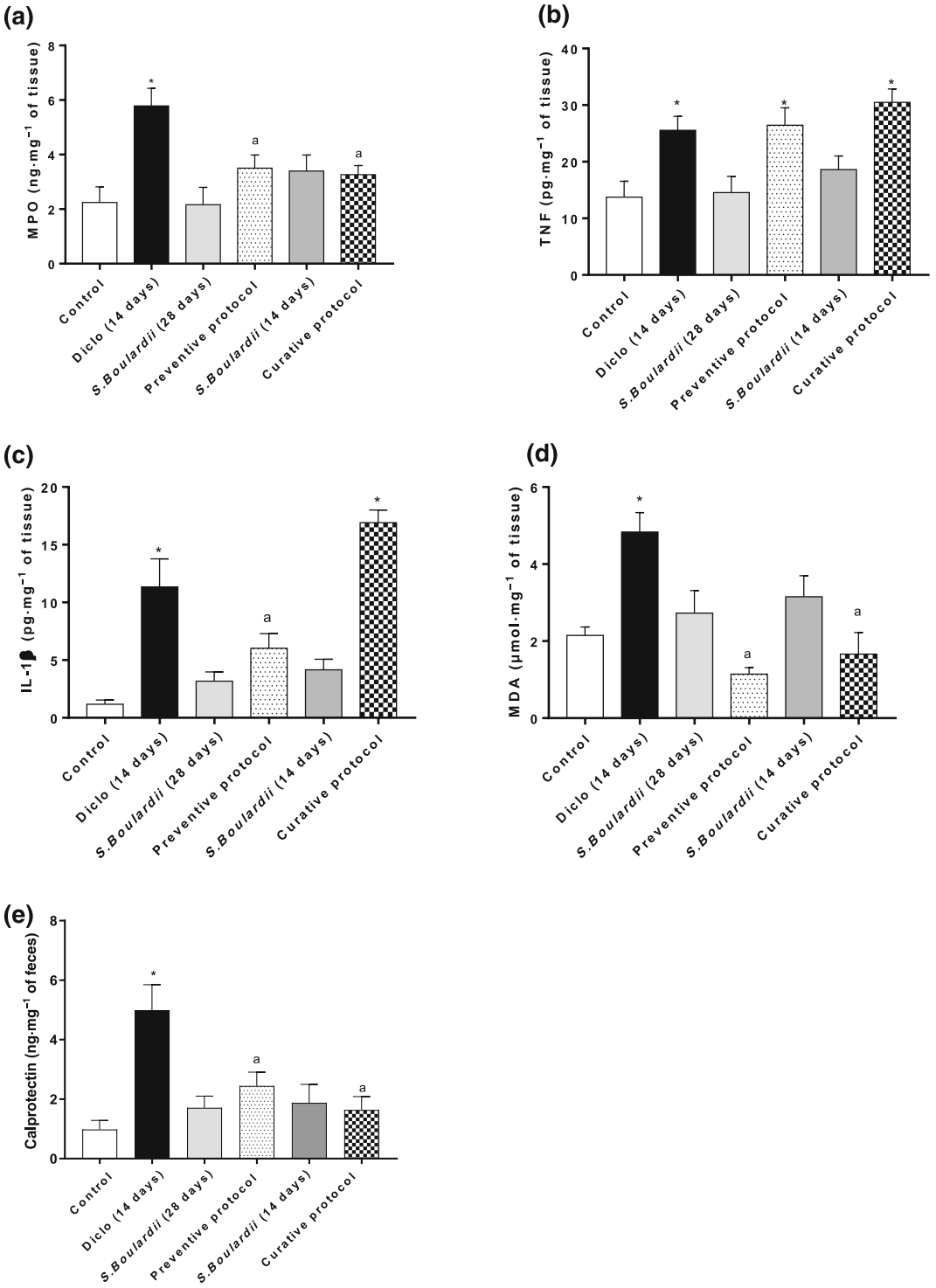

FIGURE 3 Tissue levels of myeloperoxidase (MPO) (a), tumour necrosis factor (TNF) (b), interleukin (IL)-1β (c) and malondialdehyde (MDA) (d) in the ileum from rats treated with vehicle (control), diclofenac (Diclo) (4 mg·kg^{-1} BID) for 14 days, *Saccharomyces boulardii* CNCM I-745 for 28 days, *S. boulardii* CNCM I-745 for 14 days followed by *S. boulardii* CNCM I-745 plus Diclo for 14 days, *S. boulardii* CNCM I-745 for 14 days and *S. boulardii* CNCM I-745 plus Diclo for 14 days. (e) Calprotectin levels in feces harvested at T_{+14} from rats treated with vehicle (control), Diclo (4 mg·kg^{-1} BID) for 14 days, *S. boulardii* CNCM I-745 for 28 days, *S. boulardii* CNCM I-745 for 14 days followed by *S. boulardii* CNCM I-745 plus Diclo for 14 days, *S. boulardii* CNCM I-745 for 14 days and *S. boulardii* CNCM I-745 plus Diclo for 14 days. Each column represents the mean ± SEM from 10–15 animals. *$P < 0.05$, significant difference versus control; $^{a}P < 0.05$, significant difference versus Diclo alone.

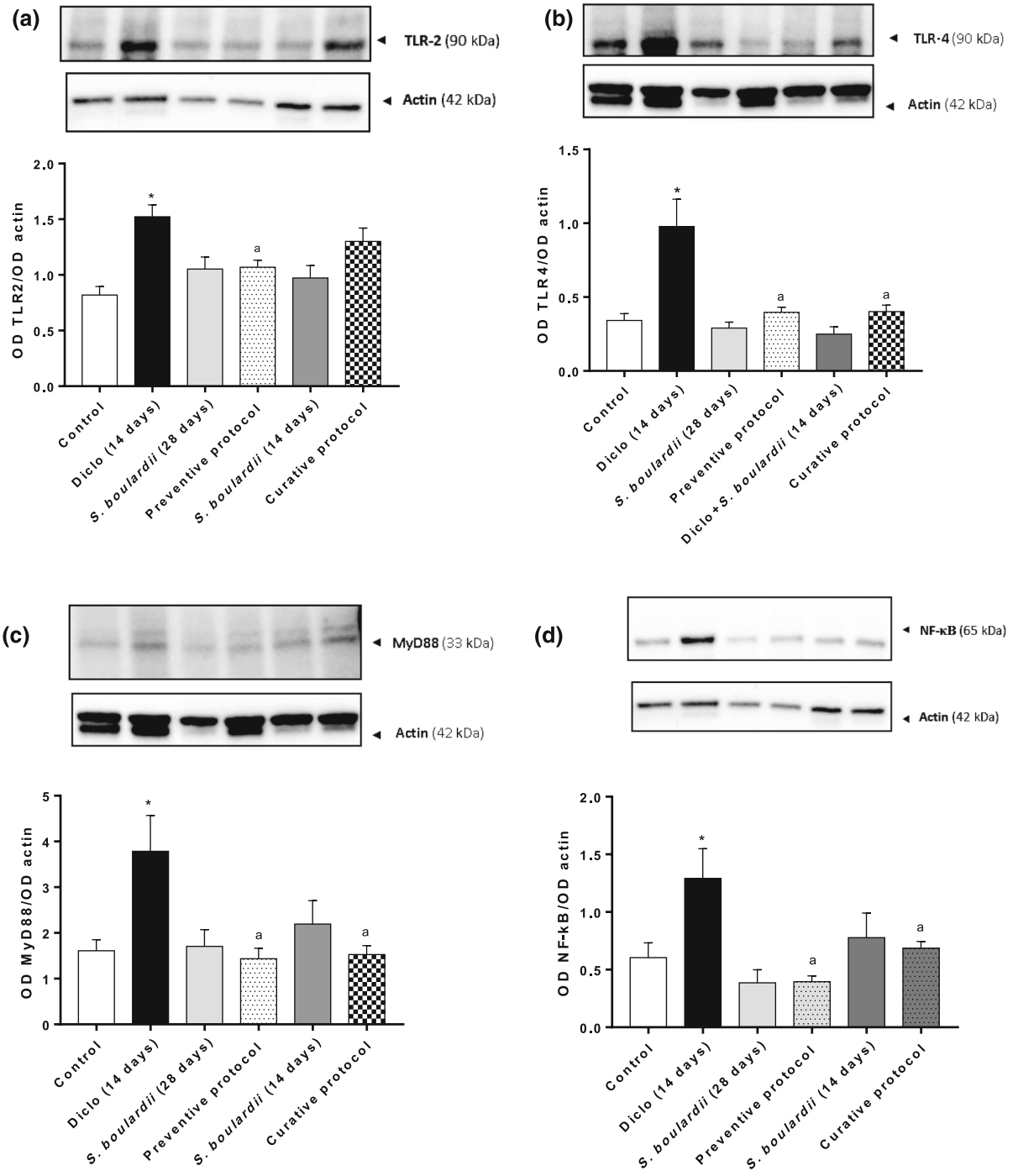

FIGURE 4 Representative blots and densitometric analysis of the expression of toll-like receptor 2 (TLR2) (a), toll-like receptor 4 (TLR4) (b), myeloid differentiation primary response 88 (MyD88) (c) and nuclear factor κB (NF-κB) (d) assessed by Western blot assay in the ileum from rats treated with vehicle (control), diclofenac (Diclo) (4 mg·kg^{-1} BID) for 14 days, *Saccharomyces boulardii* CNCM I-745 for 28 days, *S. boulardii* CNCM I-745 for 14 days followed by *S. boulardii* CNCM I-745 plus Diclo for 14 days, *S. boulardii* CNCM I-745 for 14 days and *S. boulardii* CNCM I-745 plus Diclo for 14 days. For (a) and (d), the membrane was cut for the detection of TLR2 (90 kDa), NF-κB (65 kDa) and actin (42 kDa). Therefore, the actin is the same for TLR2 and NF-κB. Likewise, for (b) and (c), the membrane was cut for the detection of TLR4 (90 kDa), MyD88 (33 kDa) and actin (42 kDa). Therefore, the actin is the same for TLR4 and MyD88. Each column represents the mean ± SEM from 7–14 animals. *P < 0.05, significant difference versus control. [a]P < 0.05, significant difference versus Diclo alone.

MCT1 expression, as compared with rats treated with diclofenac (Figure 6c). By contrast, treatment with *S. boulardii* CNCM I-745 plus diclofenac for 14 days did not exert any effect on MCT1 expression. In rats treated with *S. boulardii* CNCM I-745 alone for 28 or 14 days, no significant changes in MCT1 expression were detected, as compared with the value recorded in controls (Figure 6c).

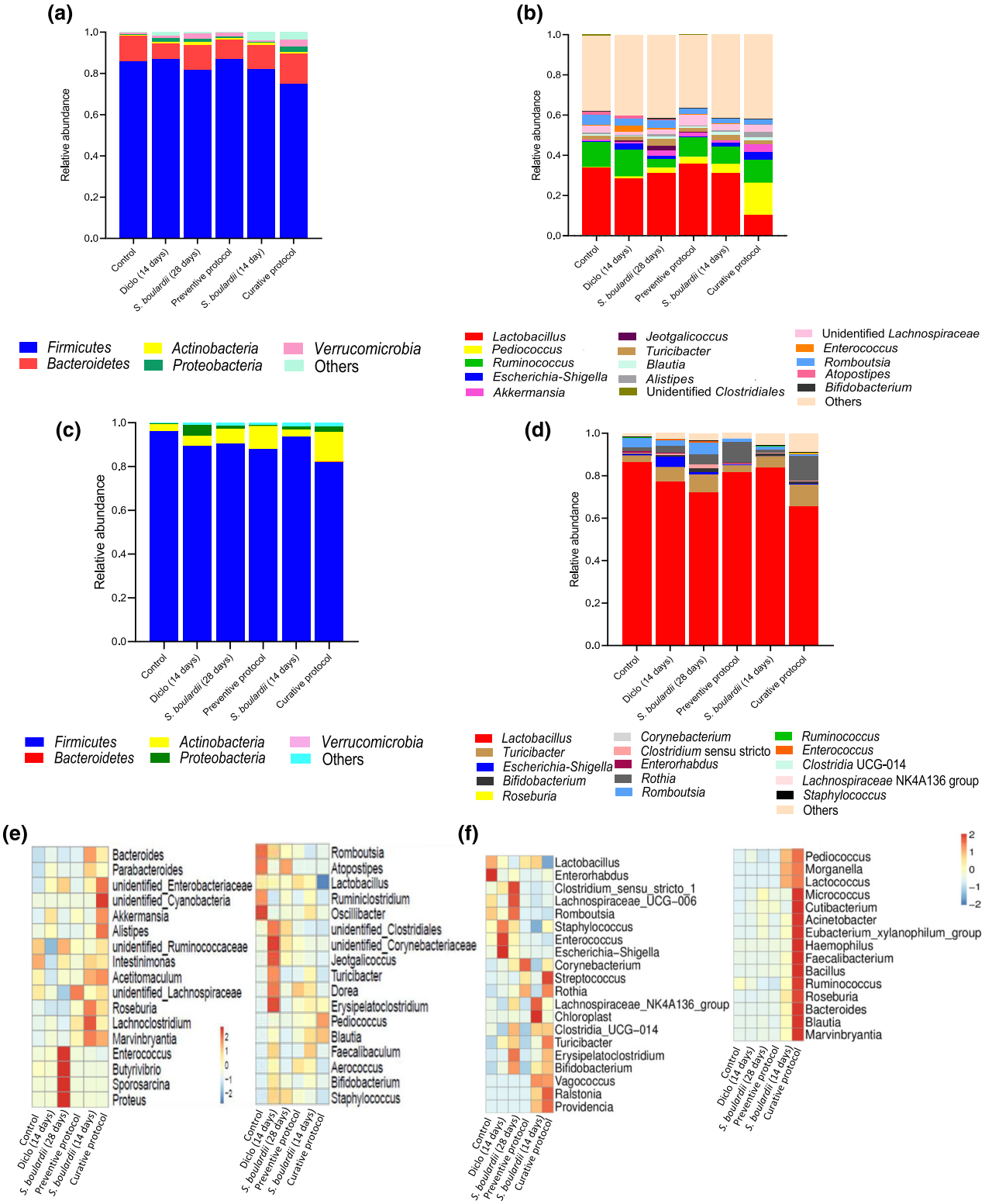

FIGURE 5 Analysis of bacterial populations at both phylum and genus levels in the faeces (a, b) and in the ileal tissues (c, d) harvested at T_{+14} from rats treated with vehicle (control), diclofenac (Diclo) (4 mg·kg^{-1} BID) for 14 days, *Saccharomyces boulardii* CNCM I-745 for 28 days, *S. boulardii* CNCM I-745 for 14 days followed by *S. boulardii* CNCM I-745 plus Diclo for 14 days, *S. boulardii* CNCM I-745 for 14 days and *S. boulardii* CNCM I-745 plus Diclo for 14 days. Analysis of bacterial populations at genus level in the faeces (e) and in the ileal tissues (f) harvested at T_{+14} from rats treated with vehicle (control), Diclo (4 mg·kg^{-1} BID) for 14 days, *S. boulardii* CNCM I-745 for 28 days, *S. boulardii* CNCM I-745 for 14 days followed by *S. boulardii* CNCM I-745 plus Diclo for 14 days, *S. boulardii* CNCM I-745 for 14 days and *S. boulardii* CNCM I-745 plus Diclo for 14 days. Differences between the corresponding values represented by colours indicate the fold changes between groups in the relative abundance of the bacterial strain considered.

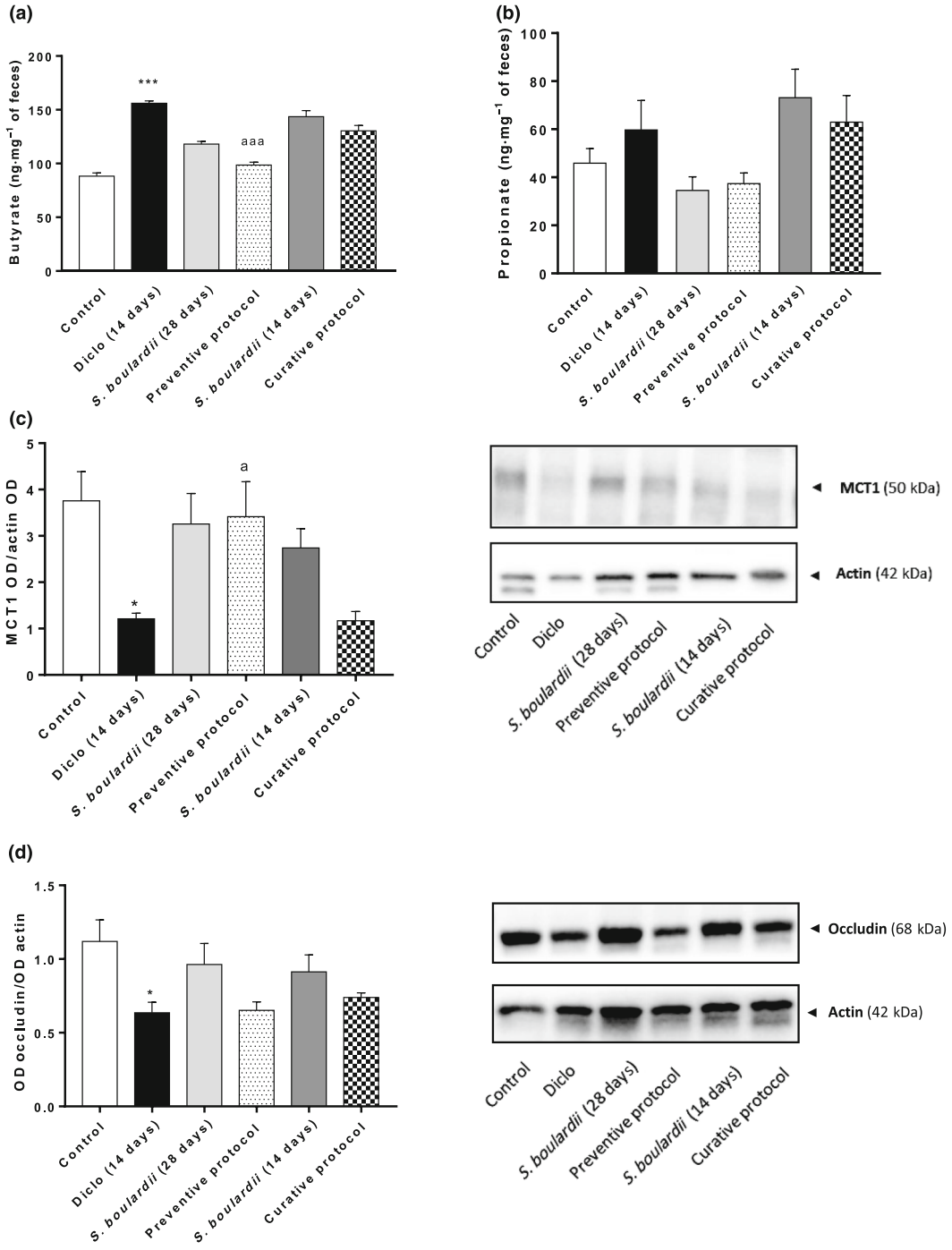

FIGURE 6 (a) Butyrate and (b) propionate levels in the faeces harvested at T_{+14} from rats treated with vehicle (control), diclofenac (Diclo) (4 mg·kg^{-1} BID) for 14 days, *Saccharomyces boulardii* CNCM I-745 for 28 days, *S. boulardii* CNCM I-745 for 14 days followed by *S. boulardii* CNCM I-745 plus Diclo for 14 days, *S. boulardii* CNCM I-745 for 14 days and *S. boulardii* CNCM I-745 plus Diclo for 14 days. Representative blots and densitometric analysis of the expression of monocarboxylate transporter 1 (MCT1) (c) and occludin (d) assessed by Western blot assay in the ileum from rats treated with vehicle (control), Diclo (4 mg·kg^{-1} BID) for 14 days, *S. boulardii* CNCM I-745 for 28 days, *S. boulardii* CNCM I-745 for 14 days followed by *S. boulardii* CNCM I-745 plus Diclo for 14 days, *S. boulardii* CNCM I-745 for 14 days and *S. boulardii* CNCM I-745 plus Diclo for 14 days. For (d), the membrane was cut for the detection of occludin (68 kDa) and actin (42 kDa) before the antibody incubation in order to detect multiple signals within the same samples. Each column represents the mean ± SEM from 5–14 animals. *P < 0.05, significant difference versus control; [a]P < 0.05, significant difference versus Diclo alone.

3.13 | Expression of occludin in the intestinal mucosa

In rats with enteropathy induced by diclofenac, the expression of occludin (integral plasma-membrane protein located at the tight junctions) was significantly reduced, as compared with the control group (Figure 6d). Treatment with *S. boulardii* CNCM I-745, in both preventive and curative protocols, did not exert any effect as compared with rats treated with diclofenac. In rats treated with *S. boulardii* CNCM I-745 alone for 28 or 14 days, no significant changes in occludin expression were detected compared with controls (Figure 6d).

4 | DISCUSSION

NSAIDs are very effective medications and remain the most largely employed drugs worldwide (both by prescription or by automedication), even though their use is associated with a broad spectrum of gastrointestinal adverse reactions, including enteric damage and occult bleeding, so-called enteropathy. Currently, in the clinical setting, no effective and specific therapeutic interventions are available as protective strategies for the prevention of NSAID-induced enteropathy, thus spurring researchers to find novel therapeutic options. In this regard, growing evidence supports the contention that the administration of probiotics could represent a valuable tool to counteract the intestinal damage and chronic inflammation associated with NSAID therapy (X. Chen et al., 2013; Fornai et al., 2020; Montalto et al., 2010; Ocón et al., 2013; Pothoulakis, 2009; Satoh & Takeuchi, 2012; Soyturk et al., 2012; Suzuki et al., 2017). In this setting, although there is no clinical evidence on *S. boulardii* CNCM I-745, a number of clinical studies have well documented the beneficial effects of bacterial probiotics in protection against NSAID-induced enteropathy (Chen et al., 2022; Endo et al., 2011; Engel et al., 2022; Gotteland et al., 2001; Montalto et al., 2010; Mortensen et al., 2019; Suzuki et al., 2017; Yoshihara et al., 2020). However, it is worth noting that the use of yeast probiotics has been proposed as a better therapeutic option than bacterial probiotics. This is due to their ability to continue acting during antibiotic treatment and withstand the stomach acids, bile and pancreatic juices, and still favour the breakdown of undigested carbohydrates and increase in SCFAs (Czerucka et al., 2007).

Based on these premises, the present study was designed to evaluate the efficacy of *S. boulardii* CNCM I-745 administration in counteracting and preventing intestinal mucosa injury and the onset of enteric inflammation associated with NSAID therapy in a rat model of diclofenac-induced enteropathy. Our experiments showed three major and novel findings: (1) *S. boulardii* CNCM I-745 administration is able to counteract the mucosal inflammation and the small intestinal damage induced by diclofenac, reducing tissue infiltration by polymorphonuclear cells and oxidative stress as well as blunting the activation of the pro-inflammatory pathway TLR4/MyD88/NF-κB, (2) such positive effects are likely to depend on the ability of the probiotic to improve the tissue butyrate bioavailability and its transport by the enteric mucosa and (3), *S. boulardii* CNCM I-745 administration modulates the microbiota composition, promoting the proliferation of enteric microorganisms recognized as having a beneficial effects on intestinal mucosa.

The experimental model of diclofenac-induced enteropathy closely mimics the bowel damage observed in patients receiving long-term NSAID treatment (Colucci et al., 2018; Fornai et al., 2020; Zhang et al., 2022). In accordance with previous reports (Colucci et al., 2018; Fornai et al., 2020), our experimental model is characterized by mucosal injury and tissue inflammation as well as intestinal bleeding, oxidative stress and increased intestinal epithelial barrier permeability, as documented by a significant decrease in occludin expression. In addition, variations in the bacterial community composition in faecal and ileal tissues in rats with diclofenac-induced enteropathy were also observed. In this situation, administration of *S. boulardii* CNCM I-745, in both preventive and curative protocols, resulted in a significant reduction in small bowel damage, as documented by a significant decrease in type 1–3 lesions, suggesting a protective effect on intestinal mucosa against NSAID-induced enteropathy. In addition, *S. boulardii* CNCM I-745 administration was effective in counteracting the decrease in blood haemoglobin (regarded as an indirect index of enteric bleeding [Bjarnason et al., 2018; Fornai et al., 2014, 2016, 2020; Lim et al., 2020]) associated with NSAID treatment. Thus providing the first experimental demonstration that such therapeutic intervention could represent a valuable tool to reduce digestive bleeding associated with NSAID-induced intestinal injury.

As the occult bleeding is a possible consequence of tissue damage and intestinal inflammation (Bjarnason et al., 2018; Fornai et al., 2016, 2020), we went on to investigate the effect of *S. boulardii* CNCM I-745 administration on intestinal inflammation associated with NSAID treatment. We observed that probiotic supplementation blunted the increment of both MPO and IL-1β tissue levels and prevented completely the increment of faecal calprotectin levels induced by diclofenac. These results are in keeping with the protective activity of *S. boulardii* against intestinal inflammation and damage and therefore suggest the involvement of anti-inflammatory mechanisms (Cristofori et al., 2021; Pothoulakis, 2009; Soyturk et al., 2012). Consistently with this data, in our experimental model we observed that the administration of the probiotic yeast significantly reduced the expression levels of TLR4 and related downstream signalling molecules (MyD88 and NF-κB p65), thus highlighting that the anti-inflammatory effects of *S. boulardii* CNCM I-745 are likely to depend, at least in part, on its ability to blunt the pro-inflammatory pathway TLR4/MyD88/NF-κB. In line with this evidence, Gao et al. (2021) demonstrated an inhibitory effect of *S. boulardii* on NF-κB activation and NF-κB-mediated inflammatory gene expression in a mouse model of dextran sodium sulfate (DSS)-induced colitis (Gao et al., 2021).

Of interest, previous studies have reported that oxidative stress is a condition commonly associated with intestinal damage induced by NSAID treatment (Fornai et al., 2014; M. Zhang et al., 2022). In line with this view, we observed that treatment with diclofenac resulted in a significant increase in MDA concentration in the ileum. In this setting, *S. boulardii* CNCM I-745, in both the preventive and curative

protocols, counteracted completely the increase in MDA levels induced by diclofenac. Of note, the antioxidant properties of *S. boulardii* have been documented in other experimental inflammatory conditions (Gao et al., 2021; Roy Sarkar et al., 2021). Indeed, Gao et al. (2021) demonstrated that *S. boulardii* administration was able to restore the antioxidant system through the increase of antioxidant enzyme levels, such as superoxide dismutase, **catalase** and **haem oxygenase 1 (HO1)**, and to blunt the MDA increase in experimental model of dextran sodium sulfate-induced colitis (Gao et al., 2021). Thus, based on these findings, it is conceivable that the activation of antioxidant mechanisms can play a significant part in the protective effects caused by *S. boulardii* CNCM I-745 against the intestinal injury induced by diclofenac. Taken together, our data provide convincing evidence that the protective effects exerted by *S. boulardii* CNCM I-745 against diclofenac-induced enteropathy could be ascribed to its anti-inflammatory and antioxidant activities.

It is well known that in the healthy bowel, commensal bacteria play a pivotal role in maintaining the intestinal homeostasis through the inhibition of pathogens growth, modulation of the immune system and regulation of epithelial integrity (Parada Venegas et al., 2019). Particularly important appear to be bacterial species that ferment non-digestible dietary fibres and produce metabolites, which exert positive effects on the intestinal mucosa, such as SCFAs, mainly acetate, propionate and butyrate (Parada Venegas et al., 2019). In this regard, several studies have reported that the chronic administration of NSAIDs induce changes in gut microbiota composition and SCFAs concentrations, with the consequent alterations of intestinal mucosa function and integrity (Colucci et al., 2018; Lai et al., 2015; Wang et al., 2021). Consistently with this evidence, in our experimental conditions, we observed a shift of bacterial taxa in both faecal and ileal samples from rats treated with diclofenac, with reduced levels of microbial genera. This is recognized to exert beneficial effects on intestinal mucosa (i.e. *Lachnospiraceae*) and increasing proportions of microorganisms, which may result high abundances of harmful bacteria (i.e. *E. coli* and *Enterococcus*) (Arthur et al., 2012; Mahnic et al., 2020; J. Zhang et al., 2019). Interestingly, treatment with *S. boulardii* CNCM I-745 partially reverted the diclofenac-induced shift of bacterial community composition in both faeces and ileal tissue. In particular, the preventive protocol was found to be more effective in maintaining microbial community proportions similar to those of the control group, counteracting the diclofenac-induced dysbiosis and having no negative impact on already present beneficial microbes (i.e. *Lactobacillus* and *Ruminococcus*). Moreover, analysis of the bacterial populations revealed a novel and interesting correlation between the administration of *S. boulardii* CNCM I-745 and that of genus *Pediococcus*. In fact, *P. pentosaceus* was recently recognized as a beneficial bacterium with probiotic properties (Jiang et al., 2021) and was found to be enriched following *S. boulardii* administration, thus allowing us to hypothesize a synergistic interaction between the two microorganisms. In addition, in our experimental model we observed that treatment with diclofenac was associated with an increase in faecal butyrate levels along with a reduction in the tight junction protein, occludin, expression in the ileal tissue, as previously observed (Choi et al., 2021; Colucci et al., 2018; Lai et al., 2015; Wang et al., 2021). Interestingly, administration of *S. boulardii* CNCM I-745 induced a normalization of faecal butyrate levels. Butyrate represents the primary energy source employed by intestinal epithelial cells to maintain intestinal homeostasis through anti-inflammatory actions and regulating enterocyte proliferation and differentiation (Huang et al., 2021; Parada Venegas et al., 2019). Physiologically, the absorption of butyrate is facilitated by luminal transporters, including MCT1 (the main butyrate luminal transporter) (Parada Venegas et al., 2019). Once internalized into intestinal epithelium, butyrate enters in tricarboxylic acid cycle (TCA cycle) as energy source and preserves the epithelial barrier homeostasis (Parada Venegas et al., 2019). In our study, we hypothesize that the marked luminal presence of butyrate in diclofenac-treated animals was ascribable to disruption of the butyrate MCT1 transporter. Indeed, in the presence of inflammation it has been reported there is a significant reduction in MCT1 expression levels, resulting in an impaired uptake and utilization of butyrate (De Preter et al., 2012; Thibault et al., 2007; Villodre Tudela et al., 2015). Consistent with this evidence, our hypothesis, supported by our experiments, demonstrated a reduced expression of MCT1 in ileal tissues from rats with diclofenac-induced enteropathy. Interestingly, *S. boulardii* CNCM I-745 administration induced a normalization of MCT1 expression levels, thus indicating for the first time the ability of this probiotic to restore and correct uptake of butyrate by enterocytes. Of note, treatment with *S. boulardii* CNCM I-745 failed to restore the altered expression of occludin elicited by diclofenac, thus suggesting that in the present model this probiotic does not directly act on the tight junction reorganization.

Taken together, these results demonstrated that treatment with *S. boulardii* CNCM I-745 is able to exert beneficial effects on tissue butyrate bioavailability, typically altered in inflammatory conditions. Such positive effect is ascribable to the ability of *S. boulardii* CNCM I-745 to improve the transport of butyrate by the enteric mucosa, as shown by the reduction of faecal butyrate levels and the increased expression of MCT1 transporter. It is noteworthy that this effect would explain the ameliorative effect of this probiotic on the intestinal inflammation induced by diclofenac. Indeed, it is well recognized that beyond a trophic activity on epithelial cells, butyrate has a remarkable anti-inflammatory effect, promoting the functionalities of M2 macrophages and follicular regulatory T-cells, and inhibiting infiltration by neutrophils (J. Chen & Vitetta, 2020; Furusawa et al., 2013).

5 | CONCLUSIONS

The result of the present study demonstrate for the first time that the administration of the probiotic yeast *S. boulardii* CNCM I-745, widely available, exerts protective effects against diclofenac-induced enteropathy, improving butyrate bioavailability in the enteric mucosa. Such beneficial effect is likely to be related to a decrease in enteric inflammation and oxidative stress. Of note, the beneficial effects exerted by *S. boulardii* CNCM I-745 administration were mainly observed in preventive protocol, as documented by its higher efficacy in

counteracting the most important signs of diclofenac-induced enteropathy, thus suggesting that administration of this probiotic should be started before a scheduled therapy with NSAIDs. Overall, we have provided original evidence that *S. boulardii* CNCM I-745 administration may represent a suitable therapeutic intervention for the prevention of NSAID-induced small bowel damage.

AUTHOR CONTRIBUTIONS

Vanessa D'Antongiovanni: Investigation (equal); methodology (equal); project administration (equal); writing—original draft (lead). **Luca Antonioli:** Conceptualization (equal); data curation (equal); formal analysis (equal); writing—review and editing (equal). **Laura Benvenuti:** Data curation (equal); formal analysis (equal); methodology (equal). **Carolina Pellegrini:** Software (equal); supervision (equal). **Clelia Di Salvo:** Formal analysis (supporting); methodology (supporting). **Marco Calvigioni:** Methodology (supporting); software (supporting). **Adelaide Panattoni:** Methodology (supporting); software (supporting). **Larisa Ryskalin:** Formal analysis (supporting); methodology (supporting). **Gianfranco Natale:** Formal analysis (equal); software (equal); validation (equal). **Sebastiano Banni:** Data curation (supporting); formal analysis (supporting); methodology (supporting). **Gianfranca Carta:** Software (equal); supervision (equal); validation (equal). **Emilia Ghelardi:** Data curation (equal); supervision (equal); validation (equal). **Matteo Fornai:** Conceptualization (equal); project administration (equal); writing—review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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SUPPORTING INFORMATION

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