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Protective effects of *Saccharomyces boulardii* CNCM I-745 in an experimental model of NSAID-induced enteropathy

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) induce a broad spectrum of gastro-intestinal adverse effects, including ulceration and bleeding. The pathophysiology of NSAID enteropathy is complex and incompletely understood, but some evidence showed that NSAIDs impair the intestinal barrier and cause a gut dysbiosis. Identifying new treatments aiming to reverse or attenuate NSAID-induced adverse effects would have a significant impact on a high number of patients. The aim of this work is to assess the effects of the probiotic yeast *Saccharomyces boulardii* CNCM I-745 (Sb) on a model of NSAID-induced enteropathy. Four groups of mice were tested: Control, Indomethacin, Sb, and Sb + Indomethacin. A clinical score was evaluated throughout the experiment. Faecal calprotectin, microbiota and haemoglobin analyses were performed. At the end of the treatments, the small intestine, colon, and caecum lengths, and intestinal permeability were measured. Sections of ileum and jejunum were observed to assess a histological score and ileal cytokines were measured by immunoassay. Indomethacin-treated animals showed an increase in their clinical scores, reflecting a worsening of their general state. Mice co-treated with Sb and indomethacin displayed an improvement of their clinical score in comparison with mice treated with indomethacin alone. Sb prevented the indomethacin-induced shortening of the small intestine and caecum, and significantly attenuated the severity of intestinal lesions. Sb also prevented the increase in faecal calprotectin, reduced faecal haemoglobin, and prevented the increase of intestinal permeability in mice treated with indomethacin. Sb also counteracted the increase of faecal bacteria associated with the pathogenesis of NSAID-enteropathy. In conclusion, our results show a protective effect of Sb in a model of indomethacin-induced enteropathy. Sb improved the intestinal barrier function and exerted a positive action on gut microbiota composition.

Keywords: enteropathy, intestinal epithelial barrier, nonsteroidal anti-inflammatory drugs, *Saccharomyces boulardii*, probiotics

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to relieve pain and fever. Despite their clinical utility, NSAIDs are associated with a broad spectrum of gastro-intestinal adverse effects, including ulceration, increased intestinal permeability, and bleeding (Lanas and Sopeña, 2009). The side effects of long-term NSAIDs use cause thousands of hospitalisations and are responsible of 16,500 deaths per year among patients affected by chronic forms of arthritis (Wang *et al.*, 2021). Small intestinal injuries are observed in approximately 70% of chronic NSAIDs users; however, the pathophysiology of these injuries is complex and has not been fully elucidated yet (Abraham and Graham, 2005). NSAIDs suppress prostaglandin synthesis by inhibiting cyclooxygenase (COX-1 and COX-2), impair mucosal cells via direct damage to the membrane brush border, uncouple mitochondrial oxidative phosphorylation, and increase the intestinal permeability (Bjarnason *et al.*, 2018). These actions facilitate mucosal exposure to luminal aggressive factors, such as bile, enzymes, and intestinal bacteria, thereby triggering inflammation and injuries in the small intestine (Bjarnason *et al.*, 2018; Jacob *et al.*, 2007). The enterohepatic re-circulation of NSAIDs further enhances its direct cytotoxic action on enterocytes (Blackler *et al.*, 2015; Wallace, 2013).

Gut microbiota is also implicated in the pathogenesis of NSAIDs enteropathy. Indeed, germ-free mice are resistant to indomethacin-induced small intestine injuries but can become sensitive when colonised with commensal microbiota (Robert and Asano, 1977; Uejima *et al.*, 1996). Antibiotics have also been shown to reduce indomethacin-induced small intestine injuries in rats, which support the role of enteric bacteria in NSAID-induced enteropathy (Fornai *et al.*, 2016; Koga *et al.,* 1999; Uejima *et al.*, 1996). Several studies also showed that NSAIDs cause dysbiosis in the small and large intestine in rodents (Maseda *et al.*, 2019; Reuter *et al.*, 1997; Terán-Ventura *et al.*, 2014; Watanabe *et al.*, 2009), and humans (Rogers and Aronoff, 2016). This dysbiosis may exacerbate the mucosal damage via activation of Toll-like receptor 4 (TLR4). TLR4 signalling activates nuclear factor-κB through the MyD88-dependent pathway, causing an infiltration of inflammatory cells, ulceration of the small intestine mucosa, and increase of pro-inflammatory cytokines (Chen *et al.*, 2018; Maseda and Ricciotti, 2020; Watanabe *et al.*, 2008). Further supporting a role for gut microbiota in enteropathy, probiotics has shown some beneficial effects in different experimental models of NSAIDs-induced enteropathy (Montalto *et al.*, 2013).

Probiotics are defined as living microorganisms that affect human health in a positive manner, and thus can be a potential treatment to prevent NSAID-enteropathy. *Saccharomyces boulardii* CNCM I-745® (Sb), a probiotic yeast, has been extensively investigated over the past decades as a treatment for several gastrointestinal diseases (Dinleyici *et al.*, 2014; Feizizadeh *et al.*, 2014; McFarland *et al.*, 1994). The main mechanisms of action of Sb include antimicrobial and anti-toxin activities (Castagliuolo *et al.*, 1996; Czerucka and Rampal, 2019; Jawhara and Poulain, 2007), trophic effects upon the intestinal mucosa (Buts and Keyser, 2006), and anti-inflammatory properties (Brun *et al.*, 2017; Sougioultzis *et al.*, 2006). Some studies also show that Sb has a positive effect on intestinal dysbiosis induced by antibiotics in mice (Barc *et al.*, 2008; Collignon *et al.,* 2010) or humans (Kabbani *et al.*, 2017), and several studies showed that Sb plays a crucial role in the preservation and/or restoration of gut barrier function in different disorders (Terciolo *et al.*, 2019).

Because of their relatively low cost and high effectiveness to manage pain, NSAIDs will continue to be particularly prevalent. It would thus be clinically relevant to co-administer probiotics with NSAIDs to reduce the risk of intestinal damage. Nevertheless, little attention has been paid to the impact of Sb on NSAID-induced enteropathy. Given its beneficial action on experimental colitis models, we aimed to explore the effects of Sb on small intestinal injuries caused by indomethacin in a widely used animal model of NSAID-induced enteropathy associated with gut microbiota modifications.

2. Materials and methods

Animals

Four-week-old specific-pathogen-free male C57Bl/6J mice were purchased (Charles River, L'Arbresle, France) and allowed to acclimate for 3 weeks in our conventional animal facility. Mice were group-housed with *ad libitum* access to food (A04, Safe, Augy, France) and autoclaved water in a temperature-controlled (22±2 °C) and humidity-controlled (55±10%) environment. Lights were on a 12-h light/dark cycle (lights on at 7:00 am). Experiments were carried out in strict accordance with the European Community regulations for animal use in research (EC, 2010), and all protocols were approved by the local Ethical Committee (C2EAn°72) and received authorisation from the French Ministry of Research prior to initiation of the research (APAFIS#21571-2019041814235846v13).

Experimental design

Mice were randomly divided into four groups (Figure 1A and Table 1): All animals received a total of 2 daily oral treatments from day -14 to day 10. All treatments were administered orally by an experimented technician with disposable flexible plastic feeding tubes (FTP 20-

Table 1. Treatment groups.[1]

Groups	Treatments from day -14 to day -1	Treatments from day 0 to day 10
Vehicle – Vehicle [V-V] (n=10)	autoclaved tap water (twice daily, 10 ml/kg)	autoclaved tap water (once daily, 10 ml/kg), PBS 1× (once daily, 10 ml/kg)
Vehicle – Indomethacin [V-I] (n=15)	autoclaved tap water (twice daily, 10 ml/kg)	autoclaved tap water (once daily, 10 ml/kg), Indomethacin (once daily, 7.5 mg/kg, 10 ml/kg)
Sb – Vehicle [S-V] (n=10)	Sb (twice daily, 3g/kg, 10 ml/kg)	Sb (once daily, 3 g/kg, 10 ml/kg), PBS 1× (once daily, 10 ml/kg)
Sb – Indomethacin [S-I] (n=15)	Sb (twice daily, 3 g/kg, 10 ml/kg)	Sb (once daily, 3 g/kg, 10 ml/kg), Indomethacin (once daily, 7.5 mg/kg, 10 ml/kg)

[1] Sb = *Saccharomyces boulardii* CNCM I-745; PBS = phosphate buffered saline.

38, Phymep, Paris, France). Due to expected mortality in the indomethacin group, the number of mice was more important in groups treated with indomethacin. Body weight was evaluated daily. Fresh stools were collected to assess microbiota composition and calprotectin. At the end of the procedure, mice were euthanatised after intestinal permeability assessment. Intestinal length and macroscopic damage score were then determined. Small intestine samples were taken for microscopic analysis and cytokine immunoassay. Fresh faecal materials were taken for haemoglobin concentration.

Clinical assessment of indomethacin-induced effects

A clinical score was determined daily from day 0. Body weight loss (0: less than 5%; 1: less than 10%; 2: less than 20%). Appearance (0: normal; 1: lack of grooming apparent, soft stools; 2: hypomotility, unkempt coat, diarrhoea; 3: abnormal posture, irregular respiration rate; 4: total immobility). Behaviour (0: normal; 1: decreased exploration; 2: very few or no exploration, piloerection; 3: no exploration, hunched, dyspnoea). Cut-off criteria for reaching end point were a clinical score equal or higher than 4 or a weight loss greater than 20%.

Faecal microbiota analysis

Faecal microbiota analyses were performed on stool samples collected in all animals at day -21 (baseline), day 0 and day 7, immediately frozen and stored at -80 °C until assay. Bacterial DNA was extracted from 100 mg of stool sample following a protocol with a double lysis based on Maxwell automate (Promega, Charbonnières-les-Bains, France). DNA concentration was then measured by fluorimetry with Qubit® (Invitrogen by ThermoFisher Scientific, Waltham, MA, USA) and DNA solution were stored at -20 °C until library construction. PCR amplification was performed using 16S universal primers targeting the V3-V4 hypervariable region of the bacterial 16S ribosomal genes (Klindworth *et al.*, 2013). PCR products were cleaned up, barcoded, and normalised according to Illumina guideline and protocol. Sequencing was performed with a MiSeq instrument (Illumina®, San Diego, CA, USA) with the V2 chemistry.

Macroscopic damage scores

Macroscopic scores were given based on the following criteria upon laparotomy (adapted from (Xiao *et al.*, 2017)): (A) abdominal distension (0, absent; 1, present), (B) presence of adherence (0, no adherence; 1, some local adherence; 2, extensive and generalised adherence), (C) appearance of the small intestine (0, normal; 1, moderate signs of congestion and/or distension; 2, clear and generalised alterations), (D) appearance of bowel contents (0, normal; 1, reduced content with some fluid or mucus; 2, no content but with fluid or mucus, and blood).

Faecal haemoglobin concentration

Fresh stool was diluted 20 times with ultrapure water, vortexed, and centrifugated. The supernatant was used for haemoglobin concentration assay (Hemoglobin Assay Kit, MAK 115, Sigma-Aldrich, Saint-Quentin-Fallavier, France), based on the improved Triton®/NaOH method in which haemoglobin is converted to a colorimetric product measured at 400 nm. Optical density was measured using a multiplate reader (Dynex MRXe, Serlabo Technologies, Entraigues-sur-la-Sorgue, France).

Faecal calprotectin levels

Calprotectin is a calcium binding protein of neutrophil granulocytes that correlates with neutrophil infiltration into the intestinal mucosa. A murine calprotectin ELISA kit (HK214, Hycult Biotech, Uden, the Netherlands) was used according to the manufacturer's protocol. Briefly, 20 mg of faeces was homogenised in extraction buffer (0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10 mM $CaCl_2$, 0.1 M citric acid monohydrate, 5g/l bovine serum albumin (BSA) and 0.25 mM thimerosal [pH 8.0]). The homogenate was centrifugated at 10,000×*g* at 4 °C during 20 min and the supernatant used according to manufacturer's instructions.

Intestinal permeability assessment

In vivo intestinal permeability was measured using fluorescein isothiocyanate (FITC)-dextran method. Briefly, on the day of euthanasia, mice were deprived of food for 3 h, then given FITC-dextran (4000 Da, Sigma-Aldrich, 60 mg/100 g of body weight) intragastrically in 0.1 ml phosphate buffered saline (PBS) 4 h before their euthanasia. Serum samples were then collected. Fluorescence intensity was measured spectrophotometrically (excitation 470 nm, emission 520 nm, fluorescence microplate reader BioSpectrometer®, Eppendorf, Montesson, France). FITC-dextran concentrations were calculated through standards concentration prepared in PBS. Intestinal permeability positively correlates with fluorescence intensity.

Microscopic damage scores

Upon removal, the intestinal tract was immediately cleaned of faecal content with cold PBS 1×, and 1 cm of ileum and jejunum were fixed in 10% formalin. Tissue samples were collected according to a standardised dissection procedure to rule out any bias. Paraffin sections (4 µm) of tissue samples were stained with haematoxylin-eosin following standard procedures. Thirty to forty sections from each animal were scored in a blinded fashion using an optical microscope (Leica DM 4000, Leica Biosystems, Nanterre, France) coupled to a camera (Sony XCD-U100CR, Sony, Tokyo, Japan) and to an acquisition system (Archimed, Microvision Instruments, Lisses, France). All images were

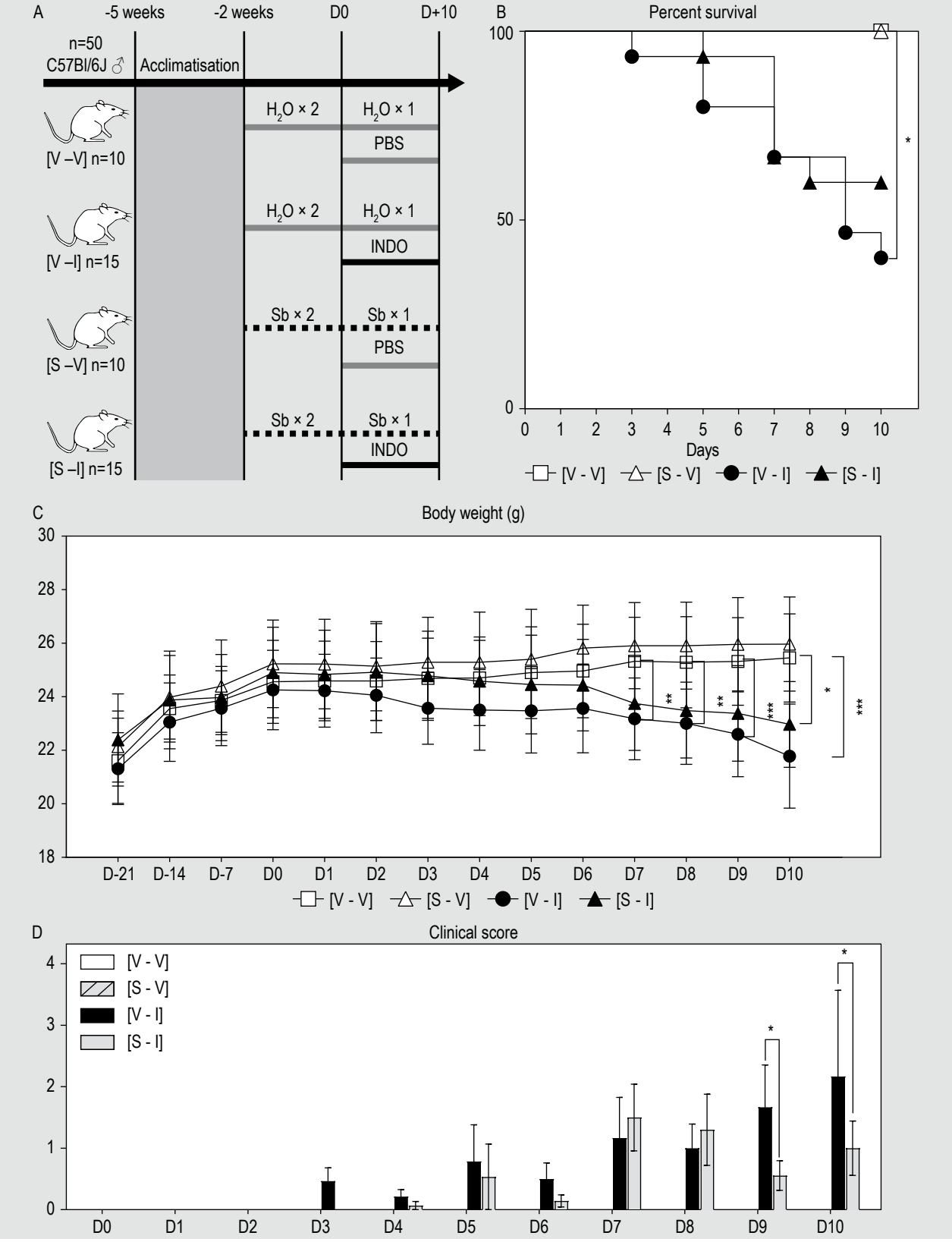

Figure 1. (A) Experimental design. Four groups of male mice were tested: one control group treated with vehicle [V-V] (n=10), one group treated with indomethacin alone [V-I] (n=15, 7.5 mg/kg, daily), one group treated with *Saccharomyces boulardii* CNCM I-745 alone [S-V] (n=10, 6 g/kg, daily), and one group co-treated with *Saccharomyces boulardii* CNCM I-745 plus indomethacin [S-I] (n=15). (B) Survival rate was recorded daily and analysed by log-rank test. (C) Body weight was recorded daily (mean ± standard error of the mean (SEM)). (D) Clinical score was measured daily (mean ± SEM). * *P*<0.05, ** *P*<0.01 and * *P*<0.001.**

acquired using a 20× objective lens. The histological score (0 to 9, adapted from (Terán-Ventura *et al.*, 2014)) was calculated for each section by summing the epithelial structure (0: normal; 1: mild alterations; 2: moderate alterations; 3: severe alterations), the presence of ulcerations (0: absent; 1: one ulcer observed; 2: several small ulcers observed; 3: numerous small and large ulcers observed), and the inflammatory infiltrate (0: absent; 1: mild localised inflammatory infiltrate; 2: moderate diffuse infiltrate; 3: severe generalised infiltrate). For villus length-to-crypt depth ratio measurement, ten villi and ten intestinal crypts per slide were measured.

Cytokine immunoassay

Ileal samples were washed, harvested, homogenised in a lysis buffer (RIPA buffer, Abcam, Cambridge, UK) containing protease and phosphatase inhibitor cocktail (Pierce™ protease and phosphatase inhibitor, ThermoFisher Scientific) and frozen. Homogenates were centrifuged twice at 1,500 rpm for 15 min at 4 °C. All assays were performed according to the manufacturer's instructions, in duplicates, and without modifications to the recommended standard curve dilution. The concentration of 7 cytokines/chemokines (interleukin (IL)-1β, IL-6, IL-10, IL-17a, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, and monocyte chemoattractant protein (MCP)-1) was determinated by Bio-Plex Pro™ mouse cytokine assays (Bio-Rad Laboratories, Hercules, CA, USA) in 500 µg of total protein of ileum homogenates (Pierce BCA protein assay kit, ThermoFisher Scientific). Briefly, magnetic beads conjugated with cytokine antibodies were loaded into the wells of a 96-well plate. After washing, standards and samples were added into wells and incubated for 30 min at room temperature (RT) on a shaking platform. The beads were washed and incubated with biotinylated detection antibody for 30 min at RT. Following the removal of excessive detection antibodies, streptavidin-phycoerythrin conjugate compound was added and allowed to incubate for 10 min at RT. Cytokine's concentrations were determined by Bio-Plex Manager™ MP software version 6.1 and calculated against the standard curve.

Data and statistical analysis

The results are presented as mean ± standard error of the mean (SEM) and show individual values, boxplots were used for bacterial populations. Survival rate was analysed by log-rank test. Metagenomic data were analysed using R-language software (R Core Team, 2018) for α and β diversity. Raw sequencing reads were analysed using bioinformatics pipeline based on Dadaist2 toolkit (Ansorge *et al.*, 2021). Basically, after demultiplexing of the barcoded Illumina paired reads, single read sequences were paired for each sample into longer fragments and cleaned. After quality-filtering and sequencing error modelling, Amplicons Variants Sequences (ASVs) were obtained. A taxonomic assignment of these ASVs were performed to determine bacterial community profiles at genus level.

Tools for bioinformatics pipeline and corresponding parameters are as follows:

- FastQC v0.11.8: quality control reports of NGS sequencing data.
- Dadaist2 Software version: 1.1.0.
- Database version used for taxonomy: RDP release 18.

Other results were analysed using two-way analysis of variance (ANOVA) (Sb or Vehicle pre-treatment) X (Indomethacin or Vehicle treatment) (with repeated measures for bodyweight and clinical score) with post hoc Tukey's multiple comparison test. ANOVA were performed using SigmaPlot® software v 12.5 (Systat Software Inc., San Jose, CA, USA). Significance was set at $P<0.05$.

3. Results

Survival rate, body weight, and clinical score

To identify the optimal indomethacin dose, we performed preliminary experiments with indomethacin doses ranging from 0.1 to 10 mg/kg. We observed a survival rate of 100% for mice treated with doses ranging up to 5 mg/kg for 10 days, whereas all mice treated with the dose of 10 mg/kg died (data not shown). The intermediate dose of 7.5 mg/kg was associated with a survival rate of 40% in mice after 10 days of treatment whereas mice treated with Sb + indomethacin displayed a 60% survival rate (Figure 1B). Based on these data, we performed the following experiments using 7.5 mg/kg of indomethacin.

While control mice showed a linear increase in body weight over time, indomethacin treated mice showed an impairment of their body weight gain: starting from the 7th day of treatment, they weighed significantly less than control mice. Mice treated with Sb alone or Sb + indomethacin did not differ from control group until the last day of treatment (Figure 1C).

Starting from day 7, indomethacin-treated animals showed a significant increase in their clinical scores (reflecting a worsening in their general state) in comparison with control group or mice treated with Sb ($P<0.001$). Sb + indomethacin mice also displayed a significant increase in their clinical score, although it remained significantly lower in comparison with indomethacin group on day 9 and 10 ($P<0.05$) (Figure 1D).

Faecal microbiota analysis

Rarefaction curves confirmed an adequate depth of sequencing (Supplementary Figure S1). Shannon and observed ASV indexes did not show significant inter- or intra-group differences (Supplementary Figure S2, Supplementary Table S1 and S2). Principal coordinates analysis (PCoA) revealed that treatments with Sb, indomethacin, or both, did not show major impact on the microbial community (Supplementary Figure S3). At the 3 time points and in all groups, faecal *Firmicutes* and *Bacteroidetes* were the most abundant phyla, in accordance with several microbiome studies. After 7 days of treatment with indomethacin, *Bacteroidetes* were significantly increased in comparison with control group ($P<0.05$, 21% for [V-V] vs 34% for [V-I]) while *Firmicutes* decreased, but the difference was not significant ($P=0.07$, 58% for [V-V] vs 47% for [V-I]). *Actinobacteria* were significantly reduced in the indomethacin group ($P<0.001$, 14% for [V-V] vs 3% for [V-I]), whereas *Proteobacteria* were significantly increased ($P<0.001$, 0.8% for [V-V] vs 4.3% for [V-I]). Treatment with Sb prevented this *Proteobacteria* increase ($P<0.001$, 1.4% for [S-I]) (Figure 2A and 2B).

At the genus level, after 7 days of treatment, indomethacin significantly reduced abundances of *Turicibacter* ($P<0.001$, 14% for [V-V] vs 0.03% for [V-I]) and *Bifidobacterium* ($P<0.001$, 9.5% for [V-V] vs 1.4% for [V-I]), and significantly increased abundances of *Bacteroides* ($P=0.018$, 1.5% for [V-V] vs 6.2% for [V-I]), *Ligilactobacillus* ($P=0.006$, 0.7% for [V-V] vs 5% for [V-I]), *Muricibaculum* ($P=0.027$, 0.8% for [V-V] vs 2.2% for [V-I]) and *Escherichia/Shigella* ($P=0.007$, 0% for [V-V] vs 2.5% for [V-I]) in comparison with vehicle treatment. Sb treatment alone had no effect on the most abundant genus, but Sb co-treatment with indomethacin significantly increased *Akkermansia* ($P<0.01$, 10.9% for [S-V] vs 17.9% for [S-I]) and prevented the increase of *Escherichia/Shigella* due to indomethacin ($P=0.018$, 2.5% for [V-I] vs 0.4% for [S-I]) (Figure 3A and 3B).

Macroscopic score

Macroscopic assessment of the abdominal cavity and the gastrointestinal tract revealed that mice treated with indomethacin displayed a significantly higher macroscopic score, as compared with control mice ($P<0.001$). The main alterations observed were abdominal distension and adherences. Pre-treatment with Sb before and during indomethacin administration significantly reduced the macroscopic score in animals treated with indomethacin ($P=0.019$) (Figure 4A). The length of the small intestine, caecum and colon in indomethacin-treated mice was significantly shorter than that in control animals ($P<0.001$). Sb prevented the shortening of the small intestine and caecum, as compared with mice treated with indomethacin ($P<0.01$) (Figure 4B).

Faecal haemoglobin concentration and faecal calprotectin levels

Indomethacin-treated mice had a significantly higher faecal haemoglobin concentration compared to [V-V] mice (908.6 mg/dl for the [V-I] group vs 516.4 mg/dl for the [V-V] group, $P=0.01$) while the presence of Sb significantly reduced haemoglobin concentration in faeces (518.6 mg/dl for the [S-I] group vs 908.6 mg/dl for the [V-I] group, $P=0.015$) (Figure 5A).

At day 9, indomethacin-treated mice showed a significant increase in faecal calprotectin levels compared to control animals (1,138 ng/mg vs 226 ng/mg, $P<0.001$). Mice treated with Sb + indomethacin had a weaker increase of faecal calprotectin levels compared to indomethacin-treated mice, although this effect just failed short for statistical significance ($P=0.052$) (Figure 5B).

Intestinal permeability assessment

Indomethacin treatment significantly increased the plasma FITC-dextran concentrations (1.5±0.4 μg/ml in the [V-I] group vs 0.5±0.2 μg/mL in the [V-V] group, $P=0.01$), indicating that indomethacin increases intestinal epithelial permeability. Treatment with Sb alone and Sb + indomethacin did not change the plasma FITC-dextran concentration in comparison with the control group. No statistically significant difference was observed between the groups treated with indomethacin alone and Sb + indomethacin (Figure 6).

Microscopic damage score

Histologic damage score was significantly increased in ileum ($P=0.004$) and jejunum ($P=0.01$) of mice treated with indomethacin compared to control group. The more frequent histopathological modifications observed were alteration of the epithelial structure (especially the destruction of the villi), and the presence of inflammatory cells such as lymphocytes and neutrophils. Pre-treatment and treatment with Sb during indomethacin administration significantly improved the severity of intestinal lesions in ileum ($P=0.009$). Histological damage score in the Sb + indomethacin group was not different from the control group for the jejunum, indicating no damage. The villus/crypt ratio was significantly decreased in ileum ($P=0.004$) and jejunum ($P=0.01$) of mice treated with indomethacin compared to control mice. This ratio was not different from control group in mice treated with Sb + indomethacin (Figure 7).

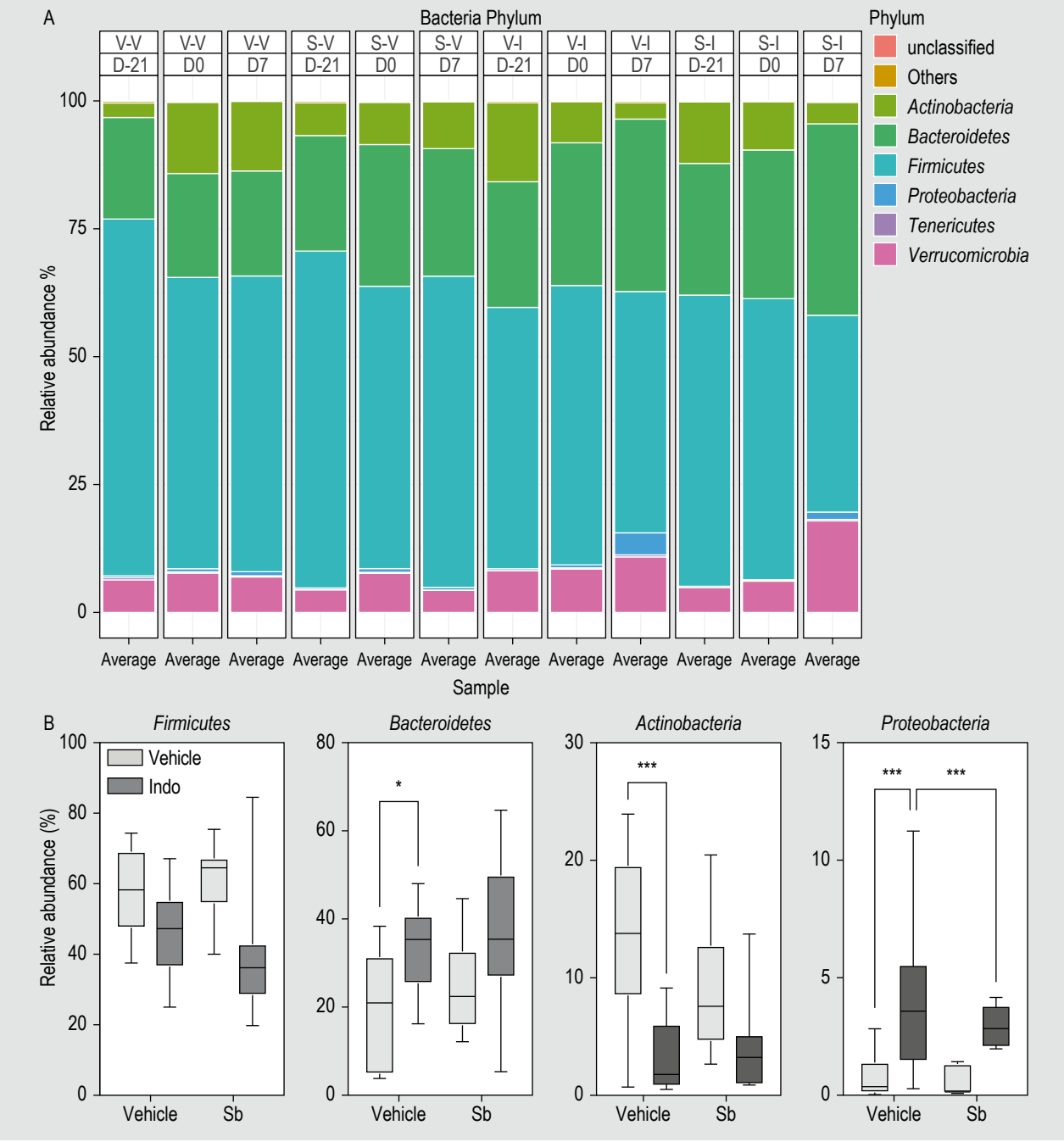

Figure 2. (A) Relative abundance of faecal bacterial populations at the phylum level from mice treated with vehicle [V-V] (n=10), *Saccharomyces boulardii* CNCM I-745 [S-V] (n=10, 6 g/kg, daily), indomethacin [V-I] (n=12, 7.5 mg/kg, daily) or *Saccharomyces boulardii* CNCM I-745 plus indomethacin [S-I] (n=12) at day 7. (B) Relative abundance of Firmicutes, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* from mice treated with vehicle [V-V], *Saccharomyces boulardii* [S-V], indomethacin [V-I] or *Saccharomyces boulardii* CNCM I-745 plus indomethacin [S-I] at day 7.

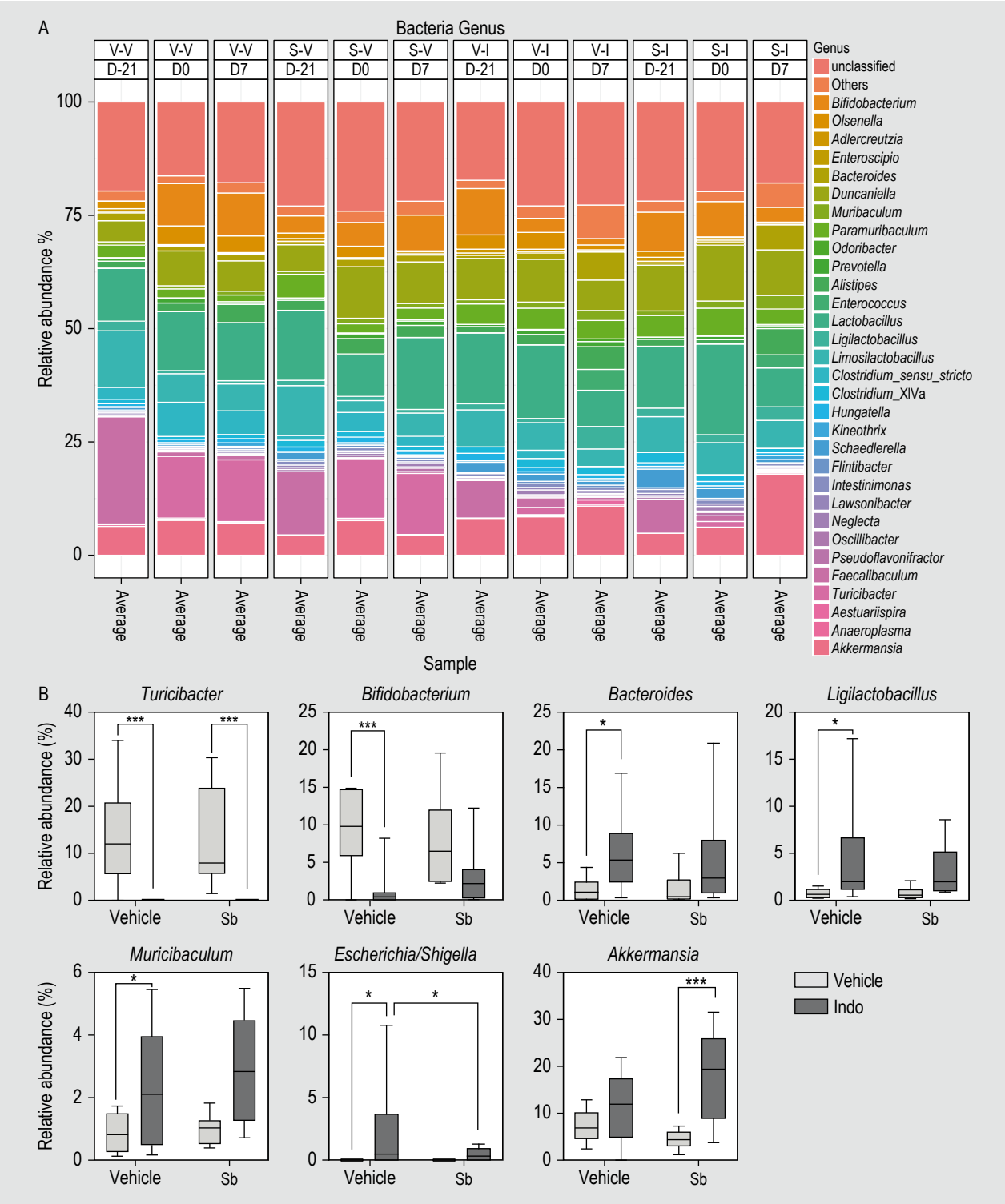

Figure 3. (A) Relative abundance of faecal bacterial populations at the genus level from mice treated with vehicle [V-V] (n=10), *Saccharomyces boulardii* CNCM I-745 [S-V] (n=10, 6 g/kg, daily), indomethacin [V-I] (n=12, 7.5 mg/kg, daily) or *Saccharomyces boulardii* CNCM I-745 plus indomethacin [S-I] (n=12) at day 7. (B) Relative abundance of Turicibacter, *Bifidobacterium*, *Bacteroides*, *Ligilactobacillus*, *Muricibaculum*, *Escherichia*/*Shigella*, and *Akkermansia* from mice treated with vehicle [V-V], *Saccharomyces boulardii* [S-V], indomethacin [V-I] or *Saccharomyces boulardii* CNCM I-745 plus indomethacin [S-I] at day 7.

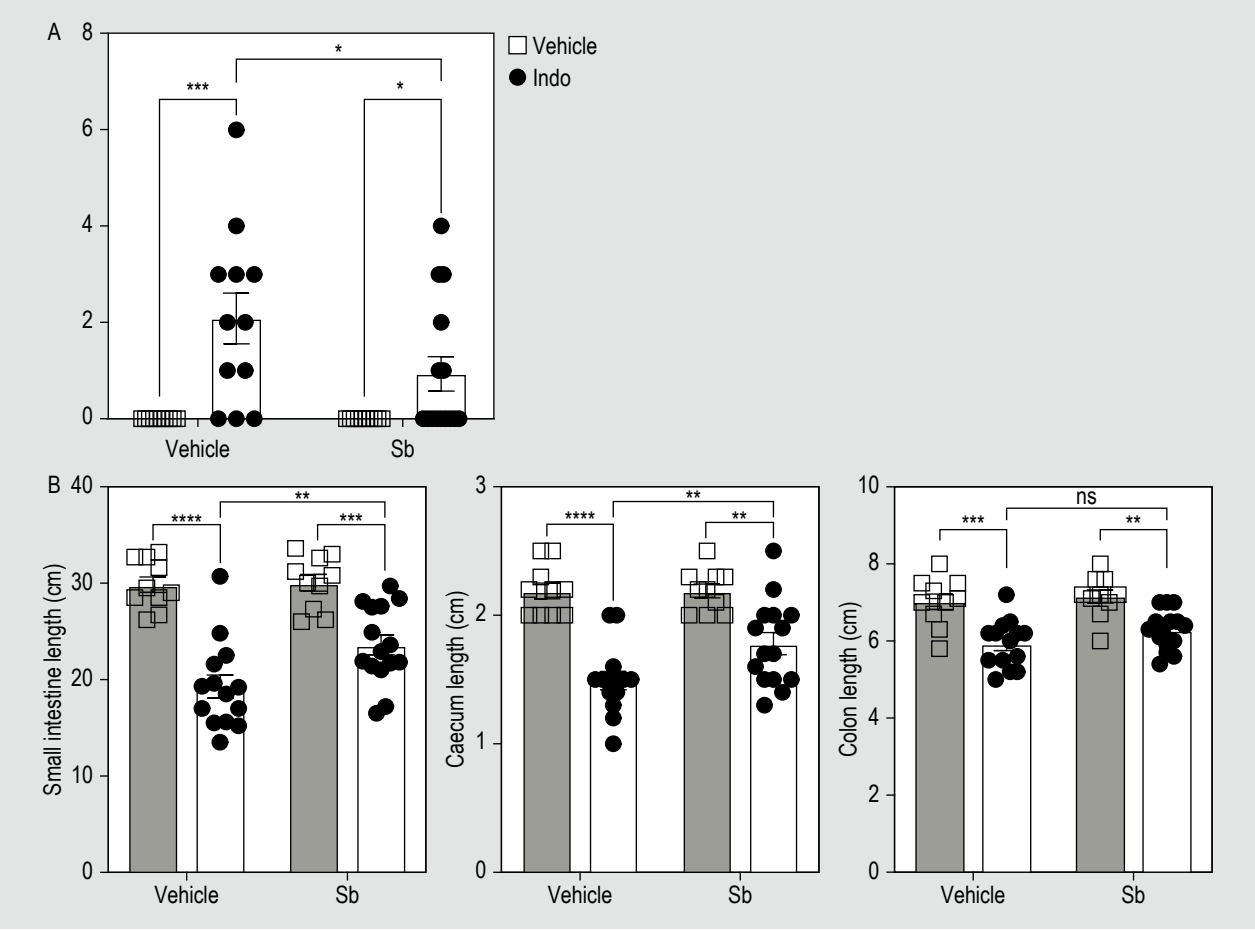

Figure 4. (A) Macroscopic score of mice treated with vehicle, indomethacin (Indo, 7.5 mg/kg, daily), *Saccharomyces boulardii* CNCM I-745 (Sb, 6 g/kg, daily), or both Sb and indomethacin. (B) Small intestine, caecum, and colon length (cm). * *P*<0.05, ** *P*<0.01,* *P*<0.001, and **** *P*<0.0001.**

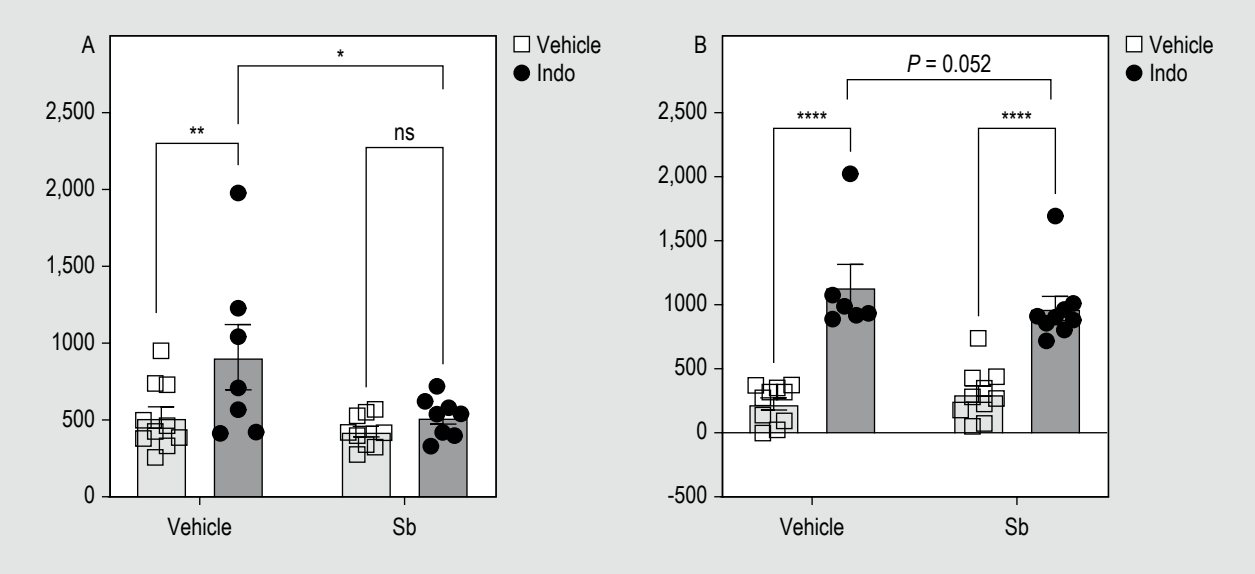

Figure 5. (A) Faecal haemoglobin concentration (mg/dl) of mice treated with vehicle, indomethacin (Indo, 7.5 mg/kg, daily), *Saccharomyces boulardii* CNCM I-745 (Sb, 6 g/kg, daily), or both Sb and indomethacin. Faecal haemoglobin concentration was determined on fresh faecal samples at the end of treatments. (B) Faecal calprotectin levels (ng/mg of faeces) were determined by ELISA on fresh faecal samples collected at day 9. * *P*<0.05, ** *P*<0.01,* *P*<0.001, and **** *P*<0.0001.**

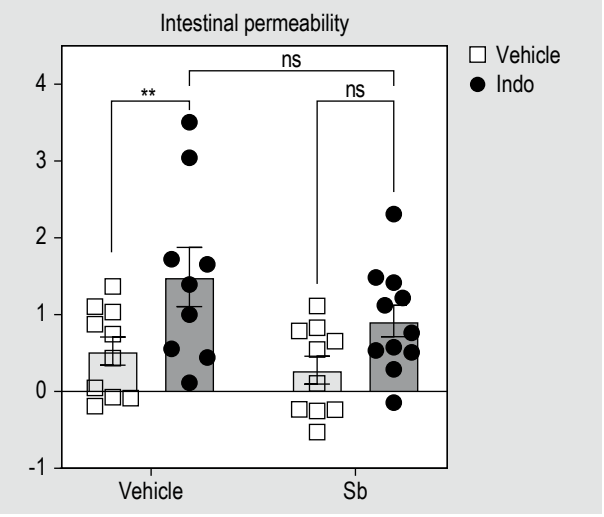

Figure 6. Plasma FITC-dextran concentration of mice treated with vehicle, indomethacin (Indo, 7.5 mg/kg, daily), *Saccharomyces boulardii* CNCM I-745 (Sb, 6 g/kg, daily), or both Sb and indomethacin. ** *P*<0.01, and * *P*<0.001.**

Cytokine immunoassay

Sb treatment alone did not change the levels of ileal cytokines and chemokines compared to control group. In mice treated with indomethacin, the levels of the proinflammatory cytokine IL-6 and the chemokine MCP-1 were significantly higher in comparison with control mice. Sb co-administered with indomethacin had no effect on these levels (Supplementary Figure S4).

4. Discussion

NSAIDs are largely used for their anti-inflammatory, antipyretic, and analgesic actions, but their use is associated with adverse effects affecting the gastrointestinal tract. Currently, there are no therapeutic strategies for the prevention of NSAID-induced enteropathy. Systemic administration of indomethacin in rodents induces an enteropathy that mimics intestinal damage observed in patients. In accordance with other studies (Horibe *et al.*, 2016; Tanigawa *et al.*, 2013; Whitfield-Cargile *et al.*, 2016), our experimental model shows impaired epithelial barrier function, increased intestinal permeability, inflammation, and intestinal bleeding. Furthermore, in our model, indomethacin induces modification in the faecal bacterial composition. In this context, the use of probiotics could represent a therapeutic intervention to avoid intestinal complications associated with NSAIDs medication. For example, *Lacticaseibacillus casei* strain Shirota prevents indomethacin-induced small intestinal injury by suppressing neutrophil infiltration and gene expression of inflammatory cytokines (Watanabe *et al.*, 2009). *Bifidobacterium longum*

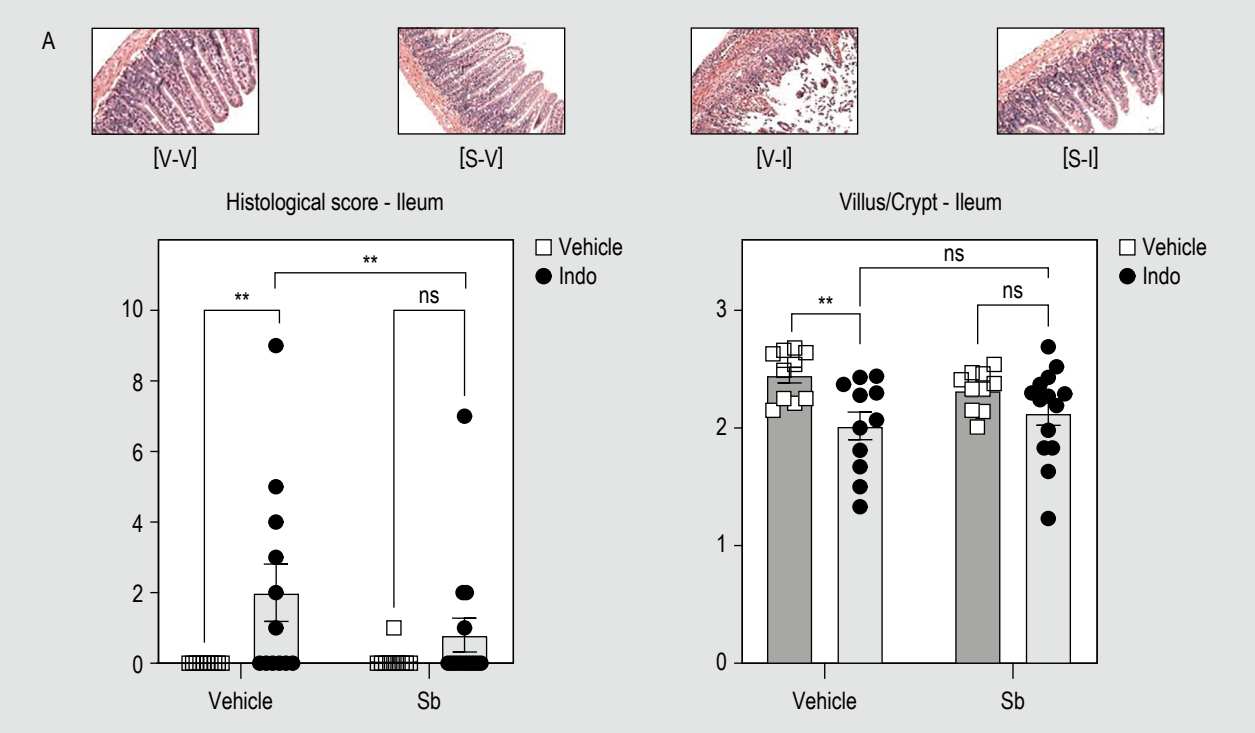

Figure 7. Protective effects of Sb treatment against indomethacin-induced small intestine damage. Pathological changes were observed by H&E staining in ileum and jejunum (magnification ×10). Representative images of ileal (A) and jejunal (B) samples. Histological score of ileal (A) and jejunal (B) samples. Villus length-to-crypt depth ratio measurement of ileal (A) and jejunal (B) samples. ** *P*<0.01, and * *P*<0.001.**

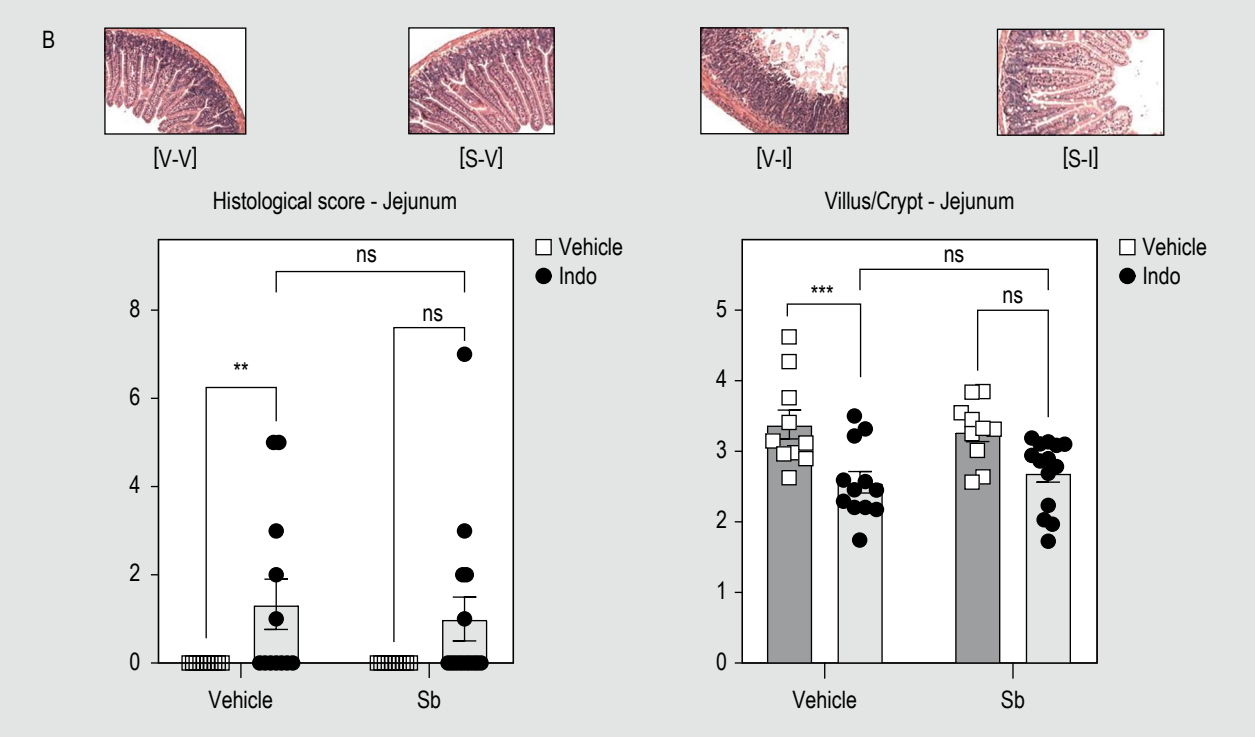

Figure 7. Continued.

BB536 have beneficial effects on an experimental model of colitis (Ocón *et al.*, 2013). *Bifidobacterium infantis* reduces tissue injury and inflammation in a rat model of necrotizing enterocolitis (Underwood *et al.*, 2014). *B. longum* associate with lactoferrin, a prebiotic, exerts a protective effect on a model of NSAID-induced enteropathy (Fornai *et al.*, 2020). Other studies showed that probiotics may help to restore an altered gut microbiota in NSAID-induced enteropathy. In rats, *Bifidobacterium adolescentis* reduces NSAID-induced ileal damage by preventing the unbalanced growth of aerobic bacteria and lipid peroxidation (Kinouchi *et al.*, 1998). Bifidobacteria and lactobacilli also showed beneficial effects in humans (Endo *et al.*, 2011; Montalto *et al.*, 2010).

S. boulardii CNCM I-745 (Sb) is a non-pathogenic yeast which is widely used as a probiotic in the prevention and treatment of gastrointestinal disorders (Kelesidis and Pothoulakis, 2012). Sb has been reported to improve antibiotic-associated disorders such as diarrhoea (Moré and Swidsinski, 2015) and *Clostridioides difficile* infections (Castagliuolo *et al.*, 1996). Clinical studies have also confirmed that Sb can improve the quality of life and symptoms in irritable bowel syndrome (IBS) patients (Choi *et al.*, 2011) by exerting immunomodulatory properties (Abbas *et al.*, 2014). Sb ability to improve intestinal permeability alterations has also been extensively proven (Terciolo *et al.*, 2019). Indeed, Sb has been found to exert both direct and indirect modulatory actions that impact intestinal integrity in different models. Sb protects the epithelial morphology and maintains E-cadherin expression at the cell surface of cultured explants of colon from inflammatory bowel disease (IBD) patients (Terciolo *et al.*, 2017). Sb also improved histological damage, inflammation, and intestinal epithelial barrier dysfunction in several models of colitis (Rodríguez-Nogales *et al.*, 2018; Soyturk *et al.*, 2012). For example, Sb significantly increased the expression of mucins, tight junction proteins, and occludin, which participate in the maintenance of the epithelial integrity in a model of colitis induced by dextran sodium sulphate (DSS) in mice (Rodríguez-Nogales *et al.*, 2018). Pre-treatment with Sb also reduced intestinal damage and lesions in a rat model of *Vibrio cholerae* infection (Brandão *et al.*, 1998) and restored the claudin-1 expression and the barrier integrity in both *in vitro* and *in vivo* models of *Shigella* infection (Mumy *et al.*, 2008).

This study shows that the probiotic yeast Sb reduces the severity of small intestine damage induced by indomethacin. Two weeks of pre-treatment with Sb and concomitant treatment with Sb and indomethacin improve the general state of mice and reduce the severity of several enteropathy hallmarks. Our results show that Sb treatment prevents the shortening of the small intestine and caecum which is a characteristic of NSAIDs treatments (Horibe *et al.*, 2016). Sb prevention of indomethacin-induced small intestine damage was confirmed by a reduction of histological scores in ileal and jejunal sections. This was supported by morphological parameters which showed maintenance of villus length-to-crypt depth ratio in mice treated with

Sb plus indomethacin, confirming the restoration of the intestinal barrier. Our results also show Sb prevention of altered intestinal permeability induced by indomethacin. All these data support the beneficial role of Sb on epithelial barrier defects and on the preservation of gut morphology integrity.

Sb may alleviate intestinal permeability through its anti-inflammatory properties. Indeed, Sb cultured supernatant reduced secretion of pro-inflammatory cytokines in LPS-stimulated dendritic cells (Thomas *et al.*, 2009). Sb pre-treatment decreased *Clostridium perfringens*-induced inflammatory cytokines mRNA expression (Wang *et al.*, 2020). In a preclinical model of carbon tetrachloride-induced liver fibrosis, Sb attenuated the increased endotoxin levels and pro-inflammatory cytokines. These were associated with modifications of intestinal permeability and faecal microbial composition (Li *et al.*, 2015). In our study, Sb treatment prevents the increase of faecal calprotectin induced by indomethacin, a well-known marker of mucosal injury and inflammation induced by NSAIDs in human patients and animal models. Sb reduces stool haemoglobin induced by indomethacin. These results confirmed the potential anti-inflammatory effects of Sb.

Different mechanisms have been proposed to contribute to the beneficial effects of Sb, including the reduction of pro-inflammatory cytokines by inhibiting NF-κB signalling (Sougioultzis *et al.*, 2006), and its capacity to reduce reactive oxygen species (ROS) accumulation via the activation of Nrf2 pathway, counteracting oxidative stress (Buccigrossi *et al.*, 2014). Sb can also increase the expression of mucins and tight junction proteins which participate in the maintenance of the epithelial integrity (Rodríguez-Nogales *et al.*, 2018) and exert a trophic effect on enterocytes via the synthesis of polyamines (Buts and Keyser, 2006). Furthermore, experimental and clinical evidence have demonstrated the positive effects of Sb to create a favourable growth environment for the beneficial intestinal microbial species and a faster recovery of healthy microbiome in case of dysbiosis (Moré and Swidsinski, 2015).

Our results show that the severity of small intestine damage induced by indomethacin is associated with an increase of some faecal bacteria, such as *Bacteroides* and *Escherichia/Shigella*, and a reduction of others, such as *Bifidobacterium*. It has been shown that *Proteobacteria*, and more specifically *Escherichia*, have been directly associated with the development of indomethacin-induced enteropathy and the aggravation of colitis (Rath *et al.*, 2001). Conversely, the reduction of bacteria such as *Bifidobacterium* may be damaging because they usually exert an anti-inflammatory and mucosal protective effect (Wallace *et al.*, 2011). The beneficial effects of Sb in indomethacin-treated mice may thus be explained also by a reduction of bacteria, such as *Proteobacteria* or *Bacteroides* which are involved in the pathogenesis of NSAID-enteropathy (Maseda *et al.*, 2019; Nadatani *et al.*, 2012; Terán-Ventura *et al.*, 2014; Watanabe *et al.*, 2008), and by the promotion of the growth of beneficial bacteria, such as *Akkermansia* (Cani *et al.*, 2022; Rodrigues *et al.*, 2022).

In conclusion, the results of the present study showed for the first time a great potential for Sb to exert protective effects against indomethacin-induced intestinal damage. Sb restores the impaired intestinal barrier function, reduces inflammation, and attenuates indomethacin-induced dysbiosis. Sb may represent a promising therapeutic intervention for the prevention of NSAID-induced small intestine damage, particularly for patients affected by chronic inflammatory diseases.

Supplementary material

Supplementary material can be found online at https://doi.org/10.6084/m9.figshare.23507790.

Figure S1. Rarefaction curves for all the samples.

Figure S2. Alpha diversity (Shannon and observed ASV indexes) from faecal samples of mice treated with vehicle [V-V], *Saccharomyces boulardii* CNCM I-245 [S-V] (6g/kg, daily), indomethacin [V-I] (7.5 mg/kg, daily) or *Saccharomyces boulardii* CNCM I-745 plus indomethacin [S-I].

Figure S3. Principal coordinates analysis (PCoA) plots of 16sRNA sequencing of the faecal microbiota at the phylum level and at the genus level from mice treated with vehicle [V-V], *Saccharomyces boulardii* CNCM I-745 [S-V] -6 g/kg, daily), indomethacin [V-I] (7.5 mg/kg, daily), or *Saccharomyces boulardii* CNCM I-745 plus indomethacin [S-I].

Figure S4. Ileal samples were analysed with the Bio-Plex Pro™ mouse cytokine assay for IL-1β, IL-6, IL-10, IL-17a, TNF-α, IFN-γ, and MCP-1.

Table S1. Shannon index. Faecal samples from mice treated with vehicle [V-V], *Saccharomyces boulardii* CNCM I-745 [S-V] (6 g/kg, daily), indomethacin [V-I] (7.5 mg/kg, daily), or *Saccharomyces boulardii* CNCM I-745 plus indomethacin [S-I] at day -21 (D-21), day 0 (D0) and day 7 (D7).

Table S2. Observed ASV index. Fecal samples from mice treated with vehicle [V-V], *Saccharomyces boulardii* CNCM I-745 [S-V] (6 g/kg, daily), indomethacin [V-I] (7.5 mg/kg, daily), or *Saccharomyces boulardii* CNCM I-745 plus indomethacin [S-I] at day -21 (D-21), day 0 (D0) and day 7 (D7).

Conflict of interest

All authors are present or past employees of Biocodex. There are no other conflict of interest.

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