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Comparative Effect of the I3.1 Probiotic Formula in Two Animal Models of Colitis

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Abstract Use of probiotic therapy is an active area of investigation to treat intestinal disorders. The clinical benefits of the I3.1 probiotic formula (*Lactobacillus plantarum* (CECT7484, CECT7485) and *P. acidilactici* (CECT7483)) were demonstrated in irritable bowel syndrome (IBS) patients in a randomized, double-blind, placebo-controlled clinical trial. The aim of this study was to evaluate the therapeutic effects of I3.1 in two experimental models of colitis, a dextran sulfate sodium (DSS)-induced colitis model and an interleukin (IL)-10-deficient mice model. Colitis was induced in 32 8-week-old Balb/c mice by administering 3% (*w*/*v*) DSS in drinking water for 5 days. Probiotics were administered orally (I3.1 or VSL#3, 1×10^9 CFU daily) for 10 days before the administration of DSS. Also, probiotics (I3.1 or VSL#3, 1×10^9 CFU daily) were administered orally to 36 6-week-old C57B6J IL-10(−/−) mice for 10 weeks. Body weight was recorded daily. Colon samples were harvested for histological examination and cytokine measurements. Body weight after DSS administration did not change in the I3.1 group, whereas the VSL#3 group had weight loss. Also, I3.1 normalized IL-6 to levels similar to that of healthy controls and significantly increased the reparative histologic score. In the IL-10-deficient model, both VSL#3 and I3.1 reduced the severity of colitis compared to untreated controls, and I3.1 significantly reduced the levels of IFN-γ compared to the other two groups. In conclusion, I3.1 displays a protective effect on two murine models of experimental colitis. Results suggest that the mechanism of action could be different from VSL#3.

Keywords Colitis · Probiotics · Interferon-gamma · TNF-alpha · Interleukin-6 · *Lactobacillus plantarum* · *Pediococcus acidilactici*

Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease, is a group of diseases characterized by inflammation of the small and large intestine. Although the etiology of IBD is not fully understood, it is believed to result from complex interactions between genetics, immunity, environment, and gut microbiota [1]. Microorganisms in the human gut act in symbiosis to modulate different functions, such as the stimulation-regulation of epithelial innate immunity, the competitive exclusion of pathogens, and the production of important metabolites [2]. Development of gut dysbiosis and imbalances in host-microbe relationships have been shown to contribute to the extent, severity, and chronicity of intestinal inflammation in IBD [3, 4].

The potential for the positive manipulation of the gut microbiome through the introduction of beneficial live microorganisms, also known as probiotics, is currently an active area of investigation. Interest in probiotic therapy in IBD is due in large part to an improved safety profile with fewer side

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effects when compared to traditional therapy [5]. Some effects of probiotics have been proposed to be species-specific, while others to be more strain-specific, such as the ones involving downregulation of inflammation [6]. To date, there is evidence to support the use of some probiotics for induction and maintenance of remission in UC and pouchitis, specially the probiotic cocktail VSL#3 [5, 7].

Dextran sulfate sodium (DSS)-induced colitis is one of the most common models of chemically induced colitis in mice. The mechanism by which DSS induces intestinal inflammation is unclear; however, early pathologic events consist of changes in the expression of tight junction proteins, increased expression of proinflammatory cytokines, and goblet cell loss. Regeneration of the eroded epithelium occurs over the course of several days to weeks after DSS exposure [8, 9]. Of note, intestinal commensal bacteria have been shown to have a significant protective effect in this model [10].

Interleukin (IL)-10-deficient mice spontaneously develop chronic colitis due to upregulated Th1 response mediated by CD4+ cells producing interferon (IFN)-γ [11]. Inflammation in this model strongly depends on the gut microbiota, as conventional housing conditions result in generalized enterocolitis, specific pathogen-free (SPF) conditions produce only proximal colon colitis, and germ-free mice remain disease-free [11–13]. Moreover, manipulation of gut flora with probiotics has been shown to attenuate intestinal inflammation [14, 15]. Therefore, IL-10(−/−) mice provide an excellent model for the investigation of bacteria in the pathogenesis of intestinal inflammation.

I3.1 is a novel probiotic formula that has been recently shown to produce clinical benefits in irritable bowel syndrome (IBS) patients with a diarrhea component (i.e., IBS-D and IBS-A) in a randomized, double-blind, placebo-controlled clinical trial [16]. Clinical benefits observed included a significant reduction in visceral hypersensitivity and a significant improvement in IBS-related quality of life (both measured with validated questionnaires) when compared to placebo. The formula is composed of *Lactobacillus plantarum* strains CECT7484 and CECT7485 and *Pediococcus acidilactici* strain CECT7483, all of them deposited in the Spanish Type Culture Collection (CECT). UC and IBS are chronic gastrointestinal disorders that, until recently, have been considered dichotomous conditions falling on either side of an organic-functional divide. However, growing evidence suggests that diarrhea-predominant IBS (IBS-D) may arise as the result of a combined process of low-grade mucosal inflammation, immune activation, and barrier dysfunction [17–20]. Therefore, probiotics found to be effective in one condition could potentially be useful for treating the other, and vice versa. Thus, in this paper, we aim to (i) examine the therapeutic effect of the I3.1 probiotic formula in two experimental models of colitis and (ii) compare its effect to the commercial VSL#3 formula.

Materials and Methods

Ethical Considerations

All experiments were conducted according to the Guidebook for the Use and Care of Experimental Animals and approved by the Experimental Animal Ethics Committee (Internal code HUGTIP 08/02-08/01; DARP_4208) of the Health Sciences Research Institute at University Hospital Germans Trias i Pujol (Badalona, Spain).

Probiotics

Two different probiotic treatments were used in this study: (i) I3.1 (AB-Biotics S.A, Barcelona, Spain), a blend of three lactic acid bacteria strains (*L. plantarum* strains CECT7484 and 7485 and *P. acidilactici* strain 7483); (ii) VSL#3 (Sigma-Tau, MD, USA), a blend of eight strains (*L. plantarum*, *L. casei*, *L. acidophilus*, *L. delbrueckii*, *Bifidobacterium longum*, *B. breve*, *B. infantis*, and *Streptococcus salivarius*). Both probiotic formulas were obtained from commercial lyophilizates.

DSS-Induced Colitis Model

Eight-week-old Balb/c mice (Charles River, Barcelona, Spain), were kept under SPF conditions in an isolator (Harlan Ibérica, Barcelona, Spain) at constant temperature (22 °C) in a 12-h light/dark cycle. Mice had ad libitum access to sterile diet (standard diet for maintenance; Harlan Ibérica S.A., Barcelona, Spain) and to drinking fluid.

Probiotics (or vehicle) were administered for 10 days before starting DSS administration. Mice were allocated to one of four experimental groups ($n = 8$ each): (a) I3.1, (b) VSL#3, (c) DSS-treated controls, (d) vehicle-treated healthy-controls (Fig. 1). Mice in the probiotic groups received daily 1×10^9 CFU of either I3.1 or VSL#3 in 0.1 mL of sterilized water by gavage. Non-probiotic-treated mice (DSS and healthy control groups) received the same volume of vehicle. On day 11, experimental colitis was induced by administering 3% (*w*/*v*) DSS (40 kD, Applichem Lifescience VWR, Barcelona, Spain) in drinking water for 5 days in all groups except the healthy control one. During the study, animal well-being was supervised and body weight was recorded daily.

Five days after stopping DSS administration, mice were sacrificed by anesthetic overdose of inhaled Halothane (Fluothane®, Zeneca Ltd., UK). After sacrifice, colon samples were harvested and washed in cold PBS and colon weight/length ratio was recorded. Then, colons were longitudinally divided into two equal sections for histological examination (4% buffered formalin-fixed and paraffin-embedded) and cytokine measurements (snap-frozen in nitrogen liquid).

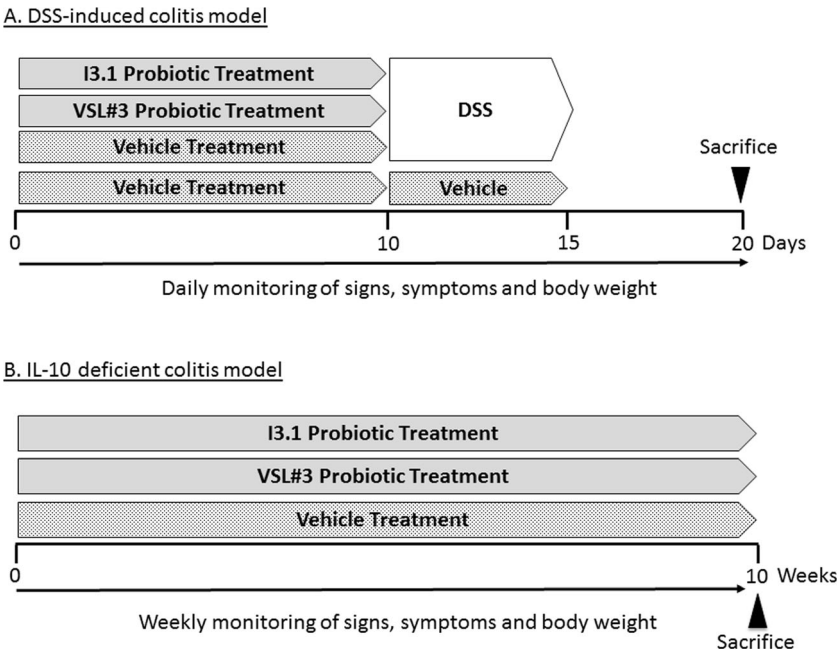

Fig. 1 Experimental design of the two animal models

IL-10-Deficient Colitis Model

Six-week-old C57B6J IL-10(−/−) mice (Charles River, Barcelona, Spain) were kept under SPF conditions as in the previous model.

Probiotics (or vehicle) were administered for 10 weeks. Mice were allocated to one of three groups (n = 12 each): (a) I3.1, (b) VSL#3, and (c) vehicle-treated controls (Fig. 1). Mice in the probiotic groups received daily 1×10^9 CFU of either I3.1 or VSL#3 in sterile drinking water. The control group received sterile drinking water alone (vehicle). Animal well-being was checked twice a week, and body weight was recorded weekly.

Sixteen-week-old mice were sacrificed by anesthetic overdose of Halothane. After sacrifice, colon samples were harvested and processed as in the DSS-colitis protocol.

Histological Scoring

Paraffin-embedded samples were sliced at 4 μm and stained with H&E for light microscopy examination. Samples were blindly analyzed by an experienced pathologist (I.O.). Intestinal injury was scored differently in the two models based on their distinctive pathophysiological characteristics. Injury in the DSS-colitis model was graded as described by Dieleman et al. [21] with some variation, thus differentiating between an inflammation score (0–10), mucosal ulceration (0–2), edema (0–1), mononuclear cells in *lamina propria* (0–2), neutrophil infiltration in *lamina propria* (0–2), lymphoid follicles (0–2), and cryptitis (0–1), and a reparative score (0–5), re-epithelialization (0–2) and fibrosis (0–3). On the other hand, injury in the IL10(−/−) colitis model was scored as previously described by Mañé et al. [22] with some variations, on a scale ranging from 0 to 20 based in the presence of mucosal ulceration (0–3), cryptitis (0–3), disruption of glandular architecture (0–3), mononuclear cells (0–3), neutrophil infiltration in *lamina propria* (0–3), lymphoid follicles (0–1), granulomas (0–1), hyperplasia (0–2), and adenoma (0–1).

Disease Activity Index and Severity of Colitis

Disease activity index (DAI), which includes animal well-being and clinical signs [23], was monitored daily in the DSS model and weekly in the IL-10(−/−) one. It was scored from 0 to 20 as a result of combining general appearance signs (unresponsive to stimuli or alert, lack of grooming (0–2); ocular/nasal discharge (0–2); hunched posture/lack of movement (0–2); alopecia (0–2); piloerection/coat changes (0–2)) and clinical signs of colitis (diarrhea (0–2), bloody feces (0–2); rectal prolapse (0–2); weight loss greater than 1% (0–4)).

Severity of colitis at the end of the study for the IL-10(−/−) colitis model was calculated as the sum of the following items as Mañé et al. [22] described previously: histological score (0–20), DAI index during the last week (0–14), presence of adherences (0–1), and presence of stenosis (0–1). Colitis was graded as follows: absent (total score ≤2), mild (total score = 3 to 10), and severe (total score >10).

Determination of Colonic Cytokine Levels

Frozen colonic samples were homogenized in 1 mL of cold PBS with inhibitor protein cocktail (Sigma-Aldrich Chem., Spain) and centrifuged (15,000×g, 10 min). Cytokine

concentrations were measured in colonic supernatant (Procarta™ Cytokine Assay Kit, Panomics, Spain) by Luminex® Platform (Luminex® Co., Austin, USA). IFN-γ, IL-10, IL-23, IL4, IL-6, and TNF-α levels were measured in the DSS model and IL-12, IL-6, IFN-γ, and TNF-α in the IL-10(−/−) colitis model. Fluorescent microbeads, pre-spotted with cytokine-specific antibodies, were incubated with 50 μL 1:5 diluted supernatant. Biotinylated secondary antibodies and streptavidin-phycoerythrin (S-PE) were sequentially added. Data were expressed as picograms per milliliter and were normalized to protein tissue content (Quick Start Bradford Protein Assay, Bio-Rad, CA, USA). All measurements were done in duplicate. The coefficient of variation for the Luminex assay was considered to be acceptable when lower than 10%, as per manufacturer specifications.

Statistical Analysis

For quantitative variables (body weight change, histological and DAI scores, and cytokine levels), because of lack of normality in several datasets, differences among all groups were assessed by Kruskal-Wallis test (i.e., non-parametric ANOVA) and pairwise differences between specific groups were assessed using Dunn's post hoc test. Reported values indicate medians and interquartile ranges. For qualitative variables (incidence and severity of colitis), differences among all groups in the experiment were assessed using Fisher's exact test for 2 × 3 tables and pairwise differences between specific groups were assessed using Fisher's test for 2 × 2 tables and corrected for multiple comparisons using Bonferroni's approach. Statistical analysis was performed with SPSS (IBM Corp., version 20.0; Armonk, NY, USA).

Results

DSS Colitis Model

Clinical Signs

Body Weight No differences in body weight increase were observed among groups during the DSS administration (not shown). Conversely, significant differences ($p = 0.002$) were observed among groups in the body weight between the end of DSS administration and sacrifice (day 16 to 21), as shown in Fig. 2a. The most pronounced difference occurred between I3.1- and VSL#3-treated mice, as weight loss in the former was non-existent, while it was moderate to severe in the latter (post hoc $p = 0.001$).

Colonic Weight/Length Ratio Colon shortening is considered as an indicator of an inflammatory process. However, no significant differences were observed among groups in our experiment ($p > 0.05$; median [IQR]: healthy control (0.025 [0.023–0.025]); DSS (0.023 [0.022–0.025]); DSS_VSL#3 (0.023 [0.020–0.024]); DSS_I3.1 (0.025 [0.024–0.03])).

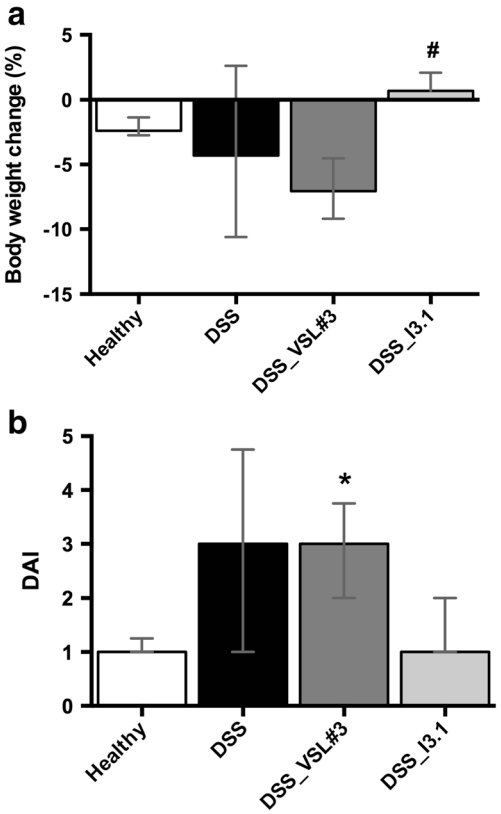

Fig. 2 **a** Body weight change (median and IQR, %) in the DSS colitis model between the end of DSS administration (day 16) and the end of the experimental period (day 21). Significant changes are observed among groups, and I3.1 was significantly different than VSL#3 (denoted by the *number symbol*). **b** DAI score at the end of the experimental period (median and IQR) in the DSS colitis model. Significant differences were noted among groups. VSL#3 was significantly different than healthy controls (denoted by an *asterisk*)

DAI Significant differences were observed among groups regarding the DAI score at the end of the experimental period, as shown in Fig. 2b ($p = 0.01$). The DAI score in the I3.1 group was similar to healthy controls, while the DAI score in the VSL#3 group was similar to the one in the DSS group and significantly higher than in healthy controls (post hoc $p = 0.025$). The difference between VSL#3 and I3.1 reached a statistical trend (post hoc $p = 0.089$).

Histological Scoring

Inflammation score is shown in Fig. 3a, and significant differences were noted among groups for this score ($p = 0.003$). Both DSS and VSL#3 groups had a higher inflammatory score than healthy controls (post hoc $p = 0.009$ and $p = 0.004$, respectively), while I3.1 displayed intermediate

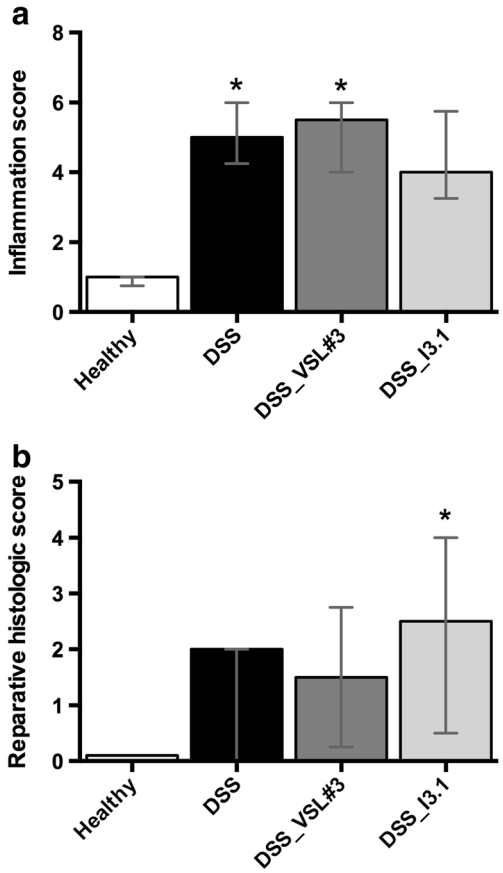

Fig. 3 **a** Inflammation score (median and IQR) in the DSS colitis model. Significant differences were noted among groups for this score. The DSS andVSL#3 had a significantly higher inflammation score compared with the healthy control group (denoted by an *asterisk*). **b** Reparative histologic score (median and IQR) in the DSS colitis model. Significant differences were noted among groups for this score. The score was higher in the I3.1 group than in healthy controls (denoted by an *asterisk*)

levels and the difference did not reach statistical significance compared to healthy controls (post hoc $p > 0.1$).

Figure 3b shows the reparative histologic score, composed of the sum of re-epithelization and fibrosis subscores, as these features are major contributors to the recovery of the disease. Significant differences were observed among groups ($p = 0.025$). In pairwise comparisons, the difference vs. healthy controls only reached significance for the I3.1 group (post hoc $p = 0.016$, denoted by an *asterisk*). This difference between the I3.1 group and healthy controls was significant for both the re-epithelization and the fibrosis subscores (post hoc $p = 0.041$ and $p = 0.018$, respectively; data not shown).

Colonic Cytokine Levels

Cytokine levels of the colonic samples are shown in Table 1. Significant differences among groups were noted for IL-6, IL-23, and TNF-α ($p = 0.024$, $p = 0.039$, and $p = 0.002$, respectively), while the difference for IFN-γ reached a statistical trend ($p = 0.055$). Conversely, although median values for IL-4 appeared to be slightly higher in healthy controls, no significant differences among groups were observed either for IL-4 or for IL-10. Treatment with I3.1 was able to normalize IL-6 to levels similar to that of healthy controls, and the difference between I3.1- and DSS-treated controls was statistically significant (post hoc $p = 0.039$), while VSL#3 failed to achieve a significant reduction of IL-6. As to IL-23, all mice in DSS-treated groups displayed higher levels than healthy controls, the pairwise difference vs. healthy controls reaching a statistical trend for both the VSL#3 group and the DSS-treated controls ($p = 0.080$ and $p = 0.066$, respectively), but not for I3.1-treated mice. Conversely, although all mice receiving DSS displayed higher levels of TNF-α than healthy controls, this difference was significant only for DSS-treated controls and I3.1-treated mice (post hoc $p \leq 0.01$ for both), while VSL#3-treated mice displayed intermediate levels between healthy controls and DSS-treated mice.

IL-10(−/−) Colitis Model

Onset of Clinical Signs

The appearance of clinical signs of colitis (weight loss >1%, diarrhea, bloody feces, rectal prolapse) was significantly different among groups ($p = 0.002$). Onset of symptoms was significantly delayed by both probiotic treatments compared to vehicle-treated controls (post hoc $p \leq 0.01$ for both I3.1 and VSL#3), as indicated in Table 2. No significant differences were noted between probiotic treatments. At the end of the study period, only 1 out of 12 vehicle-treated controls (8%) had not displayed any external symptom of colitis during the experimental period, compared to 6 out of 12 VSL#3-treated mice (50%) and 5 out of 12 I3.1-treated mice (42%).

Severity of Colitis at Study Endpoint

Significant differences were observed regarding the severity of colitis among groups at the end of the study period ($p = 0.009$), as shown in Fig. 4. Post hoc analysis indicates that both VSL#3 and I3.1 displayed lower severity than controls ($p = 0.038$ and $p = 0.045$, respectively, after adjusting for multiple comparisons). Thus, in each probiotic-treated group, 10 out of 12 mice (83%) were considered not to display colitis based on the combination of their histological score, DAI at the end of the study, and incidence of anatomophatological changes such as stenosis and adherences. Conversely, only 3 out of 12 control mice (25%) could be considered non-colitic based on the same criteria. As can be seen in Table 2, differences among groups were still significant when considering the DAI at the end of the study period ($p = 0.001$, post hoc $p \leq 0.01$ for both I3.1 and VSL#3 compared to controls) or the anatomophatological changes ($p = 0.031$) separately. However, although the histological score was higher in

Table 1 Cytokine levels (median and IQR, pg/ml) of the colonic samples in the DSS colitis model

	IFN-γ	IL-10	IL-23	IL-4	IL-6	TNF-α
Healthy controls	125 (110–136)	30 (17–32)	50 (35–57.5)	147 (66–231)	636 (530–1086)	80 (67–86)
DSS	194 (144–216)	27 (19–30)	78 (59–118)	84 (72–114)	1696 (1060–2415)	157 (117–184)*
DSS_VSL#3	195 (154–213)	21 (16–25)	83 (56–110)	96 (78–129)	1266 (659–1444)	113 (89–133)
DSS_I3.I	192 (134–224)	29 (20–35)	70 (63–99)	108 (78–138)	636 (265–2192)#	147 (119–182)*

Significant differences were noted among groups for IL-6, IL-23, and TNF-α. Levels of IL-6 were markedly lower in I3.1- than in DSS-treated mice (denoted by the *number symbol*), and levels of TNF-α were significantly higher in I3.1- and DSS-treated controls than in healthy controls (denoted by an *asterisk*).

vehicle-treated controls than in both probiotic-treated groups, the difference did not reach statistical significance.

Colonic Cytokine Levels at Study Endpoint

Concentrations of cytokines IFN-γ, IL-6, IL-12, and TNF-α are reported in Table 3. Significant differences among treatment groups were observed for IFN-γ only ($p = 0.0009$), where I3.1-treated mice had markedly lower levels of IFN-γ compared both to vehicle-treated controls and to VSL#3-treated mice (post hoc $p \leq 0.01$ for both). Although median values for IL-12 appeared to be higher in vehicle-treated controls, no significant differences among treatment groups were observed for IL-12, IL-6, and TNF-α, probably due to the larger variability within groups. It must be noted that a higher proportion of mice in the VSL#3-treated group had undetectable levels of TNF-α (7 of 11, 64%) than in the vehicle-treated group (4 of 11, 36%) or I3.1-treated group (2 of 12, 17%), this difference among groups reaching a statistical trend ($p = 0.078$). A similar analysis did not produce any meaningful result either for IL-6 or for IL-12.

Discussion

In the present article, the probiotic mixture of *P. acidilactici CECT7483*, *L. plantarum CECT7484*, and *L. plantarum CECT7483* (I3.1) shows protective effects on the development of colitis in two models of colitis in mice. The current study was carried out under specific pathogen-free (SPF) conditions to minimize uncontrolled environmental influences. Under these barrier conditions, colitis models often develop a low-grade colitis, less severe than under conventional environment [24]. Mouse models allow perturbations in gut microbiota to be studied in a controlled experimental setup and thus help in assessing causality of the complex host-microbiota interactions and in developing mechanistic hypotheses. In mouse models of IBD, gut bacterial diversity is found to be reduced, with certain shifts in gut microbiota profiles being observed, such as increases in Enterobacteriaceae, Bacteroidaceae, and Ruminococcaceae [25]. However, clear differences can be observed at the level of specific genus/species abundances between murine and gut microbiota [25], thus highlighting the need of conducting studies in humans to confirm the findings reported in mouse models.

Some differences were observed when compared to the well-known probiotic VSL#3. In the DSS model, I3.1 prevented body weight loss after DSS colitis induction, abrogated the increase of IL-6 levels, reduced the increase of IL-23 levels, and promoted reparative phenomena on the injured intestinal mucosa, while VSL#3 did not. In Balb/c mice, a 5-day course of DSS has been described to result in an acute colitis that becomes symptom-free within 2 weeks, and this resolution correlates well with histological reparative phenomena [26]. Conversely, VSL#3 was able to reduce TNF-α

Table 2 Histological and clinical assessment (median and IQR) in the IL-10(−/−) colitis model

	Onset (weeks)	DAI	Adherences and/or stenosis	Histologic score
Controls	10 (9.3–11.8)*	1 (0–3.5)*	3 of 12*	2.3 (1.1–3.9)
VSL#3	17 (13–17)	0 (0–0)	0 of 12	1 (1–2)
I3.1	15 (14–17)	0 (0–0)	0 of 12	1 (1–1.4)

Onset of clinical signs of colitis and DAI at the end of the experimental period (week 16) was significantly different among groups, as well as the presence of adherences and/or stenosis. Vehicle-treated controls displayed an earlier onset and higher DAI values than I3.1 and VSL#3 (denoted by an *asterisk*).

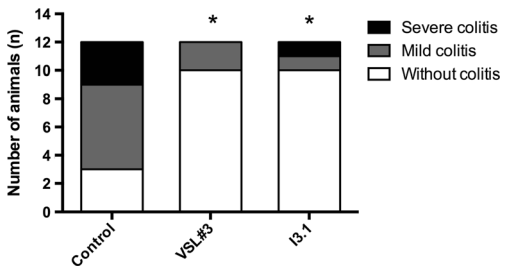

Fig. 4 Incidence and severity of colitis (median and IQR) in the IL-10(−/−) colitis model at study endpoint. Significant differences were noted among groups. Both VSL#3 and I3.1 displayed lower severity than controls in pairwise comparisons (denoted by an *asterisk*)

Table 3 Cytokine levels (median and IQR, pg/ml) in the IL-10(−/−) colitis model at study endpoint

	IFNγ	IL-12	IL-6	TNFα
Controls	5.1 (2.9–58.3)	2.8 (0.4–9.8)	0 (0–18.34)	7.6 (1.3–11)
VSL#3	4.7 (3.8–18)	0 (0–15.2)	0 (0–0)	0 (0–13)
I3.1	0.7 (0–2.4)*	0 (0–1.5)	0 (0–8.8)	13 (0.8–24.7)

Significant differences among treatment groups were noted for IFN-γ only. Levels of IFN-γ were significantly lower in I3.1 than vehicle-treated controls and VSL#3 (denoted by an *asterisk*).

to intermediate levels between DSS-treated controls and healthy controls, as opposed to I3.1. However, this effect did not translate into a reduction of the symptoms, in contrast to previous studies in the same model [27–29]. However, one of these studies used probiotic doses twice than ours, while another study used a longer administration of probiotic combined with a lower concentration of DSS and assessed DAI during the administration of DSS, not after the end of the administration. Taken together, our study further supports the inhibitory effect of VSL#3 on TNF-α production but suggests that VSL#3 may necessitate a higher dose or a longer administration than the one used in our experiment to reliably produce a clinically relevant effect.

Regarding the IL-10(−/−) model, both probiotics delayed the appearance and reduced the incidence and severity of spontaneous colitis in IL-10(−/−). However, IL-10-deficient mice receiving the I3.1 formula displayed a reduction of IFN-γ that was not observed in VSL#3-treated mice, while the latter appeared to reduce TNF-α as in the DSS model, although the effect did not reach statistical significance. Colonic levels of cytokines in this model were different from those obtained in WT healthy controls of the DSS model, because of immunologic influences and genetic background [11].

In the last decade, there has been a growing recognition of the disruption of commensal microbiota (dysbiosis) associated to IBD, obesity, IBS, type 2 diabetes, and oncogenesis [30, 31]. Several studies show abnormal profiles of enteric bacteria in IBD, increasing pathogenic *E. coli* and decreasing protective mucosa-related *Faecalibacterium prausnitzii* [32, 33], while *Bifidobacteria* were lower among IBS patients [34]. Unfortunately, identification of individual species or ecological changes as specific pathogenic effectors has not been possible. However, the above evidence suggests that the restoration of the microbiota balance could be useful as therapeutic tool. So far, the most promising approaches have been achieved with mixtures of multiple probiotic species such as VSL#3. This formula has been successfully tested in preclinical studies of intestinal inflammation and inflammation-associated colon cancer prevention [35–37]. VSL#3 has also been able to induce remission of mild to moderate flare-ups in UC and preventing pouchitis [38, 39]. Additionally, new evidences suggest that this probiotic mixture could be used for prevention of Crohn's disease post-surgical recurrence and for ameliorating symptoms of IBS in children [6, 40].

With IBD developing into a globally prevailing disease, there is an urgent need to explore new targets. Some of the most promising targets currently being explored include pro-inflammatory cytokines which are dysregulated in IBD such as IL-6, IL-12/23, IL-13, IL-18, and IL-21 [41, 42]. For instance, anti-IL-6 receptor monoclonal antibody has been shown to elicit a clinical effect in active CD [43], and a phase II clinical study is currently ongoing by Pfizer [41]. In this regard, I3.1 probiotic treatment reduced the proinflammatory cytokine IL-6 and promoted mucosal healing after DSS-colitis induction. IL-6 is produced by intestinal myeloid cells in contact with translocating microbiota or their products, stimulates intestinal epithelial cell proliferation and cell fate through mTOR and Notch signaling pathways, and is elevated in a number of chronic inflammatory diseases and gastrointestinal cancers [44]. However, IL-6-deficient mice are highly sensitive to DSS-colitis [45], and therapeutic IL-6 blockade in humans can increase bowel perforation risk [46]. Taken together, these data suggest that normalizing intestinal IL-6 expression, rather than complete blockade, could restore immune and epithelial homeostasis. In the present work, preventive I3.1 supplementation resulted in normalization of IL-6 levels, accompanied by an improvement in colon recovery and a resistance to acute DSS-colitis. Conversely, in the DSS model, VSL#3 seemed to dampen the increase in production of TNF-α, in agreement with previous studies [47, 48].

IL-10-deficient mice develop a Crohn's disease-like spontaneous enterocolitis in adulthood, in which susceptibility depends on genetic background and microbiota composition [49]. It has been shown that dysbiosis precedes colitis onset [50] both in SPF and in conventional environments. For these reasons, IL-10-deficient mice are used for studying the relationship between host immune response and differential changes of intestinal microbiota composition. Previous studies report that a 4-week course of VSL#3 probiotic mixture improved physiological transport and barrier integrity in the IL-10 gene-deficient mouse, in conjunction with a reduction in mucosal secretion of TNF-α and IFN-γ [51]. In our study, 10-week-probiotic treatment was able to reduce the incidence and severity of colitis in 16-week-old IL-10-deficient mice. However, at the end of the study period, VSL#3-treated mice displayed a mild reduction of TNF-α and no effect on IFN-γ, while I3.1 treatment drastically reduced IFN-γ levels. Inflammatory effects of IFN-γ have been described in IBD or IBS [52–54], as well as in the enteropathy that suffer IL-10(−/−) mice [15, 55, 56]. IL-10-independent beneficial effects of individual *L. plantarum* strains have been previously associated with an intestinal IFN-γ reduction in some studies [12, 57]. IL-10 deficiency causes an unregulated Th1 response, with high levels of IL-12 driving the production of

IFN-γ to maintain chronic inflammation [58]. Production of IFN-γ is modulated by microbiota composition through epigenetic and post-transcriptional mechanisms [53]. IFN-γ plays a pivotal role in orchestrating defense against infections in the gut, but its sustained production may trigger barrier dysfunction and bacterial translocation internalizing tight-junction transmembrane protein in intestinal epithelial cells [59]. Given that colitis in IL-10-deficient mice is dependent on the composition of the gut microbiota, the different cytokine profile produced by I3.1 supplementation compared to VSL#3 in IL-10-deficient mice could reflect changes in the microbial ecology of the gut different from those achieved by VSL#3.

Despite the lack of complete knowledge of the mechanism of VSL#3 anti-inflammatory activity on the intestine, current studies suggest that bacteria in this multispecies formula produce short-chain fatty acids (SCFAs) that promote epithelial barrier function, inhibit effector immune responses, and induce regulatory T cell subset through epithelial/myeloid cells axis [35, 60]. In particular, VSL#3 has been shown to increase the levels of butyrate in mice [61]. Moreover, this formula has also been demonstrated to protect the epithelial barrier by activating the p38 pathway [62] and by promoting tight junctions [28]. In this regard, the bacterial strains in the I3.1 formula have been shown to produce SCFAs from indigestible carbohydrates in vitro, mostly acetate but also butyrate, release soluble polyphosphate granules, and display strong inhibitory activity against enterobacteria (JE, personal communication). Although anti-inflammatory effects have been more widely documented for butyrate than acetate, the latter also displays anti-inflammatory activity in the gut [63, 64] and feeds beneficial butyrate-producing bacteria such as *F. prausnitzii* and *Roseburia intestinalis* [65, 66]. Besides, polyphosphate granules have been shown to protect the epithelial barrier via the integrin-p38 pathway [67, 68]. However, in this study we show in two different animal models that I3.1 inhibits the production of different cytokines than VSL#3. Moreover, at the administered doses, I3.1 was able to significantly induce reparative processes in the gut mucosa and reduce clinical symptoms in the DSS model, while at the same dose VSL#3 failed to achieve the same effects. These data suggest that although the bacterial strains in I3.1 may display similar properties to VSL#3, differences must exist in their mechanisms to produce protective effects on experimental colitis.

This study shows the potential applicability of the probiotic mixtures to treat intestinal disorders. Specifically, the new tri-strain I3.1 probiotic combination shows similar effects to VSL#3 in protecting mice from colitis. However, the mechanism of action appears to be different, at least in part. Moreover, the I3.1 formula was well tolerated, as no specific adverse effects were observed in mice receiving this probiotic compared to mice receiving the VSL#3 probiotic or healthy controls. Although I3.1 is composed by three probiotic strains, compared to eight-strain VSL#3, the results of this preclinical study predict its applicability in microbiota-based therapies in IBD, especially UC. The animal models used in this study suggest that the formula could be used to reduce an active inflammatory outbreak or to delay its onset. This potential should be further explored in clinical trials.

Compliance with Ethical Standards

Conflict of Interest V. Lorén, J. Manyé, E. Cabré and I. Ojanguren declare that they have no conflict of interest. M.C Fuentes and J. Espadaler are full-time employees of AB-Biotics S.A.

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