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Lactiplantibacillus plantarum (CECT7484 and CECT7485) and *Pediococcus acidilactici* (CECT7483) enhance actin cytoskeleton and CYP1A1 expression restoring epithelial permeability alterations induced by irritable bowel syndrome mediators

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ABSTRACT

Irritable bowel syndrome (IBS) is a multifactorial condition with heterogeneous pathophysiology, including intestinal permeability alterations. The aim of the present study was to assess the ability of a probiotic blend (PB) consisting of two *Lactiplantibacillus plantarum* strains (CECT7484 and CECT7485) and one strain of *Pediococcus acidilactici* (CECT7483) to recover the permeability increase induced by mediators from IBS mucosal biopsies and to highlight the underlying molecular mechanisms. Twenty-one IBS patients diagnosed according to ROME IV criteria (11 IBS-D and 10 IBS-M) and 7 healthy controls were enrolled. Mucosal mediators spontaneously released by IBS and HC biopsies were collected and incubated with/without the PB (10^4 and 10^6 CFU/ml). Paracellular permeability was assessed by evaluating the amount of sulfonic-acid-conjugated to fluorescein passing through the Caco-2 monolayer. RNA was extracted from Caco-2 cells and used to perform qPCR analyses, to evaluate the expression of ZO-1 and β-actin, and RNAseq to evaluate the transcriptomic profile. Untargeted metabolomics was used to characterize metabolites produced by the PB. The PB significantly reduced paracellular permeability after 3 h of incubation. Both doses of the PB significantly recovered the increase in paracellular permeability induced by IBS mediators. qPCR analyses showed that both doses of the PB co-incubated with IBS mediators induced a significant increase in beta-actin expression compared to IBS mediators alone. Concerning IBS subtypes, the high dose of the PB recovered the increase of permeability induced by IBS-D mediators. Transcriptomic analyses, confirmed by qPCR, showed that the high dose of the PB significantly increased CYP1A1 compared to IBS mediators alone. The PB produced a high amount of indole-3-lactic acid. The PB recovers the permeability increase induced by IBS mediators inducing the up-regulation of β-actin. In addition, the PB up-regulates the expression of CYP1A1, known to be involved in the metabolism of xenobiotics, possibly through the production of the indole-3-lactic acid.

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Introduction

IBS is one of the most common disorders of the gut-brain axis, characterized by recurrent abdominal pain associated with defecation or changes in bowel habits. According to the ROME IV criteria, four types of IBS can be distinguished: IBS with predominant diarrhea (IBS-D), IBS with predominant constipation (IBS-C), IBS with mixed bowel habits (IBS-M), and unclassified IBS (IBS-U).[1] The pathophysiology of IBS is multifactorial and not completely understood. It includes increased intestinal permeability,[2–4] microbiota imbalance,[5] and mucosal immune cell infiltration and activation, characterized particularly by mast cells.[6–8] Additionally, altered neuro-immune interactions at the mucosal level[7,9] lead to low-grade inflammation and altered sensory perception.[10]

The intestinal barrier is the first line of defense against harmful microorganisms and antigens while ensuring the passage of nutrients and water. A central role in maintaining this delicate balance

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is played by the tight junctions (TJs), which regulate paracellular permeability. TJs are a multiprotein complex composed of the transmembrane proteins, such as occludin and claudins, which interact with zonula occluden (ZO) proteins. These proteins bind directly to the actin cytoskeleton to control paracellular permeability.[11] Furthermore, the phosphorylation of myosin light chain (MLC), catalyzed by MLC-kinase (MLCK), induces contraction of the cytoskeleton.[12]

Mounting evidence suggests that intestinal permeability is altered in patients with IBS,[11] principally due to alterations in the localization and expression of TJ proteins.[13,14]

Interestingly, in a reverse-translational model-based Caco-2 cells, incubation with mediators spontaneously released by IBS biopsies induces an increase in paracellular permeability, compared to healthy subjects, and the rate of increase positively correlated with the score of abdominal pain severity complained by IBS patients.[4]

Among the approaches used to manage IBS patients, an adjuvant treatment is the supplementation with probiotics.[15] Probiotics are defined as live microorganisms that, when administrated in adequate amounts, confer health benefits on the host.[16] It has been hypothesized that one of the mechanisms by which probiotics act, inducing symptom improvement, is by reinforcing the epithelial barrier through the modulation of TJs[17,18]

A probiotic blend consisting of two strains of *Lactiplantibacillus plantarum* (CECT7484 and CECT7485, formerly known as *Lactobacillus plantarum*) and one strain of *Pediococcus acidilactici* (CECT7483) was used in a previously randomized, double-blind, placebo-controlled clinical trial. This study showed that the active treatment induced an improvement in IBS-related quality of life in patients with IBS-D as compared to placebo.[19] A subsequent randomized, placebo-controlled clinical trial confirmed that this probiotic blend improves quality of life in IBS and that its combination with an antispasmodic agent improved abdominal pain more than the probiotic blend alone, which was still more effective than placebo.[20] Furthermore, the probiotic blend had a protective effect in two murine models of colitis.[21]

The aim of the present study was to evaluate the in vitro effect of the probiotic blend, consisting of *L. plantarum* CECT7484 and CECT7485, and *P. acidilactici* CECT7483, on Caco-2 permeability alterations induced by mediators released by IBS mucosal biopsies compared to healthy controls, and to elucidate the underlying molecular mechanisms.

Material and methods

Subject enrollment, clinical phenotyping and symptom questionnaires

Non-constipated IBS patients, i.e., IBS-D and IBS-M ($n = 21$; 12F; mean age 41 yrs; range 23–61; IBS-D = 11, IBS-M = 10), and healthy controls (HC, $n = 7$; 3F; mean age 52 yrs; range 48–55) were enrolled at the Department of Medical and Surgical Sciences, University of Bologna (Bologna, Italy). IBS patients were diagnosed according to Rome IV criteria; HC were selected among subjects undergoing colonoscopy for colorectal cancer screening, or follow-up after polypectomy, and who did not complain any gastrointestinal symptoms. Exclusion criteria for both IBS patients and HC were the following: IBS with predominant constipation (IBS-C) or unclassified (IBS-U) diagnosis; patients with any relevant organic, systemic, or metabolic disease; patients with intestinal organic diseases, including celiac disease or inflammatory bowel diseases (Crohn's disease, diverticular disease, ulcerative colitis, infectious colitis, ischemic colitis, microscopic colitis); previous major abdominal surgeries; patients consuming probiotics or in topical or systemic antibiotic therapy during the last month; the use of corticosteroids, non-steroidal anti-inflammatory drugs, mast cell stabilizers, tricyclic antidepressants, 5-HT-selective reuptake inhibitors, serotonergic agents, including 5-HT_3 receptor antagonists (i.e., granisetron, ondansetron) and 5-HT_4 receptor agonists (i.e., prucalopride).

During colonoscopy, four mucosal biopsies were obtained from the proximal descending colon to collect mediators spontaneously released.

Informed consent was signed by all subjects, and protocol was approved by the Ethic Committee of Area Vasta Emilia Centro (CE-AVEC, approval

identification n°: 240/2019/Sper/AOUBo) and conducted in accordance with the Declaration of Helsinki.

In order to evaluate the frequency and severity scores of abdominal pain and distension over the last 2 weeks before enrollment, a modified Italian version of Bowel Disease Questionnaire (BDQ) was used.[3] Symptom severity and frequency were graded 0–4 according to its impact on patients' daily activities (0, absent; 4, extremely severe, precluding daily activities) or to its weekly frequency (0, absent; 1, up to 1 d/week; 2, 2 or 3 d/week; 3, 4–6 d per week; 4, daily), respectively. A 0–10 visual analogue scale was used to assess the quality of life (0 = "I have never felt so bad", 10 = "I have never felt so good").

Collection of mucosal mediators spontaneously released by colonic biopsies

A previously validated method[6] was used to collect mediators spontaneously released from colonic biopsies. Briefly, biopsies, immediately after they were taken, were put in 1 ml of Hepes-Krebs buffer solution (pH 7.4), weighed, and volume adjusted to incubate 15 mg of biopsies in 1 ml of buffer. After 25 min of incubation at 37°C in continuous oxygenation, samples were centrifuged at 200 g at 4°C for 7 min, and supernatants containing mediators (from now on called supernatants) were aliquoted and stored at −20°C until the assay. Before treatment, the supernatants were filtered with 0.22 µm filters (Millex-GP, Millipore, Milan, Italy).

Probiotic preparation

The powder of a commercially available capsule containing a total of 3×10^9 living cells, defined using a counting chamber, of *Lactiplantibacillus plantarum* CECT7484, *Lactiplantibacillus plantarum* CECT7485, and *Pediococcus acidilactici* CECT7483, from now on called probiotic blend (PB), supplied by AB-Biotics SA (Barcelona, Spain), was suspended in DMEM without supplements in order to obtain two concentrations of PB: 10^6 and 10^4 CFU/ml of DMEM.

Caco-2 cell culture

A previously published protocol was used to establish the in vitro model of permeability based on Caco-2 cells.[4] Transepithelial cell resistance (TER) was measured every 2 d in order to follow the formation and differentiation of Caco-2 monolayer, using a volt-ohm-meter (Millicell® ERS-2 Millipore).

Assessment of the pH of caco-2 growth medium

Cells were seeded into 24-well plates, at a density of 70.000 cells/well and cultured until confluence. Cells were washed with PBS and incubated with DMEM without supplements before adding the PB (10^6 and 10^4 CFU/ml). The pH of the medium was assessed using litmus/acid test paper for up to 30 h. In particular, the pH was assessed immediately after incubation, then every 2 h for the first 8 h, and subsequently at 24 and 30 h. Caco-2 cells incubated only with DMEM were used as control. Each condition was tested in triplicate.

Assessment of caco-2 vitality

Caco-2 vitality was assessed by using the sulforhodamine B colorimetric (SRB) assay as previously described.[4] The absorbance at 540 nm was directly proportional to the protein content and therefore to the number of live cells present in each well. Cell vitality was assessed after 6 h of incubation with the two concentrations of probiotics or only with DMEM (control).

Each condition was tested in triplicate.

In vitro permeability assay

Basolateral aliquots of 150 µl were taken and the amount of Fluorescein-5-(and-6)-Sulfonic Acid (FITC 0.1 mg/ml; absorption/emission peak: 485/535, Invitrogen) was measured using a 96-well fluorescent plate reader (TECAN). After the measurement, the aliquots were re-added to the basolateral side. Absorbance evaluations were performed at time 0 (immediately after the addition of treatments), every 30 min for the first 2 h, and then each hour for the subsequent 4 h. A standard curve was used to convert absorbance

results in concentrations of FITC (μg/ml). The FITC values were directly proportional to the paracellular permeability changes of Caco-2 monolayer.

IBS and HC supernatants were incubated with Caco-2 cells for 6 h with or without the simultaneous incubation of PB. In case of co-incubation, PB was added to Caco-2 cells at the same time of the supernatants. Each supernatant was tested in duplicate.

qPCR analyses

At the end of paracellular permeability experiments Caco-2 cells were washed in PBS and harvested in RNAlater buffer until RNA extraction (RNeasyMinikit, Qiagen, Milano, Italy) and reverse transcription (Quantitect reverse transcription kit, Qiagen). qPCR (SYBR Green Quantitect PCR kit, Qiagen) was used to assess the expression of the following genes: Zonula Occludens-1 (ZO-1) and β-actin. The expression of each gene was normalized on the expression of the reference gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The following primers were used: *ZO-1*: forward 5'-gaatgatggttggtatggtgcg-3', reverse 5'-tcagaagtgt gtctactgtccg-3'; *β-actin*: forward 5'-catgtttgagacct tcaacac-3', reverse 5'-ccaggaaggaaggctggaa-3'; *GAPDH*: forward 5'-cagcaagagcacaagaggaag-3', reverse 5'-caactgtgaggaggggagatt-3'.

Amplification conditions were as follows:

- *ZO-1* gene: 15 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 53°C, 30 s at 72°C;
- *β-actin* gene: 15 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 53°C, 30 s at 72°C;
- *GAPDH* gene: 15 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 53°C, 30 s at 72°C.

Melting curve data were analyzed at the end of each reaction. The relative gene expression was calculated considering the corresponding condition without the PB as calibrator.

A negative control for PCR reaction (1 μl of water instead of cDNA) and a no-reverse transcription control were added to each real-time PCR plate. Each sample was run in duplicate, and the mean threshold cycle (Ct) was determined from the two runs. The relative gene expression was calculated as ΔΔCt. The gene expression was expressed as fold difference ($2^{-\Delta\Delta Ct}$).

RNAseq

Considering the protective effect of the PB on permeability alterations induced by IBS supernatants, we performed RNAseq analysis in order to highlight differences in the expression profile of genes likely involved in IBS pathophysiology. The analysis has been carried out for RNA extracted from Caco-2 cells treated with HC/IBS supernatants with/without the higher dose of the PB (10^6). Macrogen KOREA performed the analysis. Briefly, RNA samples with RIN quality greater than 7.0 on Tape Station RNA Screen Tape assay (Agilent Technologies Inc., Santa Clara, USA), were used for RNA library construction. Libraries were prepared with Illumina TruSeq Stranded mRNAkit (Illumina Inc, San Diego, USA), quantified using KAPA Library Quantification kit for Illumina (Roche AG, Basel, Switzerland) and qualified using Tape Station D1000 Screen Tape assay (Agilent Technologies Inc., Santa Clara, USA). Indexed libraries were then sequenced with Illumina NovaSeq (Illumina, Inc., San Diego, CA, USA). Transcripts were assembled and quantified with the StringTie (v.2.1.3) package. The raw counts were normalized with the edge R package (function calcNormFactors) as "count per million" (cpm). Differential expression (DESeq) analysis was performed with the DESeq2 package[22] using this filtered dataset, producing regularized logs (rlogs) of expression fold change (FC), as well as adjusted p-values using the False Discovery Rate (FDR) approach and count-based independent filtering.

Validation of RNAseq analysis by qPCR

Among the genes that showed a greater difference in expression in Caco-2 cells incubated with IBS supernatants and PB compared with supernatants alone, we focused on the CYP1A1 gene. The validation of RNAseq results by qPCR was carried by

using TaqMan® Gene Expression Assays (GAPDH: Hs03929097_g1, VIC-MGB; CYP1A1: Hs00153120_m1, FAM-MGB; Life Technologies) in TaqMan® Fast Advanced Master Mix (Life Technologies, Milano, Italy) on a QuantStudio Real-Time PCR Systems 5 (Applied biosystem system, Life Technologies).

A negative control for PCR reaction (1 µl of water instead of cDNA) and a no-reverse transcription control were added to each real-time PCR plate. Each sample was run in duplicate. The mean threshold cycle (Ct) was determined from the two runs. The relative gene expression was calculated as ΔΔCt. The gene expression was expressed as fold difference ($2^{-\Delta\Delta Ct}$). The expression of each gene was normalized on the expression of the reference gene (GAPDH).

Metabolomic profiling of bacterial strains

Hundred microliters from an o/n culture were inoculated in 10 ml of fresh MRS medium and grown in sealed jars with Anaerocult. The strains usually grow to 1E + 9 CFU/ml after o/n culture (aprox. 18 h). Medium was not pre-reduced. Strains were grown in an MRS medium at 37°C for 48 h in anaerobiosis. Supernatants were recovered by centrifugation, filtered (0.22 µm), and stored at −80°Cuntil analysis.

Semi-polar metabolites were measured at MS-Omics (Denmark) using the UPLC system Vanquish coupled to an Orbitrap Exploris 240 mass spectrometer (both from Thermo Fisher Scientific) following the protocol described by Doneau et al.[23] Samples were analyzed in a randomized order. An electrospray ionization interface was used as ionization source. Analysis was performed in polarity switching ionization mode. Peak areas were extracted using Compound Discoverer 3.2 (Thermo Scientific). Compounds were identified at four levels: level 1, identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra; level 2a, identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3ppm); level 2b, identification by accurate mass (with an accepted deviation of 3 ppm) and MS/MS spectra; and level 3, identification by accurate mass alone (with an accepted deviation of 3 ppm). Compounds related to indole metabolism were manually searched at levels 1 and 2a. The significance of the change over control (MRS medium) was established at a False Discovery Rate of 0.05. This multiplicity-adjusted p-value was established considering the 153 semipolar molecules reliably identified (i.e., levels 1 and 2a) by the LC-MS analysis

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was carried out with the computer-assisted PrismGraphPad program (Prism version 8.1; GraphPad Software, San Diego, CA). Kruskal–Wallis test was used for multiple comparison; Mann–Whitney U-test was used for paired comparisons; and the Spearman rank test was used for correlation analysis. Two-tailed p values < 0.05 were considered to be statistically significant.

Results

Demographic characteristics of study subjects

Table 1 shows the demographic characteristics of subjects enrolled in the study. Groups were

Table 1. Demographic and clinical characteristics of the study subjects.

	HC (*n*=7)	IBS (*n*=21)	IBS-D (*n*=11)	IBS-M (*n*=10)	*P* values
Age	51.9±0.96; 52 [50–55]	41.3±2.7; 40 [29.5–52.5]	41.3±3.7; 40 [29–53]	41.4±4.2; 39.5 [29.3–53.5]	0.299[a]
Female percentage	3 (42.9%)	12 (57%)	7 (63.6%)	5 (50%)	0.663[a]
Abdominal pain severity	–	2.3±0.2; 2 [2–3]	2.5±0.3; 2 [2–3]	2.2±0.2; 2 [2–3]	0.597[b]
Abdominal pain Frequency	–	2±0.2; 2 [1–3]	2.4±0.2; 3 [2–3]	1.6±0.2; 1.5 [1–2]	0.040[b]
Abdominal distention severity	–	2.3±0.2; 2 [2–3]	2.5±0.3; 3 [2–3]	2.1±0.3; 2 [1.8–2.3]	0.282[b]
Abdominal distention frequency	–	2.3±0.3; 2 [1–3.5]	2.7±0.4; 3 [1–4]	1.9±0.4; 1.5 [1–2.5]	0.163[b]
Quality of life	8.7±0.2; 9 [8–9]	4.6±0.4; 5 [2.5–6][c]	4.3±0.6; 5 [2–6][c]	5±0.5; 5.5 [3.8–6][c]	<0.001[a]

Data are shown as mean±SE, median and interquartile range [in brackets] or absolute and relative frequencies. [a]: Kruskal–Wallis test (HC vs IBS-D vs IBS-M) or Pearson chi[2] (for female percentage). [b]: Mann–Whitney test (IBS-D vs IBS-M). [c]: $p < 0.001$ vs HC (Mann–Whitney test).

comparable for age and female percentage. Concerning abdominal symptoms, the frequency of pain was significantly higher in IBS-D patients compared to IBS-M ones (p = 0.040), while the other symptoms were not different between the two groups. As expected, the quality-of-life score was significantly higher in the HC group compared to IBS patients ($p < 0.001$).

Assessment of pH and cell vitality of caco-2

In our previous work, we demonstrated that a subgroup of IBS supernatants induced an increase of permeability in Caco-2 cells compared to HC supernatants after 6 h of incubation.[4] To determine the appropriate doses of the probiotic blend for further testing on Caco-2 cells, ancillary experiments were conducted. These experiments aimed to identify doses that would not alter pH levels or cell viability, as changes in these parameters could influence the permeability results. After 6 h of incubation, the two doses of the PB (10^6 and 10^4 CFU/ml) did not affect the pH of the medium or Caco-2 cell vitality (Figure 1). Therefore, subsequent experiments were conducted for a maximum of 6 h.

The higher dose of the probiotic blend strengthens the caco-2 monolayer

The incubation of Caco-2 cells with the PB induced a dose-dependent reduction in the passage of FITC from the apical to the basolateral side, which indicates a reinforcement of the Caco-2 monolayers (Figure 2a). In particular, compared to Caco-2 cells incubated with medium alone, the higher dose of the PB (i.e., 10^6 CFU/ml) induced a significant reduction in the amount of FITC after 3 h of incubation (Figure 2b), while no significant difference emerged after 6 h of incubation.

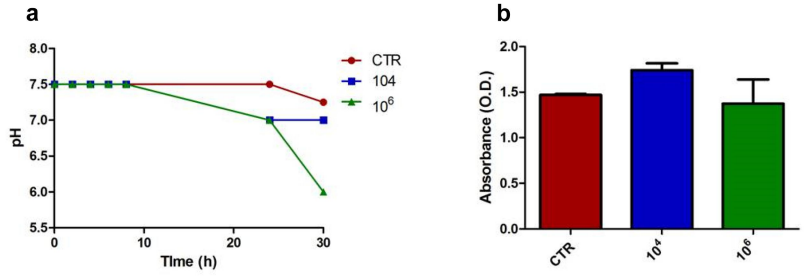

Figure 1. Evaluation of pH and vitality of Caco-2 cells incubated with two doses of the probiotic blend (PB). a, pH evaluation after 2, 4, 6, 8, 24 and 30 h of cell incubation with or without the PB. b, cell vitality assay after 6 h of incubation with or without the PB (10^6 and 10^4 CFU/ml). CTR: Caco-2 cells incubated with medium alone (n=4); 10^4: Caco-2 cells incubated with 10^4 CFU/ml (n=2); 10^6: Caco-2 cells incubated with 10^6 CFU/ml (n=2). Each condition was tested in triplicate.

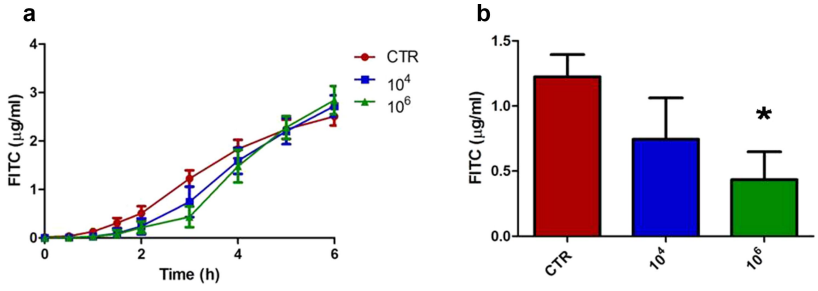

Figure 2. Effect of the probiotic blend (PB) on Caco-2 permeability. a, permeability changes during 6 h of Caco-2 incubation with/without the PB. b, focus on permeability changes after 3 h of Caco-2 incubation with/without the PB. *p<0.05vs CTR. CTR: Caco-2 cells incubated with medium alone (n=9); 10^6: Caco-2 cells incubated with 10^6 bacterial CFU/ml (n=8); 10^4: Caco-2 cells incubated with 10^4 bacterial CFU/ml (n=6).

The PB recovers permeability increase induced by IBS supernatants in caco-2 cells

Since the effect of the PB seemed maximal compared to the control after 3 h of incubation, the subsequent experiments were conducted at this time point.

Supernatants containing the mucosal mediators spontaneously released by HC and IBS biopsies were incubated with Caco-2 cells to evaluate their effect on paracellular permeability.

Supernatants of patients with IBS induced a significant increase of paracellular permeability compared to supernatants of HC ($p < 0.05$) (Figure 3), as demonstrated by the higher amount of FITC passing through the Caco-2 monolayer after 3 h of incubation. Both IBS-D and IBS-M induced a significant increase of paracellular permeability compared to HC supernatants ($p < 0.05$ and $p < 0.01$ respectively), while no difference emerged between IBS-D and IBS-M ($p = 0.860$).

The PB (10^4 and 10^6 CFU/ml) was incubated with HC or IBS supernatants to evaluate its role in restoring Caco-2 monolayer integrity (Figure 3). The co-incubation of the PB with HC supernatants did not induce any effect compared to HC supernatants alone. On the other

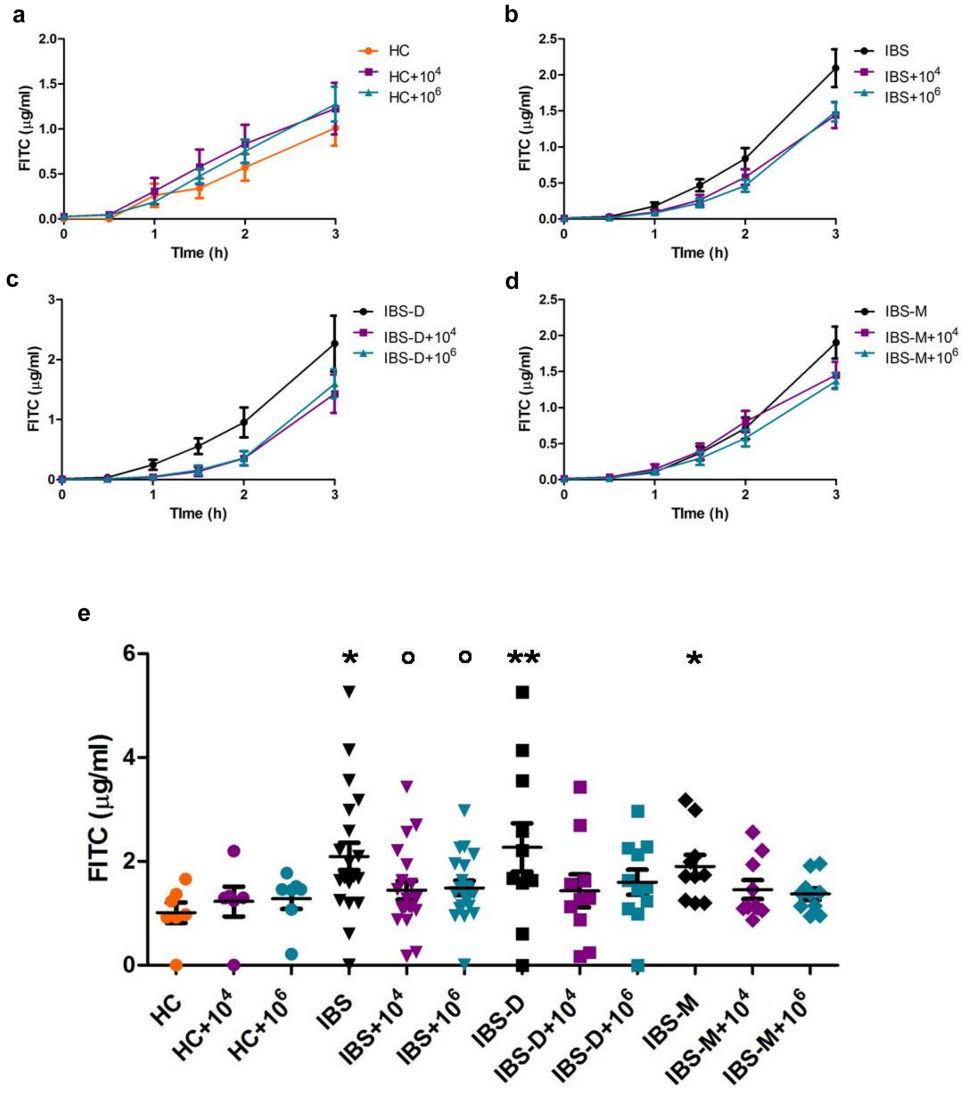

Figure 3. Effect of the simultaneous incubation of the probiotic blend (PB) and HC/IBS supernatants on Caco-2 permeability. Effect of the co-incubation of the PB and HC (A), IBS (B), IBS-D (C) and IBS-M supernatants (D) on Caco-2 permeability during 3 h of incubation. Panel E represents a focus on permeability changes after 3 h of incubation. HC: Caco-2 cells incubated with HC supernatants; IBS: Caco-2 cells incubated with IBS-D supernatant plus Caco-2 cells incubated with IBS-M supernatants, 10^4 and 10^6 CFU/ml, respectively. Statistics is according to Mann–Whitney U-test. *p<0.01 vs HC; **p<0.05 vs HC; °P<0.05 vs IBS.

hand, the simultaneous incubation of Caco-2 cells with IBS supernatants and the PB showed that both doses of the PB recovered permeability alterations induced by IBS supernatants alone ($p < 0.05$) (Figure 3e). According to IBS subtypes, co-incubation with two doses of the PB showed a decrease in the amount of FITC passed through the monolayer compared to incubation with IBS supernatants alone (IBS-M vs IBS-M + 10^4, $p = 0.063$; IBS-M vs IBS-M + 10^6, $p = 0.052$, IBS-D vs IBS-D + 10^4, $p = 0.098$; IBS-D vs IBS-D + 10^6, $p = 0.224$), although the statistical significance was not reached.

The co-incubation of the PB with IBS supernatants increases β-actin expression in Caco-2 cells compared to IBS supernatants alone

At the end of permeability experiments, RNA was extracted from Caco-2 cells to evaluate the molecular mechanisms involved in permeability alterations. Specifically, we focused on two key proteins involved in the control of permeability: a tight junction protein, zonula occludens (ZO)-1, and a cytoskeleton protein, β-actin.

The incubation of the PB did not induce any effect on the expression of ZO-1 and β-actin, except for a slight increase in β-actin with the PB 10^6 dose (Figure 4a,b).

IBS supernatants induced a significant reduction in ZO-1 expression compared to HC supernatants and the same was for IBS-D and IBS-M supernatants when considered separately (Figure 4c). A significant reduction in ZO-1 expression was induced by IBS-M compared to IBS-D supernatants. The simultaneous incubation of the PB with HC or IBS supernatants did not induce any difference compared to supernatants alone. The higher dose of the PB co-incubated with IBS-D supernatants induces an increase of ZO-1 expression, although the statistical significance was not reached (Figure 5c).

IBS supernatants did not change β-actin expression compared to HC supernatants. Interestingly, the co-incubation of both doses of the PB with IBS supernatants induced a significant increase in β-actin expression compared to supernatants alone (Figure 4d), whereas no effect was observed when co-incubated with HC supernatants (Figure 4d). Looking at IBS subtypes, the higher dose of the PB co-incubated with IBS-D supernatants induced a significant increase in β-actin expression compared to IBS-D supernatants alone (Figure 4d).

The PB alters the transcriptomic profile of caco-2 cells and induces the expression of CYP1A1

In order to further evaluate the effect of the PB on transcriptomic profile, we performed RNA-seq on Caco-2 cells incubated with IBS supernatants with or without the PB. On the basis that the higher dose of the PB induced a stronger recovery of Caco-2 permeability compared to the lower dose, together with the complexity of RNA-seq analysis, we decided to exclude from this kind of analysis Caco-2 cells incubated with the lower dose of the PB (10^4).

Clustering based on expression profile clearly separated untreated samples from probiotic-treated ones (Figure 5).

The co-incubation of Caco-2 cells with PB and IBS supernatants changed the expression of a number of genes, compared to supernatants alone (Figure 6). In particular, after calculating FDR-adjusted p-values, we focused on CY1A1 (cytochrome P450 family 1 subfamily A member 1) a canonical target of the aryl hydrocarbon receptor (AhR), whose expression was increased by the PB (adjusted $p < 0.001$). The full list of genes displaying a statistically significant change in their expression after FDR correction can be found in Supplementary Table S1.

We used qPCR to validate the results obtained from RNA-seq analysis and confirmed that the co-incubation of Caco-2 cells with the PB induced a significant increase in the expression of *CYP1A1* ($p < 0.05$, Figure 7).

Metabolomics: the PB produces indole-3-lactic acid

On the basis that CYP1A1 expression is induced by the activation of the AhR, we evaluated the

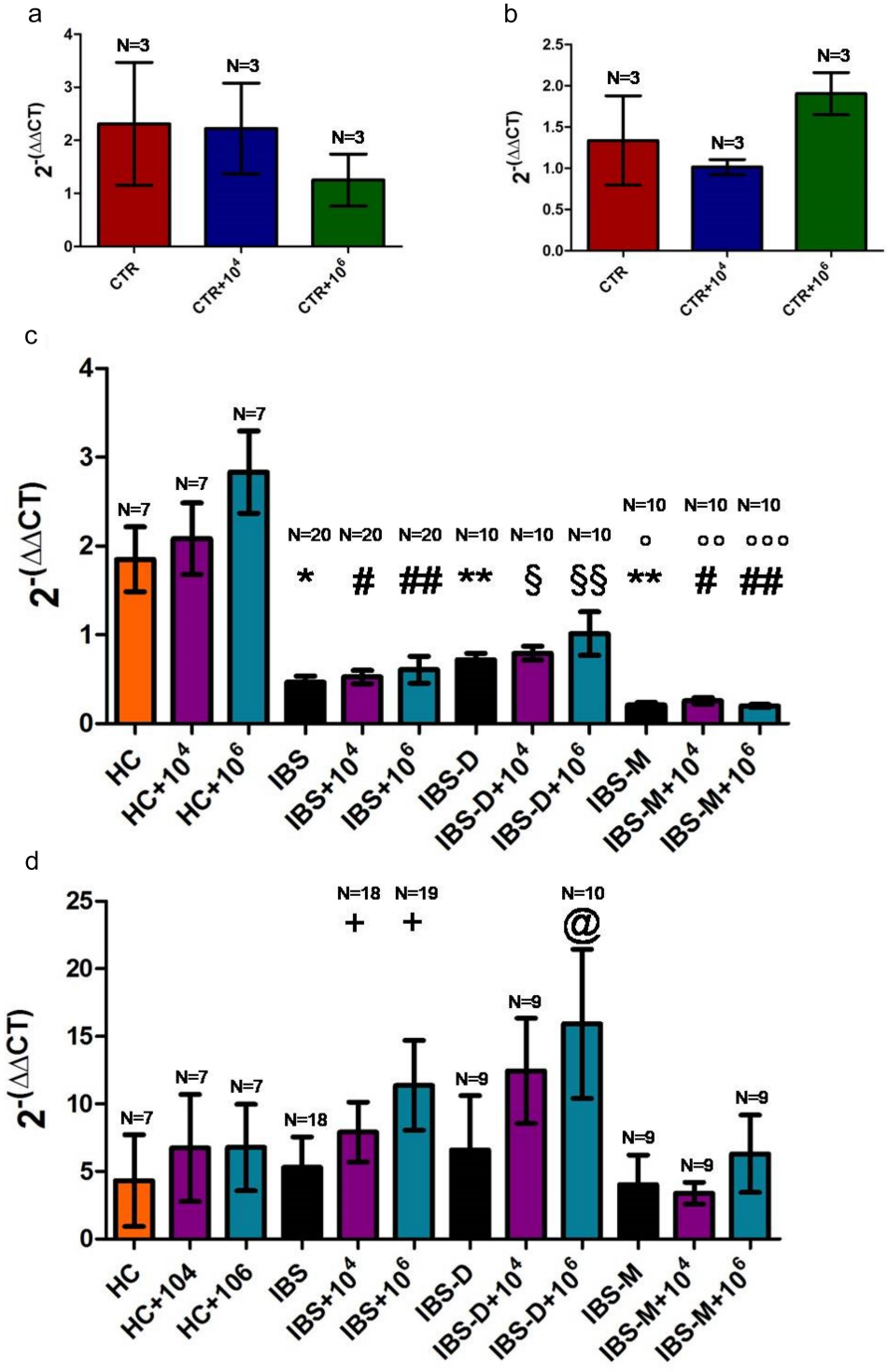

Figure 4. Effect of 3h incubation of Caco-2 cells with the probiotic blend (PB) with/without HC/IBS supernatants on ZO-1 and β-actin expression. Effect of 3-h incubation of Caco-2 cells with the PB on ZO-1 (A) and β-actin gene expression (B). Effect of the incubation of Caco-2 cells with HC/IBS supernatants with or without the PB on the expression ZO-1 (C) and β-actin (D) genes. CTR: Caco-2 cells incubated with medium alone. HC, healthy controls; IBS: Caco-2 cells incubated with IBS-D supernatant plus Caco-2 cells incubated with IBS-M supernatants; 10^4 and 10^6, CFU/ml, respectively. The expression was calculated considering the corresponding condition without the PB as calibrator. Sample number in each group (N) is indicated above each column of the histogram. Statistics is according to Mann–Whitney U-test, (C) *p<0.1 vs HC; **p<0.5 vs HC; #p<.001 vs HC+10^4; ## p<0.001 vs HC+10^6; § p<0.01 vs HC+10^4; §§ p<0.01 vs HC+10^6; °p<0.0001 vs IBS-M; °°p<0.001 IBS-M+10^4; °°°p<0.001 vsIBS-M+10^6. (D) + p<0.05 vs IBS+10^6, @ p>0.05 vs IBS-D+10^6.

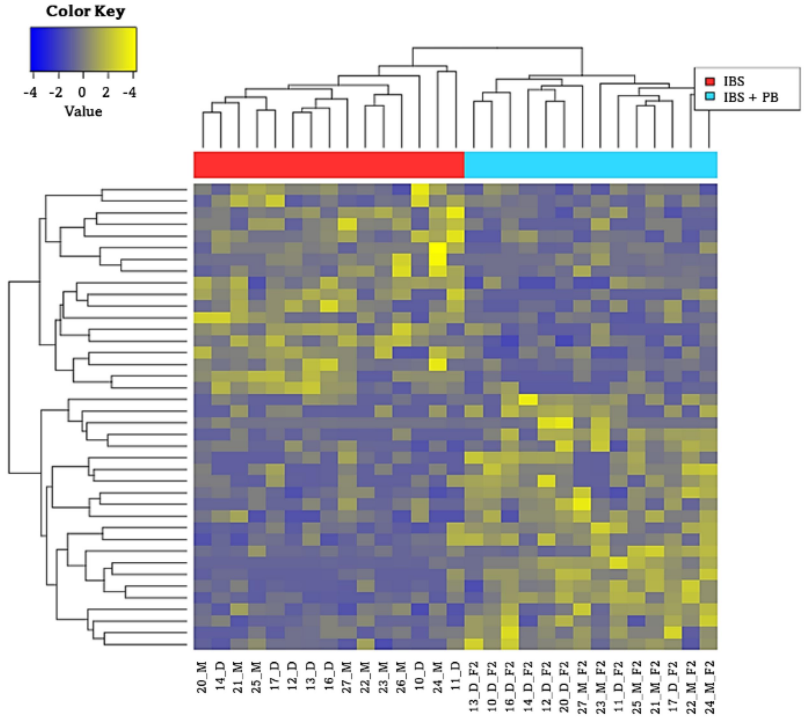

Figure 5. Two-way hierarchical clustering heatmap of samples (X-axis), on the basis of expression level (rlog, Y-axis). PB: higher dose of the probiotic blend (10^6 CFU/ml).

production of indoles, natural ligands of AhR, by the strains contained in the PB.

Five indole derivatives were detected in the supernatants of the three probiotic strains (Supplementary Table 2). Among these, indole-3-lactic acid (ILA) was the most produced compound, followed by 3-hydroxyethyl-indole, by all strains conforming the PB (Figure 8).

Relationships between permeability and symptoms

The permeability rate, expressed as the amount of FITC passing through the monolayer after 3 h of incubation of Caco-2 cells with HC/IBS supernatants, was significantly correlated with the severity and frequency of abdominal pain ($p < 0.01$ and $p < 0.05$, respectively) and bloating ($p < 0.01$ and $p < 0.05$, respectively). Looking only at the IBS group, the permeability rate is significantly correlated with abdominal pain severity and frequency ($p < 0.05$, r: 0.549 and r: 0.484, respectively) (Figure 9). Conversely, the permeability rate after 3 h of co-incubation of Caco-2 cells with HC/IBS supernatants and the two doses of the PB, did not correlate with any of the symptoms reported by patients. Looking only at Caco-2 cells incubated with IBS supernatants and the two doses of the PB, the permeability rate did not correlate with any of the symptoms evaluated.

Discussion

In the present study, we demonstrated that the higher dose (10^6 CFU/ml) of the probiotic blend composed of *L. plantarum* CECT7484 and *L. plantarum* CECT7485 and *P. acidilactici* CECT7483 strengthens the Caco-2 monolayer after 3 h of incubation. When co-incubated with the mediators spontaneously released by IBS colonic biopsies, both doses of the PB significantly recovered the increase of permeability induced by IBS mediators alone. Gene expression analysis on Caco-2 cells, incubated with and without the PB, demonstrated a significant, dose-dependent increase in actin expression compared to IBS mediators alone. In particular, when dividing by IBS subtypes, the higher dose of the PB increased actin expression compared to the IBS-D mediator alone. The higher dose of the PB also enhanced the expression of CYP1A1 when co-incubated with

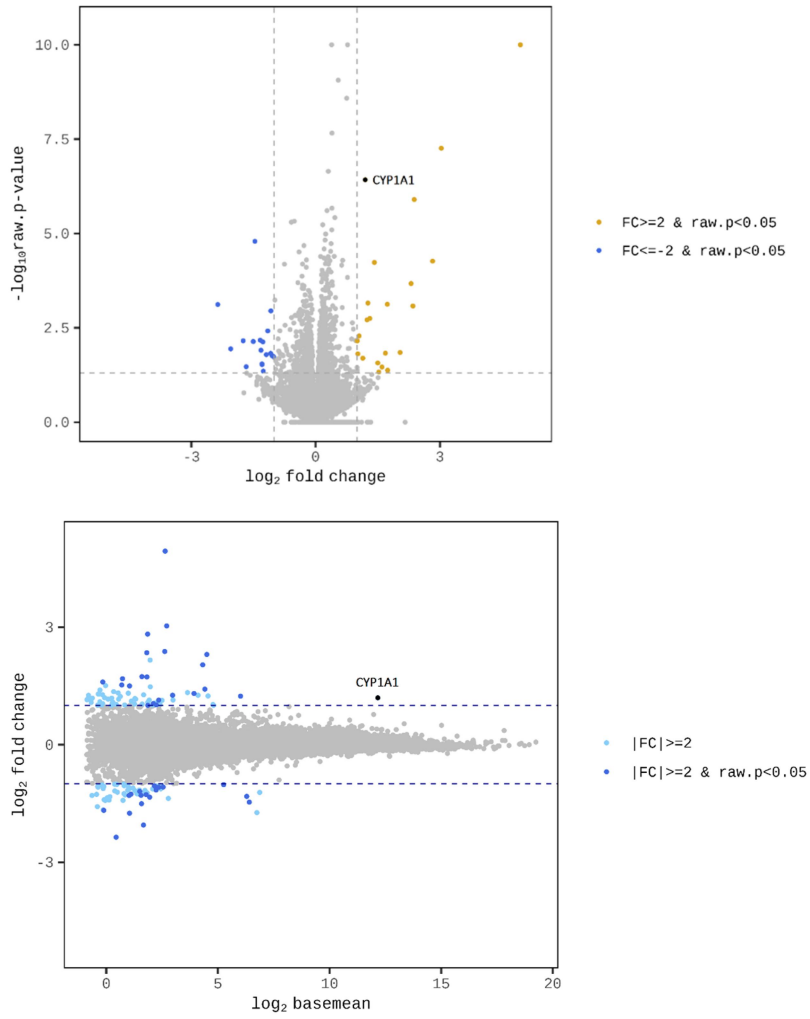

Figure 6. Differential gene expression in caco-2 cells incubated with IBS supernatants with/without the probiotic blend (PB). Top: volcano plot of log2 fold change and *p*-value obtained from the comparison of the average for each group plotted. Bottom: MA plot of average expression level (as log_2 basemean counts) and log_2 fold change.

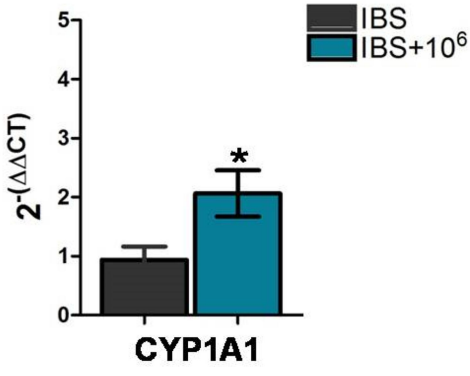

Figure 7. CYP1A1 gene expression in Caco-2 cells incubated with IBS supernatants with and without the higher dose of the probiotic blend. mRNA expression was assessed by qPCR and reported in fold changes (average plus SEM) relative to Caco-2 cells incubated with IBS supernatants alone. IBS, n=15; IBS+10^6, n=15. Statistics is according to Mann–Whitney U-test. p<0.05.

the IBS mediator alone. Finally, the strains contained in the PB produced indole lactic acid.

The role of intestinal barrier dysfunction in IBS pathophysiology is evoked for a long time. The hypothesis on the role of the intestinal barrier in IBS considers that an increase in intestinal barrier permeability facilitates the passage of luminal antigens into the lamina propria, where they can hyper-activate immune cells inducing nerve fiber sensitization.[24] Although conflicting data are reported in the literature, a recent systematic review reported 37–62% of IBS-D patients have increased intestinal permeability, suggesting the presence of this alteration, at least in a subgroup of patients with IBS.[25] As a consequence of this, alterations in immune cells in IBS have been

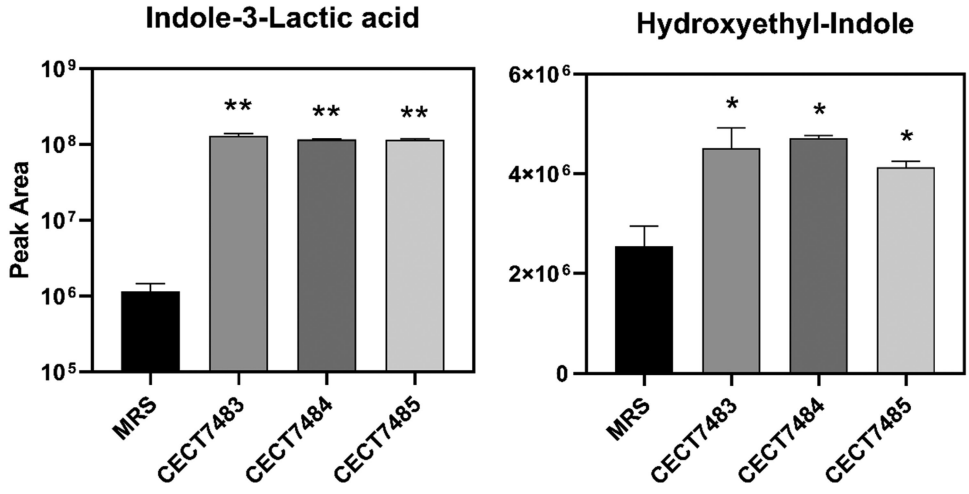

Figure 8. Metabolomic analysis of indole derivatives produced by the PB. Peak area of compounds indole-3-lactic acid (level 1) and hydroxyethyl-indole (level 2a) identified in the supernatants of *P. acidilactici* CECT 7483, *L. plantarum* CECT 7484 and *L. plantarum* CECT 7485 by LC-MS. Statistical significance vs. control (MRS medium) was set after applying a false discovery rate of 0.05. **adjusted *p*-value < 0.01; *adjusted *p*-value < 0.05.

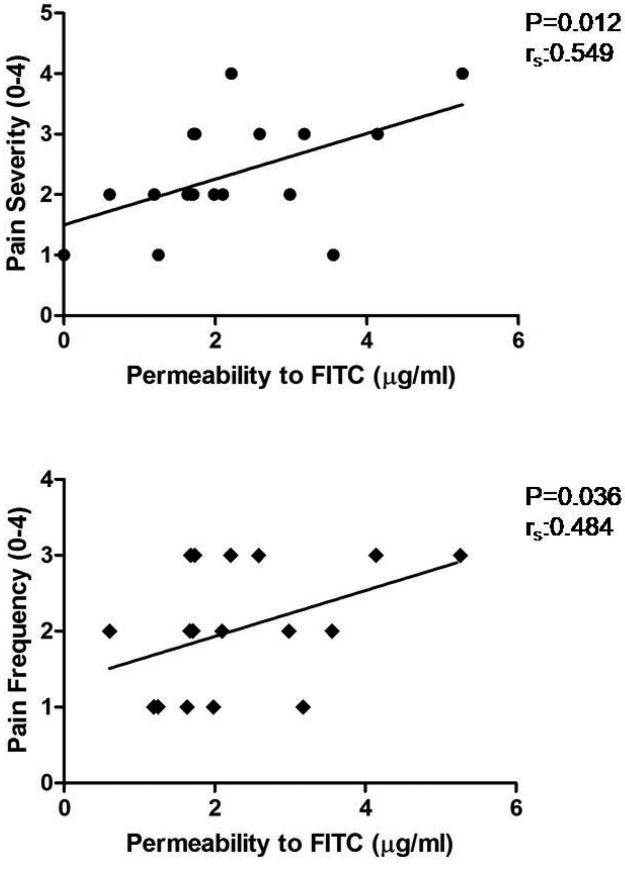

Figure 9. Relationships between permeability and symptoms. Permeability to FITC-sulfonic acid after 3 h of incubations of Caco-2 cells with IBS supernatants significantly correlated with the scores of severity and frequency of abdominal pain reported by the patients. Severity of abdominal pain: $n = 20$; frequency of abdominal pain: $n = 19$ IBS patients.

reported in the small and large bowels.[8,26] In particular, a pivotal role in the pathophysiology of IBS is likely played by mast cells, whose vicinity to nerve fibers correlates to abdominal pain referred by patients.[7] In addition, the mediators spontaneously released by IBS colonic biopsies, largely of immune origin, have a plethora of effects, from nerve excitation[27] to in vitro permeability increase, as demonstrated by a previous study showing a significant increase of Caco-2 permeability as compared with healthy subjects' mediators.[4] All together, these data suggest the presence of an increased intestinal permeability in patients with IBS and that this alteration can induce a mucosal immune activation which in turn further increases the intestinal permeability, maintaining the phenomenon.

Among the therapeutic options used to treat IBS patients, there are probiotics. Different mechanisms of action have been proposed for probiotics, including effects on intestinal permeability and inflammation.[16,28] The last systematic review and meta-analysis assessing the effect of probiotics on IBS symptoms in 82 trials involving 10,332 patients, suggested that some combinations of probiotics or strains may be beneficial in IBS.[29] The same PB used in the present study was previously employed to evaluate its effect on IBS-related

quality of life, in a multicenter, randomized, double-blind, placebo-controlled clinical trial. Eighty-four patients with IBS-D, according to Rome III criteria, were involved in the study, which demonstrated that probiotic supplementation significantly improved IBS-related quality of life compared to placebo.[19] A subsequent randomized, placebo-controlled clinical trial, including non-constipated Rome IV diagnosed IBS patients, demonstrated the PB, both alone and combined to antispasmodic agent improved IBS-related quality of life, abdominal pain, and diarrhea.[20] These results, combined with preclinical data showing a reduction in inflammation and diarrhea after PB administration in two different animal models of gut inflammation,[21] suggest a potential role for the PB in IBS, although without understanding the mechanism of action of PB. Here, we demonstrated an effect of the PB on the intestinal barrier, which is now considered an important player in IBS pathophysiology.[11] Although the intestinal barrier permeability is strictly regulated by TJs, the role of actin cannot be forgotten. The actin cytoskeleton is a highly dynamic structure that continuously rearranges through polymerization and depolymerization, regulated by various actin-binding proteins. A deregulation of these pathways, leading to increased actin depolymerization and consequently increased permeability, has been reported in the jejunal mucosa of IBS-D patients.[30] RNA-seq did not identify β-actin gene, contrarily to qPCR, but this discrepancy is likely related to technical issues.

Our in vitro results, demonstrating the ability of the PB to increase actin expression, if confirmed by future larger studies, could be of interest for the reinforcement of the intestinal barrier in IBS.

The effect of the PB on the expression of CYP1A1 is another key result of the present study. The expression of the CYP1A1 is induced by activated aryl hydrocarbon receptor (AhR). CYP1A1 leads to the detoxification and inactivation of the AhR ligands present in the diet or produced during inflammation.[31] Interestingly, the AhR has a role in immune and barrier tissue homeostasis.[32] Our metabolomics results indicate that the strains contained in the PB produce high levels of indole-3-lactic acid. This result together with the increased expression of CYP1A1 suggests that the activation of the AhR could be involved in the recovery of barrier integrity associated with the PB and is in line with a recent evidence demonstrating that indole-3-lactic acid produced by L. plantarum improves barrier integrity in mice through the AhR/Nrf2/NF-κB Axis.[33]

Our study has some limitations. First, we only enrolled IBS-D and IBS-M patients, but we cannot exclude that the arisen mechanisms could be present also in IBS-C ones; second, due to the complex methodology, RNA-seq analysis has been performed only on samples treated with the higher dose of the PB; third, the study sample was large enough for comparisons encompassing all IBS subjects, but probably underpowered to fully characterize effects according to IBS subtypes; finally, metabolomics analyses show that the PB strains have the metabolic capacity to produce indoles, although this evaluation was not carried out directly on the growth media of Caco-2 cells and PB. This discrepancy is related to the fact that when we found the PB effect on CYP1A1 from RNA seq analyses, this material was no longer available. These preliminary results suggest a role for PB in indole production and open the way for future studies to characterize this relationship in depth.

However, our study also has important strengthening points: first, we used a translational approach, in which the mucosal mediators obtained from well-phenotyped patients are used; second, the effect of IBS mediators was compared with mediators of healthy subjects; third, we employed different experimental approaches to identify possible mechanisms involved in permeability protection by the PB.

Taken together, our results support a role for the PB in the recovery of permeability alterations induced by IBS mediators. These findings highlight the potential of nutritional approach such as probiotics to favorably modulate the intestinal epithelial barrier and improve IBS symptoms and more in general consumer's health. Future studies are needed to assess the efficacy and safety of the PB in a large cohort of IBS patients.

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Disclosure statement

J. Espadaler-Mazo and M. Perez are full-time employees of AB-Biotics S.A. The other authors do not report any potential conflicts of interest.

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Data availability statement

Some data are available in the Supplementary Material. All the other data that support the findings of this study are available in Zenodo at 10.5281/zenodo.14384967.

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