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ORIGINAL CONTRIBUTION

Bifidobacterium pseudocatenulatum CECT7765 promotes a TLR2-dependent anti-inflammatory response in intestinal lymphocytes from mice with cirrhosis

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Abstract

Background Intestinal homeostasis plays an important role in bacteria-derived complications in cirrhosis. Intestinal lymphocytes are responsible for immune effector functions and can be modulated by certain probiotics. We evaluate the interaction between *Bifidobacterium pseudocatenulatum* CECT7765 and intestinal lymphocytes in mice with cirrhosis.

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Animals and methods Cirrhosis was induced by intragastrical administration of carbon tetrachloride in Balb/C mice. One week prior to laparotomy, animals received *B. pseudocatenulatum* CECT7765 (10^7, 10^9 or 10^{10} cfu/daily) or placebo. Chemokine receptor and cytokine expression were evaluated in intestinal lymphocytes. Gut permeability was studied by FITC-LPS recovery in vivo. Luminal antigens, inflammation and functional markers were evaluated in liver samples.

Results *Bifidobacterium pseudocatenulatum* CECT7765 decreased the expression of pro-inflammatory chemokine receptors CCR6, CCR9, CXCR3 and CXCR6 in intestinal lymphocytes from cirrhotic mice in a concentration-dependent manner. The bifidobacterial strain induced a shift towards an anti-inflammatory cytokine profile in this cell subset. *B. pseudocatenulatum* CECT7765-induced inflammatory modulation was TLR2-mediated, as in vitro TLR2 blockade inhibited the reduction of TNF-alpha and its receptors and the increase of IL-10 and IL-10 receptor secretion. The recovery rate of administered fluorescence-labelled endotoxin was significantly and dose-dependently lowered with the bifidobacterial strain. The reduced intestinal permeability was associated with a decreased burden of bacterial antigens in the liver of mice treated with *B. pseudocatenulatum* CECT7765. Liver function and inflammation were improved with the use of the bifidobacterial strain at the highest dose tested (10^{10} cfu).

Conclusion *Bifidobacterium pseudocatenulatum* CECT7765 improves gut homeostasis and prevents gut-derived complications in experimental chronic liver disease.

Keywords Cirrhosis · Endotoxin · *B. pseudocatenulatum* CECT7765 · Inflammation · Toll-like receptor 2 · Intestinal lymphocytes

Introduction

Bacteria-derived complications are frequent in cirrhosis [1, 2]. Most episodes of bacterial translocation (BT) are due to endogenous microoganisms coming from the gut. Since alterations in the gut microbiota composition have been described to increase inflammatory signals and the liver exposure to microbial products [3, 4], the possibility of re-shaping gut microbiota composition to improve immune homeostasis and to prevent the translocation of commensal bacteria or their products into mesenteric lymph nodes (MLNs) is of increasing interest in cirrhosis [5–7].

We have recently shown that the oral administration of a bifidobacterial strain (*Bifidobacterium pseudocatenulatum* CECT7765) to CCl$_4$-treated mice is associated with a reduced inflammatory environment and lower BT rates compared with animals not receiving the probiotic strain [8]. However, the mechanism by which this strain interacts with intestinal lymphocytes (ILs), responsible for the eventual effector functions after antigen recognition, remains unclear. This crosstalk would help in explaining homeostasis restoration and the reduction of gut permeability by *B. pseudocatenulatum* CECT7765.

The intestinal lamina propria is loaded with pre-activated T-helper cells coming from the MLNs. This expansion into the intestinal mucosa is mediated by cell adhesion molecules present on the T cell surface and plays a central role in chronic intestinal inflammation [9, 10]. One of the main receptors involved in bacterial product recognition and in regulating adaptive immune responses is the Toll-like receptor (TLR) family. For instance, *B. breve* activates intestinal dendritic cells to produce interleukin (IL)-10 and IL-27 in a TLR2-dependent manner and induce IL-10-producing regulatory T cells [11]; *C. butyricum* can suppress TLR-4 expression in intestinal epithelial cells by induction of IL-10-producing macrophages [12, 13]; and *B. fragilis* is able to restore the Th1/Th2 balance by stimulation of IL-10-producing CD4$^+$ T cells [14].

In addition, the ability of commensal bacteria to increase TLR expression has been reported [15–17], showing the susceptibility of TLR responses to intestinal microbiota products. With this background, and considering that cirrhosis is associated with a dysbiosis in the microbiota composition, we hypothesise that *B. pseudocatenulatum* CECT7765 will induce its regulatory effect by changing the TLR expression profile in intestinal T cells of mice with chemically induced cirrhosis. To evaluate this, our objective has been to determine the pathway of intestinal immune response modulation by *B. pseudocatenulatum* CECT7765 in experimental cirrhosis.

Animals and methods

Experimental model and design

Female Balb/C (Harlan, Barcelona, Spain) with 18–20 g initial body weight were included in a cirrhosis induction protocol by intragastrical administration of 2 weekly doses of CCl$_4$ during 12 weeks. Phenobarbital (0.25 mmol/L) was given in drinking water along study protocol. Mice received 2 uL of CCl$_4$ in mineral oil at the beginning of the protocol (100 uL/kg), and the dose was increased based on changes in weight 48 h after the previous dose, reaching 100 uL/mice. CCl$_4$ was administered using a sterile pyrogen-free syringe with an attached stainless steel animal feeding tube without anaesthesia as previously described [4]. One week prior to laparotomy, animals received *B. pseudocatenulatum* CECT7765 (10^7, 10^9 or 10^{10} cfu administered daily intragastrically) or placebo (vehicle). A group of naïve non-treated animals were included as a control group. Laparotomies were performed under anaesthesia with isofluorane at week 12. After laparotomies, all detectable MLNs were aseptically removed. Animals under anaesthesia were euthanised by heart injection and total blood collection. Liver, spleen, complete small intestine, content from caecum and blood were collected. The liver was perfused in situ with 6 mL of Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} at 37 °C at a rate of 1.5 mL/min as previously described [8]. Animals received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. The study was approved by the Animal Research Committee of Universidad Miguel Hernandez (Alicante, Spain).

B. pseudocatenulatum CECT7765 culture conditions

The strain *B. pseudocatenulatum* CECT7765 was obtained from Spanish Type Culture Collection (CECT). The strain was grown in MRS broth (Scharlau, Barcelona, Spain) supplemented with 0.05 % (w/v) cysteine (MRS-C Sigma, St. Louis, MO, USA) and incubated at 37 °C for 22 h (at stationary growth phase) under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK). Cells were harvested by centrifugation (6.000g for 15 min), washed twice in phosphate-buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4) and re-suspended in 10 % skimmed milk for oral administration to mice or in PBS for in vitro/culture experiment. Aliquots of these suspensions were frozen in liquid nitrogen and stored at −80 °C until used. The number of live cells was determined by colony-forming unit (CFU) counting on MRS-C agar (Biomerieux) after 48-h incubation at 37 °C. For the strains tested, more than 90 % cells were alive upon

thawing and no significant differences were found during storage time (2 months). One fresh aliquot was thawed for every new experiment to avoid variability in the viability of cultures.

Intestinal lymphocytes (IL) isolation and culture

The whole small intestine was removed from naïve and CCl_4-treated mice. Peyer's patches, fatty tissue and mesentery were dissected out. The gut was cut into small pieces (1 cm), cleaned by flushing with a syringe filled with cold phosphate-buffered saline (PBS) calcium and magnesium free (Euroclone, Milano, Italy), supplemented with 2 % foetal bovine serum (FBS; LifeTechnologies, Madrid, Spain) and open longitudinally. Intraepithelial mononuclear cells were liberated by incubating in HBSS supplemented with 5 % FBS, with dithiothreitol (DTT; Sigma-Aldrich, St Louis, MO, USA) during 20 min, twice. Epithelial cells were discarded after incubating with EDTA (BioRad, Madrid, Spain) in HBSS calcium and magnesium free (GE Healthcare Europe GmbH, Barcelona, Spain) and washing with 1,640 medium (LifeTechnologies) supplemented with 5 % FBS. Thereafter, segments were incubated during 45 min at 37 °C in RPMI, containing 2 mg/mL of collagenase D (Roche Diagnostic, Barcelona, Spain) and 1 % FBS, and later passed through a stainless steel sieve to obtain the lamina propria mononuclear cell suspension. Intraepithelial and lamina propria collected mononuclear cells were filtered through a packed nylon wool fibre column (Polysciences Europe GmbH, Eppelheim, Germany) to remove mucus and dead cells. Erythrocytes were lysed using a hypertonic solution (EasyLyse; Dako, Barcelona, Spain). Intestinal T cells were positively selected from filtered cells using CD3 MicroBeads (Miltenyi Biotec, Madrid, Spain). IL enrichment was evaluated by flow cytometry using a FACS CANTO (Becton–Dickinson, San Jose, CA) and can be followed in Supplementary Fig. 1. Cells were adjusted to 1×10^6/mL in DMEM (Euroclone) for culture experiment.

IL cells, obtained as described before, from 5 CCl_4-treated mice not receiving the bifidobacterial strain were used for in vitro assays. Cells (0.5×10^6/well) were incubated at 37 °C with 5 % CO_2 for 24 h in the following conditions: unstimulated, stimulated with LPS (0.2 ug/mL *E. coli* serotype 0111: B4), stimulated with *B. pseudocatenulatum* CECT7765 10^7 cfu/100 uL PBS and stimulated with *B. pseudocatenulatum* CECT7765 10^9 cfu/100 uL PBS. Half of all conditions were also incubated with 10 ug/mL blocking monoclonal antibody anti-TLR-2 (Antibodies Online GmbH, Aachen, Germany). Cells and supernatants were collected for gene expression analyses and cytokine secretion, respectively.

As supporting in vitro experiment, IL cells from an additional batch of CCl_4-treated mice were incubated at 37 °C

with 5 % CO_2 for 24 h in the following conditions: unstimulated, stimulated with LPS (0.2 ug/mL *E. coli* serotype 0111: B4), *B. breve*, *B. adolescentis*, *B. animalis* and *B. pseudocatenulatum* CECT7765 (in all cases 10^9 cfu/100 uL PBS). Supernatants were collected for cytokine secretion assays.

Chemokine receptor and inflammatory cytokine expression in ILs

Gene expression levels of chemokine receptors CCR6, CCR9, CXCR3 and CXCR6, as well as TNF-α, TNF receptor (TNFR) I, TNFRII, IL-10, IL-10 receptor (IL-10R), and TLRs, were quantified in total RNA from ILs using Quantitec SYBR Green (QIAgen) in an IQ5 Real-Time PCR (BioRad, Hercules, CA, USA). B2-microglobulin was used as housekeeping gene in all gene expression analyses. Primer pairs used in the study can be followed in Supplementary Table 1.

Western blots were performed in RIPA buffer-lysed cell pellets. The primary antibodies used against CCR6, CCR9, CXCR3 and CXCR6 and β-actin were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Enzyme-linked immunosorbent assays (ELISA) from R&D Systems (Minneapolis, MN, US) were performed according to the manufacturers' instructions for quantitation of IL-secreted TNF-α and IL-10 in vitro. ELISA kits were also used for determining quantitative levels of TNF-α and IL-6 in liver samples. All samples were tested in duplicate and read in a Sunrise Microplate Reader (Tecan, Mannedorf, Switzerland).

FITC-LPS permeability assay and gut barrier integrity markers analysis

Intestinal permeability was evaluated administering fluorescein isothiocyanate (FITC)-LPS from *E. coli* O111: B4 (Sigma) by gastric gavage 4 h before killing. Whole blood was obtained by retro-orbital puncture at the time of killing. Dilutions of FITC-LPS in PBS were used as a standard curve, and FITC-LPS measurements were performed in 100 μL of plasma or standard in a fluorometer at 488 nm. Gene expression levels of occludin and tight junction protein (TJP)-1 were evaluated using Quantitec SYBR Green as described above. Primers are detailed in Supplementary Table 1.

Analysis of luminal antigen translocation and liver function

Bacterial DNA was detected and identified by broad-range PCR and partial sequencing analysis in MLNs, blood and liver samples according to the methodology described elsewhere [18, 19]. The quantitative chromogenic limulus

amebocyte lysate test (BioWhittaker, Nottingham, UK) was performed to evaluate endotoxin levels in MLNs, blood and liver samples according to the manufacturer's instructions. All samples were tested in duplicate and read at 405 nm in a Sunrise Microplate Reader.

Liver function was evaluated by determining ALT and total bilirubin levels by handling ELISA kits from Cloud-Clone Corp (Houston, TX, US) and Cusabio Biotech Ltd. (Wuhan, China), respectively, according to the manufacturers' instructions. All samples were tested in duplicate and read at 450 nm.

Statistical analysis

Continuous variables are reported as mean ± SD and categorical variables as frequency or percentages. Statistical differences were analysed using the Chi-squared test for categorical data applying the Yates correction when required. The Kolmogorov–Smirnov test was used to identify normal distribution of the data. Differences in quantitative data were analysed using the Student's t test for variables with normal distribution and the Mann–Whitney U test for quantitative data without normal distribution. Multiple comparisons were analysed using analysis of variance (ANOVA) or the Kruskal–Wallis test for data with and without normal distribution, respectively, followed by pairwise comparisons using the post hoc Bonferroni correction. Bivariate correlations were calculated using the Pearson or Spearman test according to data distribution. All reported p values are two-sided. p values lower than 0.01 were considered significant. All calculations were performed using the R-2.14.1 2011 (The R Foundation for Statistical Computing)

Results

Study groups and characteristics of animals

Cirrhosis was induced by oral administration of CCl_4 in a starting series of 70 mice. Seventeen mice died during the cirrhosis induction protocol (24 %), with no statistically significant differences between groups. Causes of death were CCl_4 toxicity and liver insufficiency. Finally, 10 naïve mice, 12 mice in the CCl_4 group, 12 mice in the CCl_4 + CECT7765 1×10^7 cfu/daily, 10 mice in the CCl_4 + CECT7765 1×10^9 cfu/daily and 11 mice in the CCl_4 + CECT7765 1×10^{10} cfu/daily were subjected to laparotomy. Cirrhosis was histologically confirmed by collagen deposition and regenerative nodules in all CCl_4-treated animals, with no significant differences according to the administration of the bifidobacterial strain. Neither CCl_4 induction of cirrhosis nor *B. pseudocatenulatum*
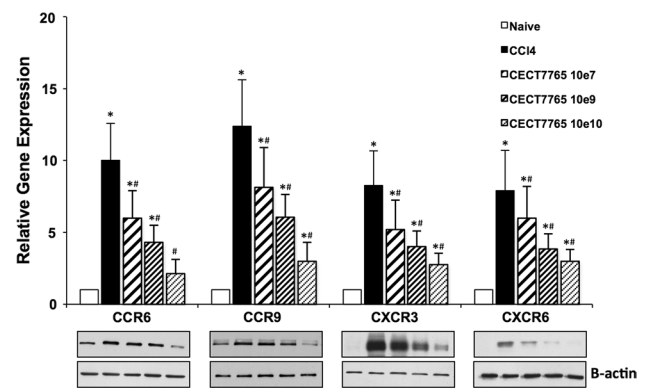

Fig. 1 Relative gene expression levels of chemokine receptors CCR6, CCR9, CXCR3 and CXCR6 in ILs from naïve and CCl_4-mice, either receiving or not *B. pseudocatenulatum* CECT7765 at different concentrations. *ILs* intestinal lymphocytes, *CCR* C-C chemokine receptor, *CXCR* C-X-C chemokine receptor. *$p < 0.01$ compared with naïve mice; #$p < 0.01$ compared with CCl_4 mice without *B. pseudocatenulatum* CECT7765 and/or the previous bifidobacterial strain concentration

CECT7765 administration had any visual effect on intestinal wall morphology (Supplementary Fig. 2). Body and spleen weights in animals from all groups are detailed in Supplementary Table 2. Liver and spleen weights in all CCl_4 animals were significantly higher compared with naïve mice.

IL chemokine receptors and cytokine response are regulated by *B. pseudocatenulatum* CECT7765

The expression of an array of chemokine receptors involved in the inflammatory balance and T cell migration from MLNs was evaluated in ILs from cirrhotic mice, either treated or not with the bifidobacterial strain. The administration of *B. pseudocatenulatum* CECT7765 significantly reduced both the gene and protein expression of all studied chemokine receptors in a dose-dependent fashion (Fig. 1).

Soluble mediators of inflammation TNF-alpha and IL-10 were also evaluated in ILs from the different study groups. Gene expression levels of TNF-alpha and its receptors TNF-RI and TNF-RII significantly decreased with the administration of *B. pseudocatenulatum* CECT7765 compared with mice not receiving the bacterial strain. In addition, this inflammatory inhibition was dependent on the *B. pseudocatenulatum* CECT7765 concentration administered (Fig. 2a). Inversely, gene expression levels of IL-10 and IL-10R were significantly increased in mice with *B. pseudocatenulatum* CECT7765, and these increments were also dependent on *the bifidobacterial* concentration administered (Fig. 2b). These results clearly show the IL response to *B. pseudocatenulatum* CECT7765 administration.
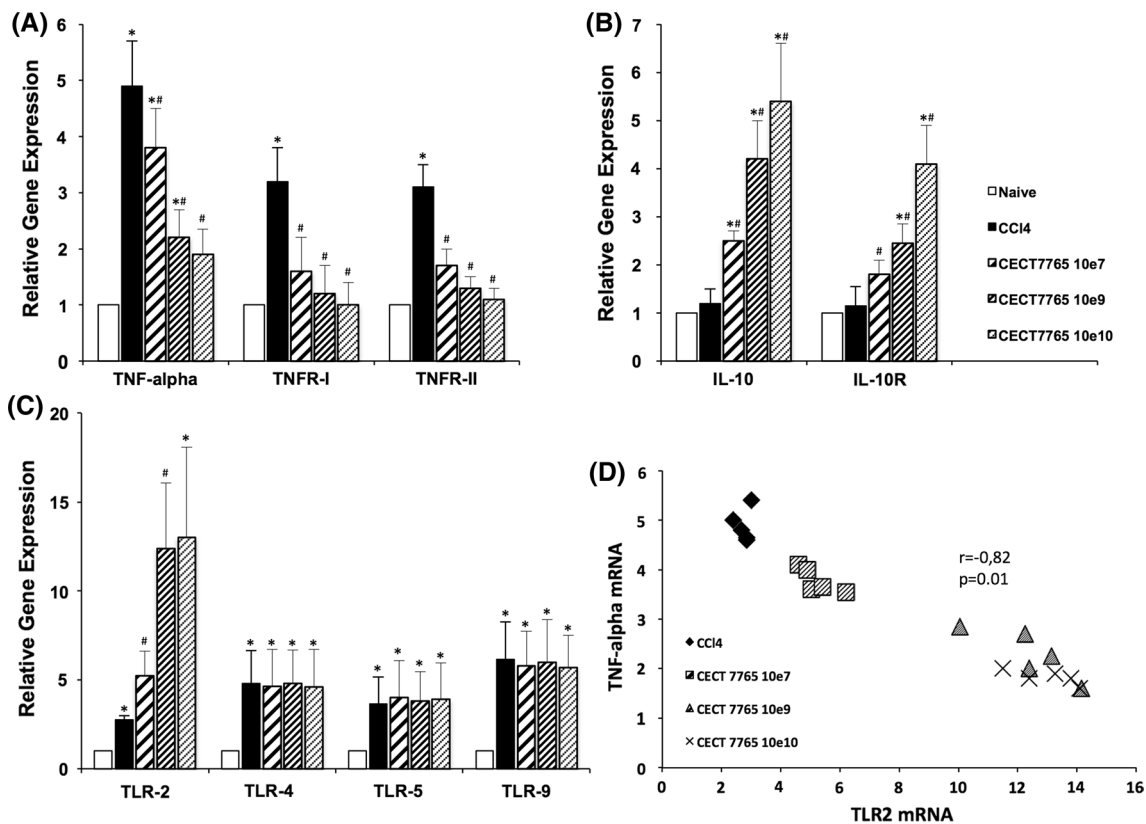
Fig. 2 Relative gene expression levels of TNF-alpha and TNF-alpha receptors (**a**), IL-10 and IL-10 receptor (**b**) and TLRs (**c**) in ILs from naïve and CCl$_4$ mice either receiving or not *B. pseudocatenulatum* CECT7765 at different concentrations. **d** Correlation between TNF-alpha and TLR-2 gene expression levels in ILs from naïve and CCl$_4$ mice either receiving or not *B. pseudocatenulatum* CECT7765 at different concentrations. *ILs* intestinal lymphocytes, *TNF-alpha* tumour

necrosis factor alpha, *TNF-RI* tumour necrosis factor alpha-receptor I, *TNF-RII* tumour necrosis factor alpha-receptor II, *IL-10* interleukin 10, *IL*-10R interleukin 10 receptor; *TLRs* Toll-like receptors. *$p < 0.01$ compared with naïve mice; #$p < 0.01$ compared with CCl$_4$-mice without *B. pseudocatenulatum* CECT7765 and/or the previous bifidobacterial strain concentration

After evaluating the homeostatic effect of *B. pseudocatenulatum CECT7765* in ILs, we aimed at delineating the mechanism by which *B. pseudocatenulatum CECT7765* might interact with this cell population. Gene expression levels of TLRs in ILs were measured to determine the signalling pathway for *B. pseudocatenulatum* CECT7765 to induce cytokine secretion. Only TLR-2 was significantly upregulated with increasing concentrations of *B. pseudocatenulatum* CECT7765 (Fig. 2c), and its expression inversely correlated with the TNF-alpha gene expression levels shown in the different groups of animals (Fig. 2d). The rest of TLRs tested showed a *B. pseudocatenulatum* CECT7765-independent expression.

B. pseudocatenulatum CECT7765 promotes a TLR-2-dependent modulation of IL cell-secreted cytokine profile

To confirm the relevance of TLR-2 signalling in the bifidobacterial strain regulatory mechanism, ILs from CCl$_4$-treated mice were isolated and cultured with LPS and *B. pseudocatenulatum* CECT7765 in the presence/absence of an anti-TLR-2 antibody. As can be followed in Fig. 3a, TNF-alpha secretion by cultured cells was significantly increased after 24-h stimulation with LPS, whereas stimulation with two concentrations of *B. pseudocatenulatum* CECT7765 did not induce TNF-alpha secretion in vitro. However, the inhibition of TLR-2 signalling significantly induced TNF-alpha secretion by ILs in the presence of *B. pseudocatenulatum* CECT7765 to the levels observed in cells from unstimulated CCl$_4$-treated mice.

On the other hand, stimulation with *B. pseudocatenulatum* CECT7765 induced significantly increased levels of IL-10 in cell supernatants compared with levels induced by LPS or in unstimulated cells (Fig. 3b). When TLR-2 was blocked, secreted IL-10 significantly decreased in *B. pseudocatenulatum* CECT7765-stimulated cells to levels observed in unstimulated cells. These data demonstrate a TLR-2-mediated anti-inflammatory effect of *B. pseudocatenulatum* CECT7765 in ILs from cirrhotic mice.
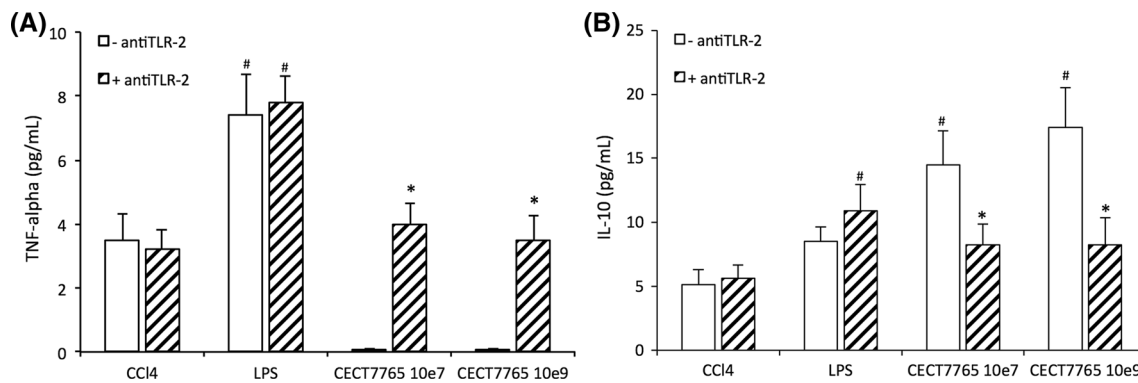
Fig. 3 TNF-alpha (**a**) and IL-10 (**b**) levels secreted by isolated ILs cultured in unstimulated conditions and after stimulation with LPS and *B. pseudocatenulatum* CECT7765 at different concentrations in the presence/absence of a blocking monoclonal antibody anti-TLR-2.

Mean values $\pm$ SD from three different experiments are represented. $^{\#}p < 0.01$ compared with cells from unstimulated CCl_4 mice; $^{*}p < 0.01$ compared with cells not receiving the blocking anti-TLR-2; *TLR* Toll-like receptor, *LPS* lipopolysaccharide

As all commensal bacteria are expected to stimulate TLR-2 at some degree, we ran an in vitro experiment to determine the relative sensitivity of *B. pseudocatenulatum* CECT7765 to TLR-2-dependent proinflammatory reduction compared with other bifidobacterial strains obtained from collections. The results are shown in Supplementary Fig. 3. Although all the bifidobacterial strains showed an increment in TNF-alpha and IL-6 after TLR-2 blockade compared with unstimulated conditions, *B. pseudocatenulatum* CECT7765 showed a significantly higher increment in both cytokine secreted levels after TLR-2 blockade compared with the rest of bifidobacterial strains.

B. pseudocatenulatum CECT7765 reduces intestinal permeability

The reduction in the intestinal inflammatory environment was significantly associated with a dose-dependent reduction of gut permeability. As can be observed in Fig. 4a, the administration of the bifidobacterial strain significantly reduced FITC-LPS recovery rates among cirrhotic animals, restoring permeability to values similar to those observed in naïve mice. Accordingly, *B. pseudocatenulatum* CECT7765 was associated with improved gut barrier integrity markers in CCl_4-treated mice, providing a possible explanation for the observed reduction in gut permeability assay (Fig. 4b). Despite these results, as commented before, no visual effects of gut barrier morphology could be observed (Supplementary Fig. 1).

B. pseudocatenulatum CECT7765 reduces liver inflammation and improves liver function

We were interested in testing whether a reduction in intestinal permeability might improve liver inflammation. Spontaneous bacterial antigen translocation into liver was significantly reduced after administration of increasing concentrations of *B. pseudocatenulatum* CECT7765. As shown in Fig. 4c, d, the oral administration of 1×10^{10} cfu/daily for a week significantly reduced the translocation rate of bactDNA and endotoxin in the liver to levels present in naïve control mice. No *B. pseudocatenulatum* CECT7765 DNA was identified in any of the bactDNA-positive samples. Translocating bacterial species belonged to the family *Enterobacteriaceae* in all cases: *E. coli* ($n = 9$), *K. pneumoniae* ($n = 4$), *E. cloacae* ($n = 2$), *S. marcescens* ($n = 1$). Liver endotoxin levels inversely correlated with the concentration of *B. pseudocatenulatum* CECT7765 administered ($r = -0.72$; $p = 0.001$).

The reduced bacterial antigen translocation rates were associated with the statistically significant downregulation of TNF-alpha (Fig. 5a) and IL-6 (Fig. 5b) levels in the liver. Also, an improved liver function was significantly associated with the administration of the bifidobacterial strain at 1×10^{10} cfu/daily, as can be observed for ALT and total bilirubin levels in Fig. 5d, respectively. Both parameters were significantly decreased compared with CCl_4-treated mice without the bifidobacterial strain, although levels remained higher than those observed in naïve mice, even after administration of the highest dose of *Bifidobacteria*.

Discussion

Intestinal homeostasis is fundamental in preventing bacterial complications, frequently present in cirrhosis. A balanced immunological response at the edge between a vast luminal commensal microbiota population and the rest of host's territories guarantees a reduced permeability environment for bacteria or their products to translocate. In the present study, we demonstrate that ILs, responsible for the local immune effector functions, are modulated by *B.*
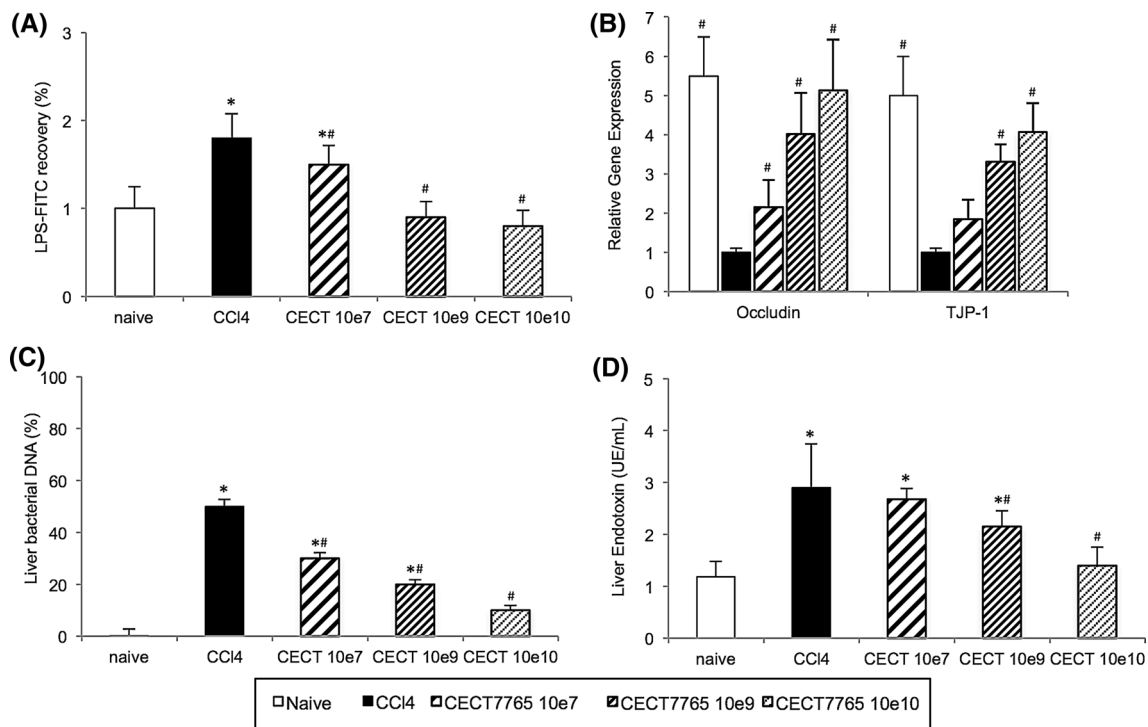
Fig. 4 **a** FITC-LPS recovery rates after LPS oral administration in naïve and cirrhotic mice with different concentrations of *B. pseudocatenulatum* CECT7765. Mean values ± SD from five different experiments are represented. Bacterial DNA translocation **b** and endotoxin levels **c** into liver samples from all naïve and CCl₄ mice either receiving or not *B. pseudocatenulatum* CECT7765 at different concentrations. Mean values ± SD are represented. *$p < 0.01$ compared with naïve mice; #$p < 0.05$ compared with CCl₄ mice without *B. pseudocatenulatum* CECT7765 and/or with the previous dose of the bifidobacterial strain. *FITC* fluorescein isothiocyanate, *LPS* lipopolysaccharide

pseudocatenulatum CECT7765, reducing their pro-inflammatory chemokine receptors expression and inducing a shift in their cytokine profile. In vitro assays demonstrate that the IL reduced pro-inflammatory cytokine secretion induced by the bifidobacterial strain is dependent on TLR2 expression.

The gut-associated lymphoid tissue (GALT), where effector CD4$^+$ T cells reside after priming at MLNs, constitutes a central element in maintaining intestinal homeostasis and host's immunity against the translocation of bacterial antigens [20, 21]. The homing signals for ILs to migrate back to the LP are chemokine receptors expressed in their surface, mainly CCR6, CCR9, CXCR3 and CXCR6 among others [22], that also account for the host defence and repair processes. We here report the *B. pseudocatenulatum* CECT7765 dose-dependent reduction of these chemokine receptors in ILs from mice with cirrhosis compared with animals not receiving the bifidobacterial strain (Fig. 1). In fact, it has been recently reported that CCR9 is increased in mononuclear cells and activated hepatic stellate cells during liver fibrosis and that CCR9 deficiency reduces liver inflammation in CCl4-treated mice [23]. Similarly, CCR6 has been shown to stay upregulated in

CD4$^+$ cells in experimental fibrosis and intrahepatically in patients with chronic liver diseases [24].

The reduced pro-inflammatory IL expansion was confirmed by the shift observed in the profile of cytokine production with the administration of the probiotic strain. Whereas IL TNF-alpha and TNFRs were significantly decreased, IL-10 and IL-10R expression was increased after the probiotic treatment in a bifidobacterial strain concentration-dependent manner. We further identified TLR2 as the IL receptor for the bifidobacterial strain-induced immune modulation (Figs. 2, 3). TLRs have been shown to induce downstream intracellular signalling and subsequent cytokine secretion in response to specific probiotics [25, 26]. In our study, the inhibition of *B. pseudocatenulatum* CECT7765 capacity to change the cytokine secretion pattern by blocking TLR-2 reveals the central role for this receptor in the bifidobacterial recognition and induction of the host's cytokine response. In fact, similar results had been obtained in the past showing the anti-inflammatory properties of TLR-2 [27–29] and the induction of a pro-inflammatory cytokine response by *B. breve* after blocking TLR-2 [30]. In contrast to this and the previously mentioned studies, an inflammatory downregulation
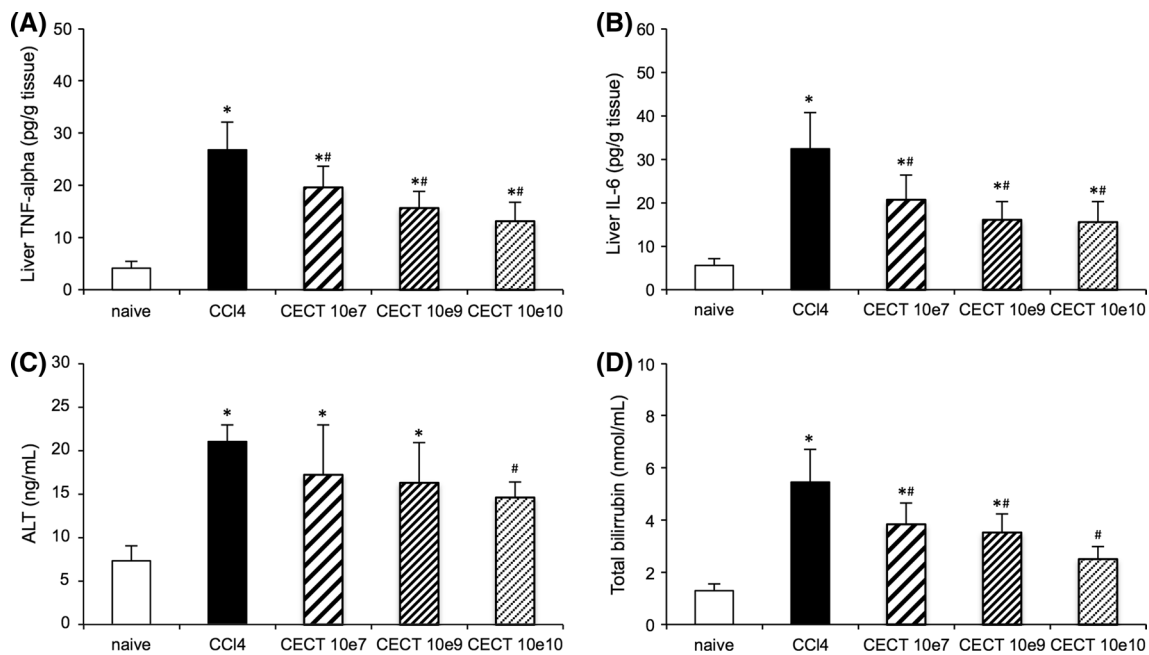
Fig. 5 Serum ALT (**a**) and total bilirubin levels (**b**) in naive and CCl$_4$-treated mice receiving or not *B. pseudocatenulatum* CECT7765 at different concentrations. Mean values ± SD are represented. *$p < 0.01$ compared with naïve mice; #$p < 0.05$ compared with CCl$_4$ mice not receiving *B. pseudocatenulatum* CECT7765 and/or with the previous dose of the bifidobacterial strain. *ALT* alanine aminotransferase

in monocytes from lamina propria in TLR2-deficient mice with bile duct ligation has been reported [31]. Several aspects, such as the distinct experimental model used and the intestinal monocyte population used rather than ILs, may explain this difference. Anyhow, considering the increment in pro-inflammatory mediators that comes along with fibrosis progression and cirrhosis [4, 32], the possibility of an immune response repolarisation by *B. pseudocatenulatum* CECT7765 becomes an appealing strategy to self-limit inflammation and derived complications that should be further pursued and confirmed in human immune cell subsets.

The bifidobacterial strain induction of a reduced inflammatory environment had an impact on gut permeability, as evaluated in vivo by measuring FITC-LPS recovery after oral administration in cirrhotic mice with different concentrations of *B. pseudocatenulatum* CECT7765. Several studies have shown the improvement in gut barrier integrity markers with the use of probiotic strains [33–37]. However, although a distorted gut barrier may facilitate BT, the study of integrity markers cannot confirm the actual passage of bacterial products. In the present study, besides an increased expression of tight junction proteins, the permeability assay demonstrates the decreased rate of LPS translocation through the gut barrier with increasing amounts of the bifidobacterial strain. Accordingly, the rate of bacterial antigens reaching the liver is also significantly reduced, as shown in Fig. 3.

Regarding liver damage, the administration of *B. pseudocatenulatum* CECT7765 did not show any significant effect on histology architecture, despite a significant reduction in the profibrogenic TIMP-1 and MMP-2 gene expression levels (Supplementary Fig. 1). Despite one study showed fibrosis amelioration with the administration of VSL#3 in an experimental model of NASH [38], results presented herein are consistent with previous findings from our group working on this bifidobacterial strain [8] and with results obtained with VSL#3 in the rat model of cirrhosis [37]. However, an interesting result of the study is that the administration of *B. pseudocatenulatum* CECT7765 at 10^{10} cfu/daily to CCl$_4$-treated mice significantly reduced liver TNF-alpha and IL-6 levels together with ALT and total bilirubin levels, suggesting an improvement in liver function also dependent on *B. pseudocatenulatum* CECT7765 concentration. Although these results have to be considered with caution and confirmed with further specifically designed studies on the bifidobacterial strain effect on liver function, the possibility of reducing liver inflammation and ameliorating functional markers such as ALT had already been reported in ob/ob mice treated with VSL#3 [39].

In summary, the present study shows that chemokine receptors expression and cytokine profile of ILs in mice with cirrhosis are modulated by the oral administration of *B. pseudocatenulatum* CECT7765. This homeostatic effect favours a reduced intestinal permeability that lowers

the burden of bacterial antigens reaching the liver. These promising results encourage further studies on the bifido-bacterial interaction with different immune cell subsets from patients with cirrhosis aimed at eventually testing *B. pseudocatenulatum* CECT7765 supplementation in patients with cirrhosis.

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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