Oral Supplementation With Selected *Lactobacillus Acidophilus* Triggers Antimicrobial Response, Activation of Innate Lymphoid Cells Type 3 And Improves Colitis

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Research Article

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Abstract

Live biotherapeutic products constitute an emerging therapeutic approach to prevent or treat inflammatory bowel diseases. *Lactobacillus acidophilus* is a constituent of the human microbiota with probiotic potential, that are illustrated by direct and indirect antimicrobial activity against several pathogens and improvement of intestinal inflammation. In this study, we evaluated the anti-inflammatory properties of the *L. acidophilus* strain BIO5768 and assessed the underlying mechanisms of action. BIO5768 was able to counteract the acute colitis that is induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS). When administered alone or in combination with *Bifidobacterium animalis* spp. *lactis* BIO5764 and *Limosilactobacillus reuteri*, BIO5768 was also able to alleviate intestinal inflammation induced by *Citrobacter rodentium* infection. Supplementation of naïve mice with either strain BIO5768 alone or as mixture, increased the gene expression of several target genes involved in immune signaling, including c-type lectin Reg3 gamma. Consistently, the ability of innate lymphoid cells to secrete IL-22 was enhanced in response to BIO5768. Interestingly, the aforementioned responses were shown to be independent of NOD2 and Th17 signaling in mice that were mono-colonized with BIO5768. In conclusion, we identify a new potential probiotic strain with the ability for the management of inflammatory bowel diseases, and provide some insights into its mode of action.

Introduction

Crohn's disease (CD) is traditionally characterized by the development of transmural inflammatory lesions leading to progressive destruction of the intestinal wall. In Europe, the combined prevalence is about 250–300 cases per 100,000 inhabitants, with about 2.2 million people affected. Several epidemiological and experimental evidences indicate that in emerging countries incidence rates are rising due to the influence of many gene-environmental interactions, such as, amongst others, tobacco smoking. While the lifetime therapeutic management of the disease is far from optimal, CD impairs patient's ability to work while also their social interaction is altered in various ways. Invasive surgery and bowel resection is required in about two-thirds of CD patients, although it does not cure the disease. Consequently, the annual economic burden of medical care of CD patients ranges between 2.1 and 16.7 billion € in Europe [1].

The gut microbiota plays a crucial role in many physiological processes from the earliest days of life, including the maturation of the gut barrier and the immune system. Recent progress in high-throughput sequencing revealed that lowered bacterial diversity is commonly observed in the gut microbiota of CD patients [2, 3]. Specifically, a decreased prevalence of *Faecalibacterium prausnitzii* was associated with a higher risk of postoperative recurrence of ileal CD [4]. Inappropriate interaction between some components of the gut microbiota and the mucosal immune system is thereby thought to influence disease initiation and progression [5, 6]. It is worth noting that clinical remission following anti-TNF therapy was significantly associated with an increased bacterial diversity of fecal microbiota and correlated with higher levels of

butyrate and substrates involved in butyrate synthesis [7]. Accordingly, prospective studies revealed that early microbiome changes can predict the response of CD patients to Vedolizumab [8] and Ustekinumab [9].

Therefore, supplementation with health-promoting probiotics is becoming particularly attractive not only to reconstitute the diversity and the functionality of patient's microbiome but also to counteract the exaggerated inflammatory responses. While probiotics showed successful impact against ulcerative colitis, none of the reported clinical trials with probiotics proved some efficacy in CD [10–13]. A possible explanation for such inefficiency is that their mode of action may rely on certain genes that are found mutated in CD patients. In agreement with this hypothesis, we provided evidence that the protective capacity of a selected strain of *Ligilactobacillus salivarius* Ls33 (*formerly known as Lactobacillus salivarius* Ls33) requires an intact nucleotide-binding oligomerization domain 2 (NOD2) signaling [14], mutations of which occur in more than one third of the CD patients in Europe and North America. We then performed a comprehensive screening of the Bioprox probiotic collection for identifying strains that are exhibiting anti-inflammatory and antimicrobial abilities. This led us to identify a *Lactobacillus acidophilus* strain inducing antimicrobial responses that do not depend on NOD2 signaling. This strain BIO5768 was originally isolated from the human gastrointestinal tract and is produced and commercialized as dietary supplements by the society Bioprox Healthcare.

In the current study, we report that supplementation with *L. acidophilus* BIO5768 enhanced the activity of innate lymphoid cells type 3 (ILC3), that are known to play an essential role in the maintenance of the barrier function and tissue repair [12], [15]. Specifically, we tested their capacity to modulate the expression of interleukin-22 (IL-22) and of antimicrobial peptides (AMPs) both *in vitro* and *in vivo*. Consequently, the expression of several IL-22-targeted genes was enhanced in response to BIO5768, as earlier observed in response to retinoic acid and aryl hydrocarbon receptor [16, 17]. Interestingly its ability to induce AMPs responses was shown to be NOD2 and IL-17-independant. Given that BIO5768 exhibited a different mode of action than the previously studied strains *Bifidobacterium animalis* spp. *lactis* BIO5764 (referred herein as BIO5764) and *Limosilactobacillus reuteri* BIO5454 (formerly known as *Lactobacillus reuteri* BIO5454, referred herein as BIO5454) [18], we compared its *in vivo* anti-inflammatory capacity when administered alone or in combination, by using a *Citrobacter rodentium* infection model and a TNBS colitis model.

Results

L. acidophilus **BIO5768 ameliorates the severity of TNBS-induced acute colitis in mice.**

The potential ability of the strain BIO5768 to limit the severity of colitis was first evaluated in an experimental murine model of TNBS-induced acute colitis. While we observed only a moderate effect of the bacterial oral supplementation on weight loss (Fig. 1a), BIO5768 administration dampened the severity of colitis as indicated by a significant decrease of the macroscopic Wallace score (Fig. 1b), confirmed by the histological analyses of colon tissue indicated by a decreased Ameho score, albeit not significant (Fig. 1c). The expression of genes encoding the pro-inflammatory markers *Tnfa* and *Cxcl2* was

also significantly downregulated in the BIO5738-treated group and to a lower extend also the genes encoding *Il1b* and *Il6* (Fig. 1d).

L. acidophilus **BIO5768 downregulates inflammatory responses in the** Citrobacter rodentium **infection model.**

A *C. rodentium* infection model in mice was used to mimic the human situation in which enteropathogenic and enterohemorrhagic strains of *Escherichia coli* contribute to the development of intestinal inflammatory responses in IBD. We evaluated the impact of the oral administration of BIO5768 on the prevention and/or limitation of transient colitis caused by this bacterium, pathogenic for mice. Despite no effect of BIO5768 on the burden of *C. rodentium* (Fig. 2a), the colon length was significantly reduced in mice infected by *C. rodentium* ($p < 0.001$), while no significant difference was measured in mice treated with BIO5768, in comparison to non-infected control mice (Fig. 2b). Hyperplasia of crypts in *C. rodentium*-infected mice was significantly increased compared to non-infected mice ($p < 0.001$). There was only a marginal effect of BIO5768 administration on the shortening of crypt length, as compared to *C. rodentium*-infected mice (Fig. 2c). Infection by *C. rodentium* significantly elevated gene expression in the colon of the pro-inflammatory markers (*Tnfa*, *Il6* and *Il1b*), as compared to non-infected mice ($p < 0.001$). BIO5768 supplementation lowered the expression of these aforementioned pro-inflammatory markers, but this effect was not statistically significant (Fig. 2d).

L. acidophilus **BIO5768 promotes antimicrobial expression** in vitro **and** in vivo, **IL-22 production and dendritic cell maturation.**

We evaluated the capacity of BIO5768 to regulate the production antimicrobial peptides by using the mouse intestinal cell line m-ICcl2 presenting a crypt phenotype, and is able to differentiate to cells that share similarities with Paneth cells [19]. BIO5768 induced the expression of the transcripts encoding defensin beta 2 (*Defb2)* and defensin alpha 4 (*defa4)*, although no effect was observed on Regenerating islet-derived protein 3 gamma (*Reg3g*) expression and on IL-22 (Fig. 3a). We then evaluated the capacity of BIO5768 to promote antimicrobial peptide expression *in vivo* in BALB/c naïve mice. BIO5768 significantly increased the expression of *Defb2* ($p < 0.05$) and to a lower extend *Reg3g* in the proximal colon while the expression of *Defa4* was not affected by BIO5768 administration (Fig. 3b). Similar results were observed in the distal colon (Fig. S1). We then evaluated the capacity of the strain to regulate the expression of the gene encoding for IL-22. Despite the absence of significant changes in Il22 and *Reg3g* expression in the proximal colon of mice treated with BIO5768, we observed a positive correlation between *Il22* and *Reg3g* expression (Fig. 3c). This result suggested to us that BIO5768 may at least modestly regulate the activity of ILC3 cells. While we observed similar frequency of IL-22-producing Natural Cytotoxicity Receptor positive (NCR+) ILC3 cells in the mesenteric lymph nodes (MLN) of treated and untreated mice (Fig. 3d), an increased expression of IL-22 within the MLN of mice supplemented by BIO5768 was observed in the NCR^- ILC3 (Fig. 3e), known to initiates IL-17 production upon IL-23 stimulation [20]. However, the BIO5768 was not able to expand the number of regulatory T cells, notably $CD4^+$ $CD25^+$ $FoxP3^+$ (data not shown). Dendritic cells (DCs) are professional antigen presenting cells that

are playing a key role in induction of cellular immunity and polarization of T helper cells. To test the capacity of BIO5768 to induce DC maturation, bone marrow derived DCs (BMDCs) were stimulated by BIO5768 for 24 hours and cell surface DC activation markers were analyzed by flow cytometry. BIO5768 significantly increased cell surface presence of MHCII ($p < 0.01$), CD40 ($p < 0.05$) and CD86 ($p < 0.01$), demonstrating a strong effect of BIO5768 on the DC maturation (Fig. 3f).

The anti-microbial abilities of L. acidophilus BIO5768 in vivo is independent of NOD2 and IL-17.

To test the possible dependence of NOD2 and IL-17 signaling on the antimicrobial abilities of BIO5768, germ-free (GF) mice deficient for Nod2 (*Nod2*$^{-/-}$), for the receptor-Interacting Protein 2 (*Rip2*$^{-/-}$) and for IL-17 Receptor A (*IL17ra*$^{-/-}$) were mono-colonized by BIO5768. Thirty days after mono-colonisation, no significant differences on gene expression of *Defb2, Ang4, Reg3g, Il22, Il10 and Il17a* was observed in the colon of the mono-colonized deficient mice, as compared to similarly treated wild type (WT) GF mice, as shown by Fig. 4a. While we observed an enhanced production of IL-22 by either natural cytotoxicity receptor positive or negative subsets of ILC3, the production of IL-22 by total CD4^{+} was also significantly increased in the large intestine of mice supplemented by BIO5768 (Fig. 4b). By contrast, such differences were not observed in the MLN from those mice (data not shown).

The mixture of BIO5768 with two other strains alleviated inflammation in the C. rodentium infection model.

We evaluated the capacity of *L. acidophilus* BIO5768 in combination (referred as mixture) with the two other strains *B. animalis* spp. *lactis* BIO5764 and *Li. reuteri* BIO5454 that were previously shown to limit the severity of colitis caused by *C. rodentium* [18]. Mice were treated orally for five consecutive days by the mixture prior to *C. rodentium* infection. Similarly to single strain application (for BIO5764 and BIO5454 see reference [18] and for BIO5768 see Fig. 2a), the mixture had no effect on the burden of *C. rodentium* (Fig. 5a) nor on the colon length (data not shown). However, the mixture supplementation was able to significantly downregulate the gene expression of *Il1b*, while having moderate effect on *Cxcl2*, *Tnfa* and *Il6* (Fig. 5b).

The mixture increased antimicrobial peptide expression and IL-22 secretion by innate lymphoid cells type 3.

The potential of the mixture to induce *in vivo* expression of antimicrobial peptides was compared to the impact of individual administration of BIO5768. Supplementation by the mixture to naïve mice induced the expression of *Defb2*, *Defa4*, *Reg3g* and *Il22*, but it was only significant for *Reg3g* (Fig. 6a). Interestingly, the effect of the mixture was higher than BIO5768 alone for *Reg3b* and *Defa4*, while the *Il22* gene expression was higher for BIO5768 alone ($p < 0.01$). The capacity of the mixture to promote IL-22 secretion by ILC3 isolated from MLN was analyzed by multiparameter flow cytometry. The mixture significantly elevated IL-22 production by all three ILC3 subpopulations, both NCR^{+} and NCR^{-} ILC3 as well as lymphoid tissue inducer cells (LTi) (Fig. 6b). Supplementation by the mixture also promoted IL-

17A$^+$RORgt$^+$ CD4$^+$ T cells (Fig. 6c), whereas BIO5768 failed to do so (data not shown). As what observed with BIO5768 alone, the mixture was not able to increase significantly the abundance of CD4$^+$ CD25$^+$ FoxP3$^+$ Tregs in MLN (data not shown), to the same extent as previously observed with BIO5464 [18].

Discussion

Crohn's disease is associated with multiple pathogenic factors including genetic polymorphisms, gut microbiota dysbiosis and broadly dysregulated adaptive immune and antimicrobial responses. Therefore probiotics able to dampen disease severity and to restore the composition of the gut microbiota are promising safe alternatives in IBD. The potential of bacterial strains to limit the severity of inflammation and the degree of intestinal damage in chemically induced colitis, has been largely reported [21–23]. Similarly, in several documented studies, different strains of lactobacilli and bifidobacteria were able to alleviate detrimental inflammatory responses in a murine model of infectious colitis induced by *C. rodentium* [24, 25]. On the contrary, Kennedy *et al*. observed no effect of *L. plantarum* 299 on TNBS-induced colitis in rat experimental model highlighting the strain-dependent effect of probiotics in different experimental models [26]. It is important to emphasize that probiotics may mediate their beneficial effect in a strain-dependent manner via distinct signaling pathways that overall remain poorly studied.

We previously reported that the anti-inflammatory capacity of selected lactobacilli relied on an intact NOD2 signaling [14]. Genome-wide association studies demonstrated that *NOD2* is the most important genetic factor linked to ileal CD. Indeed, over 30 percent of CD patients present loss-of-function polymorphisms in NOD2. NOD2 is involved in the sensing of bacteria and in the secretion of antimicrobial peptides (AMPs), making it a key player in innate and adaptive immune responses and regulation of the gut microbiota. The NOD2-dependent beneficial effect of probiotics could explain the failure of clinical trials using probiotics for CD patients, as compared to successful results observed in ulcerative colitis [10, 13]. Therefore the selection of probiotic strains able to exhibit protective effects in a NOD2-independent manner is relevant to alleviate CD outcome in patients carrying *NOD2* mutations.

L. acidophilus is one of the main commercial species of lactic acid bacteria available in different types of dairy products or dietary supplements with claimed probiotic effects [27]. In this report, we highlighted the capacity of the strain *L. acidophilus* BIO5768 to improve the severity of inflammation in two experimental models of colitis, TNBS induced colitis and transient colitis caused by the pathogenic bacterium *C. rodentium*. Similarly, *L. acidophilus* NCFM was shown to be effective in inhibiting colitis induced by *C. rodentium* [28]. Neonatal and adulthood supplementation of mice by *L. acidophilus* also limited the severity of pathology associated with the presence of *C. rodentium* [12, 29]. The strain-dependent effect among probiotic species is well known, and differences among *L. acidophilus* strains in improving intestinal epithelial barrier function was reported recently [30]. Notably, the strain of *L. acidophilus* LA1 uniquely enhanced intestinal tight junction [31] barrier function in a TLR2 dependent manner [30]. *L. acidophilus* was also reported to exhibit antioxidant and anti-inflammatory potential in an experimental model of arthritis[32]. The protective effect of *L. acidophilus* against experimental colitis was also shown

to be dose-dependent, emphasizing the importance of selecting an optimal dosing regimen [33]. A recent report also indicated that the anti-inflammatory abilities of *L. acidophilus* LA-5 depend on the matrix in which the bacterium is delivered (capsules versus yogurt) [34].

Since impaired production of AMPs in patients suffering from CD is a largely reported issue, the capacity of probiotics to promote host defence is highly desirable. We showed both *in vitro* using a murine cell line mimicking Paneth´s cells (m-ICcl2), and *in vivo* using naïve mice, that the BIO5768 strain was able to upregulate the expression of some beta-defensins and the production of IL-22. IL-22 is produced by different types of immune cells, notably ILC3 and other immune cells such as Th17, Th22, natural killer cells, γδT cells and lymphoid tissue inducer (LTi). This cytokine is important in maintaining the integrity of the epithelial barrier [31, 35, 36]. Several reports highlighted promising potential of distinct bacterial strains to renew AMP production both *in vitro* and *in vivo* [37]. Different *Lactobacillus species* and especially *Li. reuteri* were also shown to activate IL-22 production by ILC3 [38, 39]. ILC3 is a heterogeneous population consisting of three subpopulations: NCR^+ ILC3, NCR^- ILC3 and LTi. During the early postnatal period when the gut associated lymphoid tissue is developing, LTi regulates the composition of the gut microbiota and contributes to the establishment of bacterial tolerance. In adults, LTi are important for the renewal of damaged gut lymphoid tissue suggesting their critical role in IBD. Since their function is impaired in CD patients [40], ILC3 has become a potential target of novel therapeutic approaches [31, 41]. Under normal conditions, ILC3 is induced by bacterial metabolites such as SCFA or tryptophan metabolites. Recently, a protective effect of *Li. reuteri* D8 on the epithelial barrier has been documented *in vitro* using co-cultured system with *lamina propria* lymphocytes (LPLs) in an organoid model [42]. The authors demonstrated that the indole-3-aldehyde produced by the strain stimulated LPLs to produce IL-22 through Aryl hydrocarbon Receptor 43 43 and subsequently phosphorylation of signal transducer and activator of transcription 3 (STAT3), thus accelerating the proliferation of epithelial cells and the recovering of damaged intestinal mucosa in a dextran sodium sulfate (DSS)-induced colitis model [42]. Other studies have reported that supplementation with three *Lactobacillus* strains with high tryptophan-metabolizing activities were able to restore intestinal IL-22 production [44, 45]. Lactobacilli were also reported to maintain healthy gut mucosa by producing L-ornithine able to increase the level of AhR ligand, L-kynurenine, upon tryptophan metabolism in the gut epithelial cells, therefore increasing the expansion of $ROR\gamma t^+$ $IL\text{-}22^+$ ILC3 cells [46]. Here we confirmed that BIO5768 was sufficient to increase IL-22 production by different subsets of ILCs and $CD4^+$ T cells. RORγt is involved in Th17 cell development, which produces the key effector cytokine IL-17, playing a dual role in IBD. Pro-inflammatory immune responses mediated by IL-17 are known to contribute to the pathology of IBD on the one hand but on the other hand, mice with impaired IL-17 signaling typically present a worse course of colitis [47, 48]. Blocking IL-17 activity also worsen gut inflammation [49]. T_H17 cells have been shown to be capable of regulatory functions and to be crucial in maintaining mucosal immunity against specific pathogens by promoting mucosal barrier repair through the stimulation of epithelial cells and tight junction protein, as well as the induction of antimicrobial peptides[50]. Other studies also suggest that IL-17 might be a novel class of cytokines which possesses both pro- and anti-inflammatory abilities, suggesting its critical role in setting

and maintaining the gut homeostasis either by promoting barrier function [51] or dampening expression of RANTES [52]. Pleiotropism in T_h17-associated responses may be attributed to IL-22. IL-22 was indeed shown to be secreted abundantly by T_h17 cells [53]. Interestingly, *L. acidophilus* was reported to suppress the activation of the IL-23/Th17 axis associated with DSS-induced colitis [54]. In the present study, we showed that the *L. acidophilus* BIO5768 strain had a capacity to significantly increase IL-22 production together while limiting the severity of inflammation in TNBS and *C. rodentium* experimental models of colitis.

Importantly, the expression of transcripts encoding for AMPs and IL-22 was not different in BIO5768 mono-associated germ-free mice deficient for Nod2 (*Nod2*$^{-/-}$) and IL-17 (*Il17ra*$^{-/-}$) signaling. This led us to provide evidence that the selected strain BIO5768 triggers NOD2-independent AMP expression. Since between 30% and 50% of CD patients in the Western countries carry loss-of-function *Nod2* polymorphisms [55], this potential probiotic strain could therefore be an interesting candidate for further testing in preclinical models of chronic colitis, especially in models exhibiting NOD2 deficiency, in order to confirm the efficacy of the strain to treat patients with NOD2 polymorphisms.

Many studies reported improved performance of probiotic mixtures, compared to individual strains. A mixture containing *L. acidophilus* was shown to alleviate DSS-induced colitis, notably by increasing the expressions of TJs and by upregulating the number of Tregs [56]. To ensure that probiotic supplementation could indeed trigger multiple protective signaling pathways, including the capacity to promote secretion of IL-22, we evaluated the capacity of the BIO5768 strain in combination with the two other strains *B. animalis* spp. *lactis* BIO5764 and *Li. reuteri* BIO5454, previously reported to exhibit anti-inflammatory abilities in experimentally induced colitis, albeit with different modes of action [18]. Indeed, *in vitro*, strain BIO5454 efficiently triggered IL-22 secretion by ILC3 and $CD4^+$ T lymphocytes and NOD2-independent AMP expression. Likewise, BIO5764 did not require an intact NOD2-dependent signaling pathway for regulating *Defb2* and IL-22 [18]. In the *C. rodentium* infectious colitis model, the mixture with the three strains was able to downregulate the expression of inflammatory genes, in a similar manner as the BIO5768 strain alone. The mixture also promoted the expression of antimicrobial peptides in naïve mice, with the level of *Reg3g* and *Defa4* being higher than with strain BIO5768 alone, although it was lower for *Il22* and *Defb2*. Importantly, the mixture increased IL-22 and IL-17A secretion by different ILC3 subsets and by Th17, respectively. The propensity of the mixture to induce cytokines with the capacity to trigger expression of antimicrobial peptides is of high importance in the patients suffering from CD.

In conclusion, we highlighted the health beneficial abilities of *L. acidophilus* strain BIO5768 to counteract the severity of inflammation in two experimental models of colitis. The beneficial effect of this bacterium was partly mediated by its capacity to trigger antimicrobial responses and to promote the functional activity of ILC3. Interestingly, the antimicrobial capacity of this strain was shown to be NOD2-independent which could be of great interest for the treatment of CD patients with loss-of-function NOD2 polymorphisms. Combining of this strain with two other dairy strains maintained not only the anti-inflammatory and antimicrobial potential, but also triggered a Th17 response. Further studies are

warranted to clarify the mode of action of *L. acidophilus* or the mixture, notably if the protective effects are indeed NOD2-independent *in vivo*. Our results suggest that the selected strains could provide an interesting complementary therapy in maintaining remission and improving the quality of life of patients. It remains necessary to investigate their clinical efficacy and the safety of the strains, however.

Materials And Methods

Bacterial strains. The three bacterial strains were selected from the Bioprox collection: *Limosilactobacillus reuteri* BIO5454 (BIO5454), *Lactobacillus acidophilus* BIO5768 (BIO5768), *Bifidobacterium animalis* spp. *lactis* BIO5764 (BIO5764). Lactobacilli were grown at 37°C in MRS broth (Difco, Detroit, USA). Bifidobacteria were cultured in MRS media supplemented with cysteine (0.5 µg/ml) under anaerobic condition. Bacteria were grown overnight, harvested by centrifugation (10min at 4000 × g), washed twice with PBS buffer (pH 7.2). For *in vitro* stimulation, bacteria were resuspended in PBS containing 20% (v/v) glycerol to a final concentration of 2×10^9 CFU/ml and stored at − 80°C until used. For *in vivo* administration, fresh cultured bacteria were resuspended in PBS at 2.5 x 10^9 CFU/ml and were intragastrically administrated to mice (5×10^8 CFU in 200 µl) as described previously [18].

Mice. C57BL/6 and BALB/c female mice were purchased from Charles River (L´Arbresle, France) and were housed in specific pathogen-free condition in the animal facilities of the Institut Pasteur de Lille (accredited No. C59-350009). 7–8 week old mice were maintained in a temperature-controlled (20 ± 2°C) facility with a strict 12-hour dark/light cycle. Animal experiments were performed in compliance with European guidelines of laboratory animal care (number 86/609/CEE) and with French legislation (Government Act 87–848). The study was carried out in compliance with the ARRIVE guideline and was approved by local Animal Ethics Committees (Nord-Pas-de-Calais CEEA N_75, Lille, France) and the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche, France (accredited No. 201608251651940). For *Citrobacter rodentium* infection, experiments were performed in the biosafety level 2 facility as described previously [18]. Before experimentation, animals were provided a one-week acclimation period and were given *ad libitum* access to regular mouse chow and water. GF that are deficient or not for Nod2 (*Nod2*$^{-/-}$), for the receptor Interacting Protein 2 (*Rip2*$^{-/-}$), and for IL-17 Receptor A (*Il17ra*$^{-/-}$) on a C57BL/6 background were generated at TAMM/CNRS Orleans (TAAM agreement number: D-45-234-6) and were bred in isolators under strict GF conditions. Monocolonisation experiments were approved by national and local Animal Ethics Committees authorization number 1038.

Preparation of m-ICc12 epithelial cell monolayers and antimicrobial peptides induction. The murine intestinal crypt cell line m-ICc12 [19] (kindly provided by A. Vandewalle, Inserm U246, Paris, France) was maintained at 37°C, 5% CO_2 in HAMF'12/DMEM (50/50 v/v, Gibco, NY, USA) supplemented by the following reagents: insulin (5 µg/ml), dexamethasone (50 nM), selenium (60 nM), transferrin (5 µg/ml), triiodothyronine (1 nM), Hepes 20mM, glutamine 2 mM, D-glucose (0.22%), fetal calf serum (FCS, 2%) and gentamycin (50 µg/ml), all purchased from Sigma-Aldrich (Saint Louis, MO, USA), and mouse epidermal growth factor (10 ng/ml) (Peprotech, Neuilly-Sur-Seine, France). Cells were differentiated until 10 days-

culture by changing the medium every two days. On day 11, cells were stimulated with the bacteria (bacteria/cell ratio 10:1) for 4 hours. After stimulation, RNA was extracted from adherent cells using the Macherey-Nagel NucleoSpin RNA II isolation kit (Düren, Germany) and stored at -80°C until used for further gene expression quantification.

Preparation of Bone Marrow Derived Dendritic Cells (BMDC). BMDC were generated from bone marrow progenitor cells as described previously [57]. Briefly, progenitor cells were obtained by flushing tibia and femur from BALB/c mice and cultured at 37°C under 5% CO_2 in Iscove's modified Dulbecco's media supplemented by FCS (10%), gentamycin (50 µg/ml), glutamine (2 mM), β-mercaptoethanol (50 µM) and 10% of supernatant from a granulocyte-macrophage colony-stimulating factor-expressing cell line (GM-CSF transfected J588 myeloma cell line) for 10 days. On day 10, cells were stimulated by bacteria (ratio bacteria/cell: 10:1). After 24 hours stimulation, BMDC were harvested, washed and stained using mAbs anti-CD11c PE-Cy7, CD40 PE, CD80 FITC, CD86 PE-Cy5, MHCII APC (eBioscience, San Diego, CA, USA) and acquired on BD FACS Canto II (Becton Dickinson).

Preclinical model of TNBS-induced colitis. TNBS-induced murine model of acute colitis [58] was performed using BALB/c mice as described previously [18]. Briefly, anesthetized mice received an intra-rectal administration of TNBS (Sigma-Aldrich Chemical, France; 110 mg/kg) dissolved in 0.9 % NaCl/ethanol (50/50 v/v). The protective effect of the probiotic strain was evaluated by oral administration (intragastric feeding) of bacteria ($5x10^8$ CFU/ mice) starting 5 days before colitis induction. Forty eight hours after colitis induction, mice were sacrificed and the severity of colitis was graded according to the macroscopic inflammation based on Wallace scoring method [59]. Histological analysis was performed on May-Grünwald-Giemsa stained 5 µm tissue sections from colon samples and inflammation was graded according to Ameho score. Immediately after sacrifice, colonic samples were taken and stored in RNAlater storage solution (Ambion, Austin, TX, USA) at -80°C until further processed.

Preclinical model of infectious colitis. *Citrobacter rodentium* infection was performed using the kanamycin resistant DBS 120K strain as described previously [18]. Briefly, a single colony of *C. rodentium* was cultured overnight in Luria Bertani broth containing 50 µg/ml kanamycin, under agitation. Bacteria suspension was centrifuged, washed, resuspended in PBS and adjusted to $5x10^9$ CFU/ml. Mice were infected by oral administration of *C. rodentium* (10^9 CFU per mice). The potential capacity of the selected probiotic strains to limit inflammation caused by *C. rodentium* was evaluated upon intragastric administration of the bacteria or the mixture ($5x10^8$ CFU per mice, all strains were present in equal amount in the mixture) 5 days prior *C. rodentium* infection and daily following infection until termination of the experiment. Mice were sacrificed 9 days after infection. Level of infection was monitored as described previously [18]. Histological analyses were performed on May-Grünwald-Giemsa stained 5 µm tissue sections from colon samples fixed in 10% formalin and embedded in paraffin and crypt length was measured using ZEN (Zeiss, Oberkochen, Germany). Immediately after sacrifice, proximal and distal colon segments were put in RNAlater® (Ambion, Life Technologies, Foster City, CA, USA) and frozen at -80°C until RNA extraction and qRT-PCR analysis.

RNA extraction and analysis of gene expression using quantitative real-time polymerase chain reaction (qRT-PCR). Colon samples were removed at sacrifice and stored in RNAlater® storage solution (Ambion, Life Technologies, Foster City, CA, USA) at − 80°C until qRT-PCR analysis. Tissue samples were homogenized using Lysing Matrix D (MPbio, Eschwege, Germany) and total RNA from samples was extracted as described previously [18]. Briefly, Macherey-Nagel NucleoSpin RNAII isolation kit (Düren, Germany) was used for RNA extraction according to the manufacturer's recommendation. RNA quantity and quality were checked by Nanodrop (260/280 nm, 260/230 nm) and 1 µg RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Woolston Warrington, UK). Quantitative RT-PCR (qRT-PCR) was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) on ViiA 7 Real-Time PCR System (Applied Biosystems). Primers sequences can be available upon request. Results are expressed as relative gene expressions ($2^{-\Delta\Delta ct}$) values, by comparing the PCR cycle thresholds (Ct) for the gene of interest and for the house keeping gene β-actin (*bact*) (or Glyceraldehyde-3-Phosphate Dehydrogenase, *Gapdh* for mono-associated mice*)* as described previously [60].

Supplementation of naive mice and characterization of IL-22 and IL-17-producing cells and regulatory T cells in mesenteric lymph nodes by flow cytometry. Strains (5×10^8 cfu/day/mice) were administered by intragastric gavage to WT conventional BALB/c mice for 5 days. Colon samples were removed at sacrifice and stored in RNAlater® storage solution (Ambion, Life Technologies, Foster City, CA, USA) at − 80°C until qRT-PCR analysis. MLN were harvested and immediately processed for flow cytometry.

Cell suspensions of MLN (3-5x10^6 cells, in RPMI1640 supplemented by 10% FCS, 2mM L-glutamine, 2mM HEPES, 40 mg/ml gentamycine) were stimulated using the Leukocyte Activation Cocktail containing BD GolgiPlug (BD Biosciences) (1 µl/ml of cell suspension) for 5 hours. Cells were stained by mAbs anti-mouse CD11c eFluor450, CD11b eFluor 450, B220 eFluor 450, CD3 eFluor 450, CD117 Alexa Fluor 700, NK1.1 PerCP-Cy5.5, NKp46 FITC (provided by eBioscience), CD4 APC-H7, CD90.2 BV 500; CD45RB BV 605, MHCII BV 650 (provided by BD Bioscience, san Jose, CA, USA). Subsequently, cells were permeabilized and fixed using the Transcription Factor Buffer Set (BD Bioscience), and intracellular staining was performed using mAbs anti-IL-22 PE and RORgt APC (eBioscience).

Monocolonisation of axenic mice. GF WT and *Nod2*$^{-/-}$, *Rip2*$^{-/-}$, and *Il17ra*$^{-/-}$ mice (9–13 weeks old, C57BL/6 background) were mono-associated with the BIO5768 strain by a single intragastric gavage (5x10^8 CFU/ mice). After 30 days of mono-association, colon samples were removed at sacrifice and stored in RNAlater® storage solution (Ambion, Life Technologies, Foster City, CA, USA) at -80°C until qRT-PCR analysis was performed. Cell suspensions of colon and caecum from monocolonized mice were performed as described previously [61]. Cells (1x10^5 cells) were stained by mAbs anti-mouse CD11c FITC (HL3 clone), CD11b FITC (M1/70 clone), B220 FITC (RA3-6B2 clone), CD3 FITC (145-2C11 clone), NK1.1 FITC (PK136 clone), CD4 V500/ Amcyan (RM4-5 clone), CD117 APC-H7 (2B8 clone), NKp46 APC/eFluor 660 (29A1.4 clone), Il7ra V450 / PB (SB/199 clone), CD90.2 PE-Cy7 (53 − 2.1 clone). Mouse IgG2a, k (G155-178 clone) and Goat IgG (Poly5164 clone) were used as isotype controls. Subsequently, cells were

permeabilized and fixed using the Transcription Factor Buffer Set (BD Bioscience) and intracellular staining was performed using mAbs anti-IL-22 PE (1H8PWSR clone) and RORgt PerCP-Cy5.5 (Q31-378 clone).

Statistics. GraphPad Prism was employed for graph preparation and statistical evaluation. Statistical significance was determined using either non-parametric Mann Whitney test, non-parametric one-way analysis of variance (ANOVA) followed by Dunn multiple comparison posthoc test and non-parametric two-way ANOVA with Bonferroni post-tests (GraphPad Prism software). Data with p values ≤ 0.05 were considered to be significant. The Spearman correlation coefficient was used to analyze correlation between *Il22* and *Reg3g* expression.

Declarations

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Author contributions. Jiří Hrdý and Aurélie Couturier-Maillard performed most of the experiments. Jiří Hrdý, Denise Boutillier and Aurélie Couturier-Maillard did histology, qRT-PCR, Mathias Chamaillard performed statistical analysis. Aurélie Couturier-Maillard and Bernhard Ryffel contributed to studies on germ-free mice and monocolonized animals. Jiří Hrdý, Aurélie Couturier-Maillard, Corinne Grangette and Denise Boutillier performed the experiments on Citrobacter infection and Denise Boutillier and Corinne Grangette the bacterial culture and TNBS colitis experiments. Jiří Hrdý, Corinne Grangette, Mathias Chamaillard, Aurélie Couturier-Maillard and Bernhard Ryffel analyzed the data and wrote the manuscript. Carmen Lapadatescu and Philippe Blanc provided the strains. Bruno Pot, Philippe Blanc and Carmen Lapadatescu participated to the writing of the manuscript and scientific discussions. Corinne Grangette, Bernhard Ryffel Mathias Chamaillard conceived and supervised the study.

Conflict of interest statement. Carmen Lapadatescu was employed full time by the company Bioprox. Philippe Blanc is employed full time by the company Bioprox. Bruno Pot is now part-time employed by Yakult Europe. The other authors have no any conflict of interest to declare.

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Figures

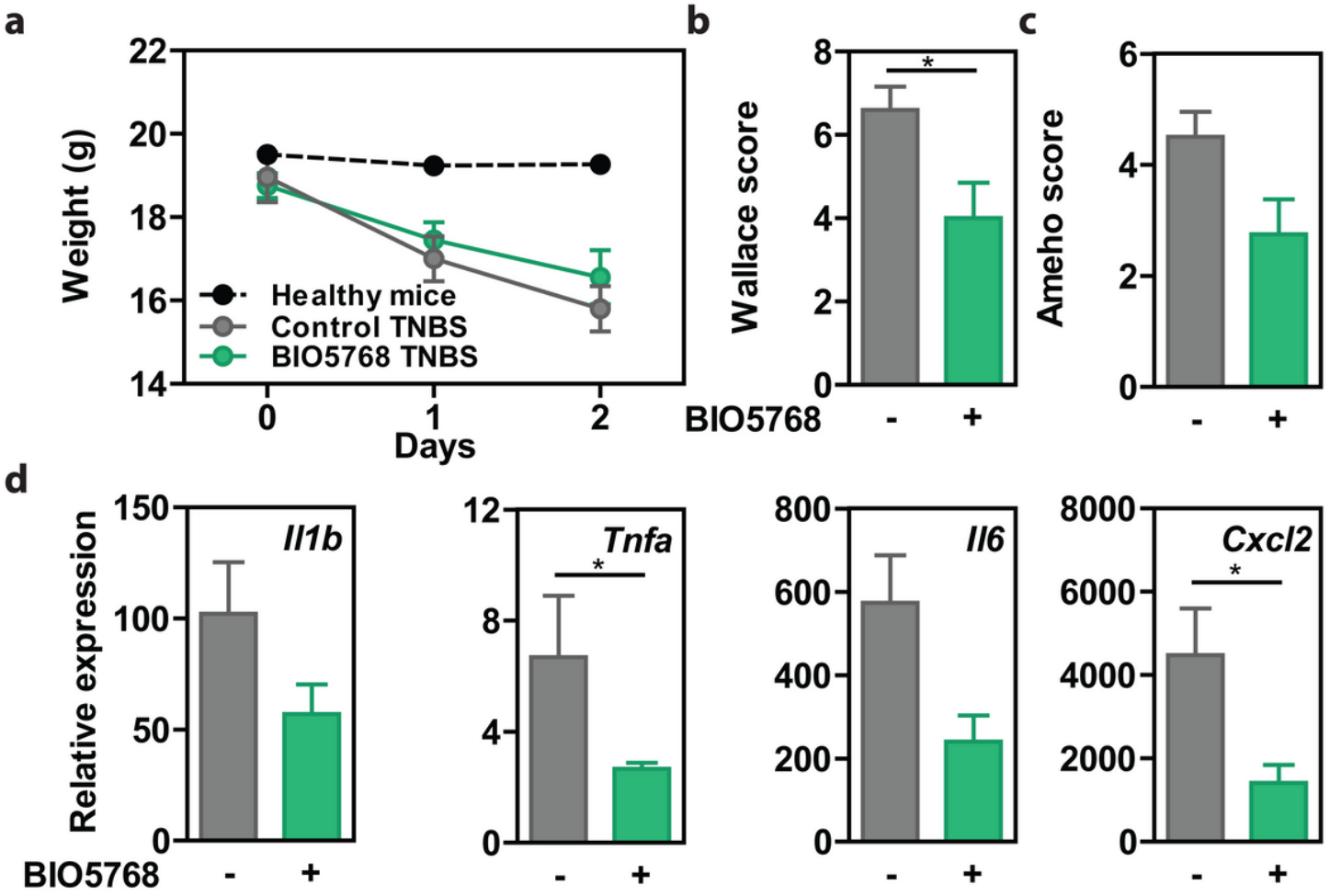

Figure 1

Protective effect of L. acidophilus BIO5768 supplementation in a mouse model of TNBS-induced colitis. BIO5768 (5x108 CFU of each) was administered to BALB/c mice for 5 consecutive days before and 1 day after TNBS induction. After 48 h, mice were sacrificed and the impact of BIO5768 was analyzed on a) weight loss, b) macroscopic grading of inflammation according to Wallace score, c) histological analyses of colon tissue according to Ameho score and d) gene expression of pro-inflammatory markers. Bars represent means ± SEM of 10 mice per group. *p < 0.05.

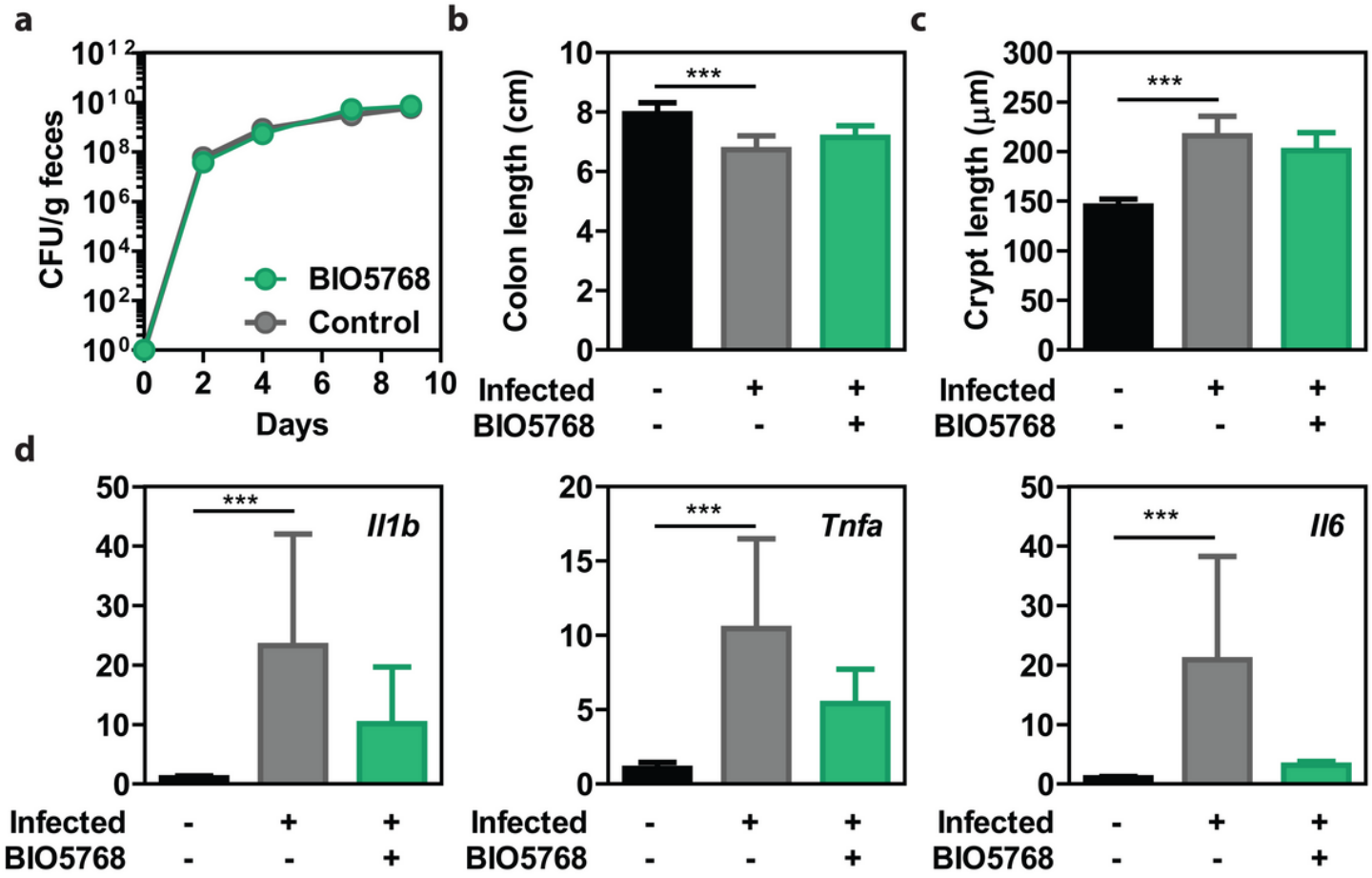

Figure 2

Impact of L. acidophilus BIO5768 supplementation on the course of colitis in an experimental mouse model of C. rodentium infection. BIO5768 (5x108 CFU) was daily administered to C57BL/6J mice 5 days prior to infection and after the infection until the day of sacrifice. Mice were orally inoculated with 1×109 CFU C. rodentium and sacrificed 9 days after infection. a) Effect of BIO5768 on bacterial load of C. rodentium determined by plating over the course of the experiment, fecal samples dilution on Luria Bertani medium containing 50 µg/ml kanamycin and compared with control infected mice. b) Effect of BIO5768 supplementation on the colon length and c) crypt length. d) Pro-inflammatory genes expression analyzed by qRT-PCR. Results are expressed as means ± SEM. *** $p < 0.001$.

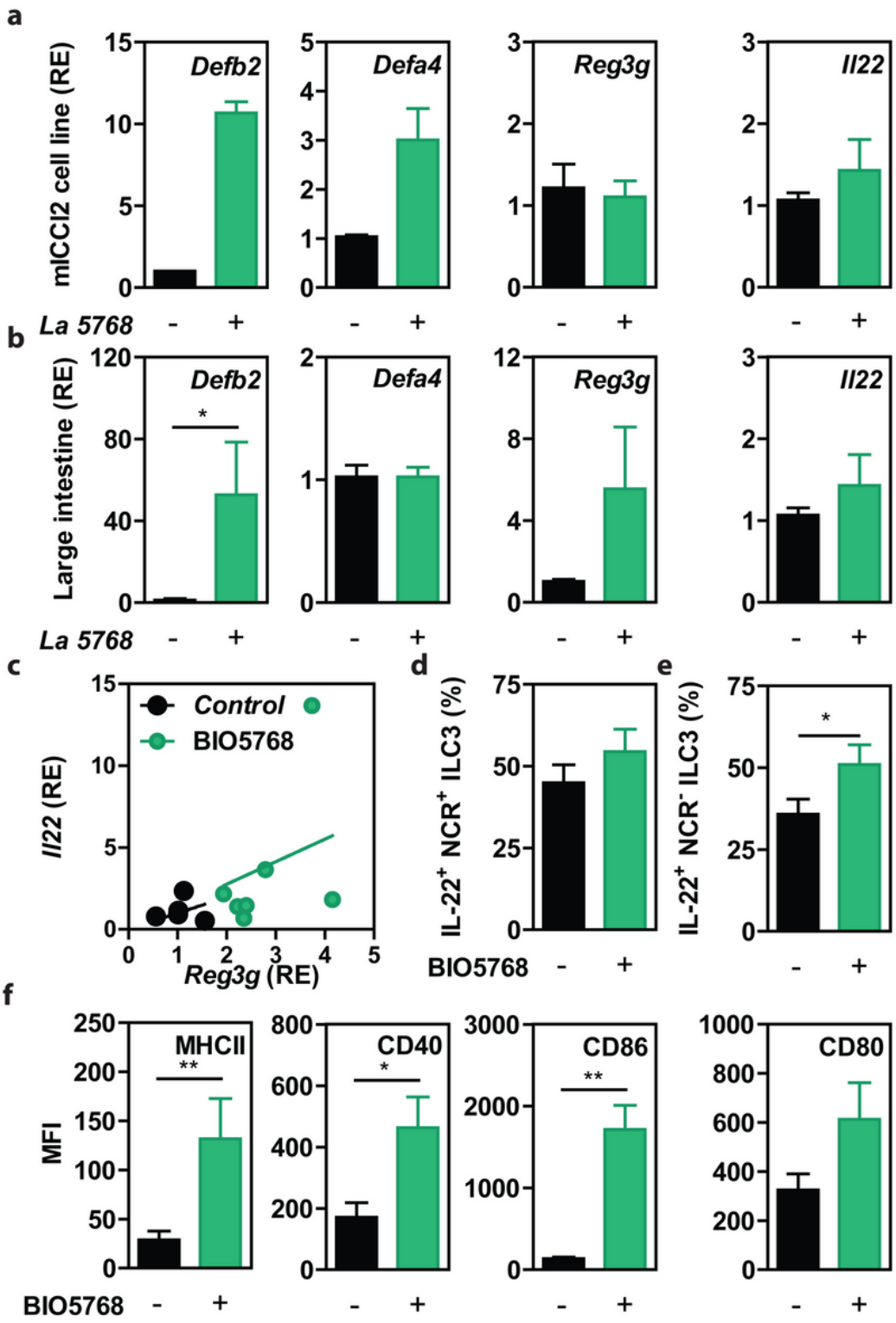

Figure 3

Capacity of the L. acidophilus BIO5768 to induce gene expression of antimicrobial peptides in vitro and in vivo, to induce IL-22 –producing cells in vivo and to activate bone marrow derived dendritic cells (BMDC) in vitro. a) Gene expression of defb2, defa4, reg3g and Il22 after 4h in vitro stimulation (or not) of mICCl2 cell line by the strain BIO5768, analyzed by qRT-PCR. Results represent the mean of three independent experiments. b) Gene expression of defb2, defa4, reg3g and Il22 in the proximal colon of BALB/c naive

mice after 5 days of BIO5768 supplementation (or not), determined by qRT-PCR. c) Correlation between Il22 and reg3g expression. Impact of BIO5768 supplementation on the percentage of d) ILC3 and e) CD4+ producing IL-22, determined by flow cytometry in MLN. f) Capacity of BIO5768 to induce BMDC maturation analyzed by flow cytometry according to the cell surface presence of activation markers MHCII, CD40, CD86, and CD80. Representative results of 6 independent experiments are shown (bars represent mean ± SEM). MFI - Mean of Fluorescence Intensity); RE – Relative Expression; * $p < 0.05$; ** $p < 0.01$.

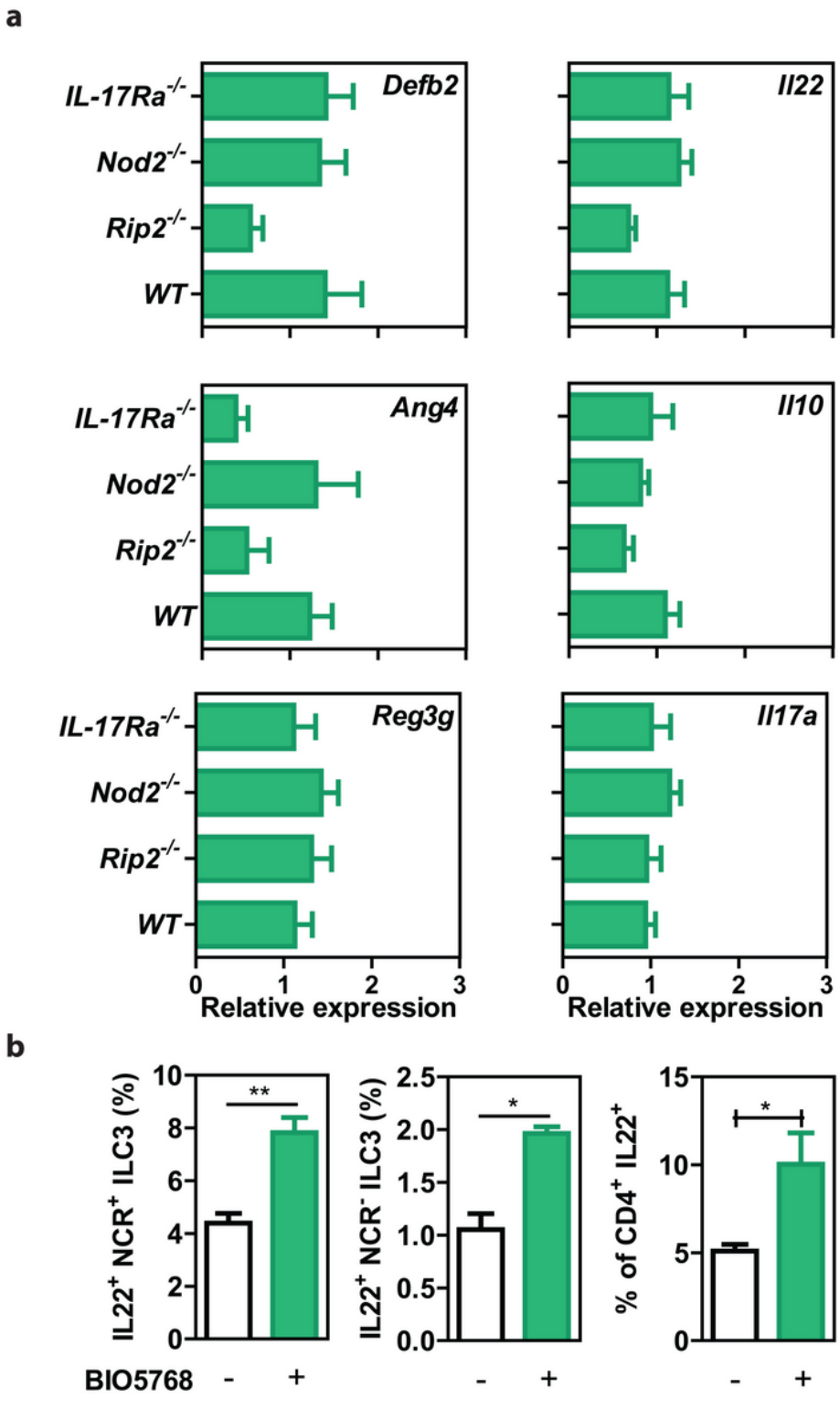

Figure 4

The anti-microbial abilities of L. acidophilus BIO5768 in vivo is independent of NOD2 and IL-17 signaling. Germ-free female C57BL/6 mice deficient for NOD2 (Nod2-/-), RIP2 (Rip2-/-), and IL-17 Receptor A (Il17Ra-/-) were mono-colonized with strain BIO5768 by a single administration (5x108 CFU/ mice) and compared to monocolonized GF WT mice (n=6 mice per group). After 30 days mono-association, no significant difference on gene expression of defb2, ang4, reg3g, IL-22, il10 and IL17a induced by BIO5768 was observed in mono-colonized deficient mice compared to mono-colonized WT mice. Values are expressed as the relative mRNA levels of mono-colonized deficient mice compared with mono-colonized WT mice and expressed as means ± SEM. b) Impact of BIO5768 supplementation on the percentage of cells producing IL-22, determined by flow cytometry in colon and caecum. NCR natural cytotoxicity receptor. Data represent means values of each group (n = 8 mice) ± SEM. * refers to the comparison of BIO5768-mon-colonized mice versus untreated mice. .* p < 0.05; **p < 0.01

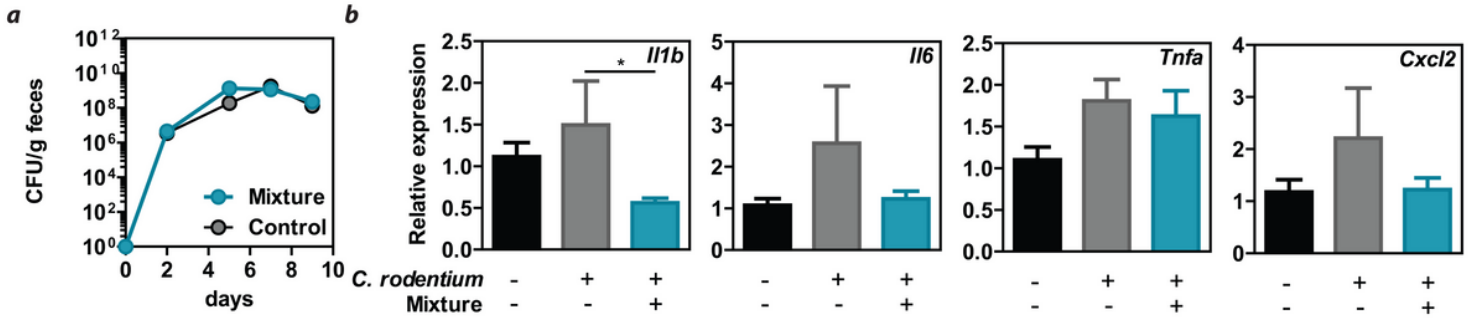

Figure 5

Capacity of the mixture to limit Citrobacter rodentium-induced colitis in C57BL/6J mice. Mixture of three bacterial strains (5x108 CFU/ day / mice, all strains were present in equal amount in the mixture) was administered by intragastric gavage for 5 days. a) Effect of mixture supplementation on C. rodentium burden, and b) gene expression of cxcl2, Il6, Il1b and tnfa in proximal colon evaluated by qRT-PCR. Values are expressed as the relative mRNA levels compared with samples from untreated mice and represent a mean of 10 mice per group ± SEM. * p < 0.05 **p < 0.01.

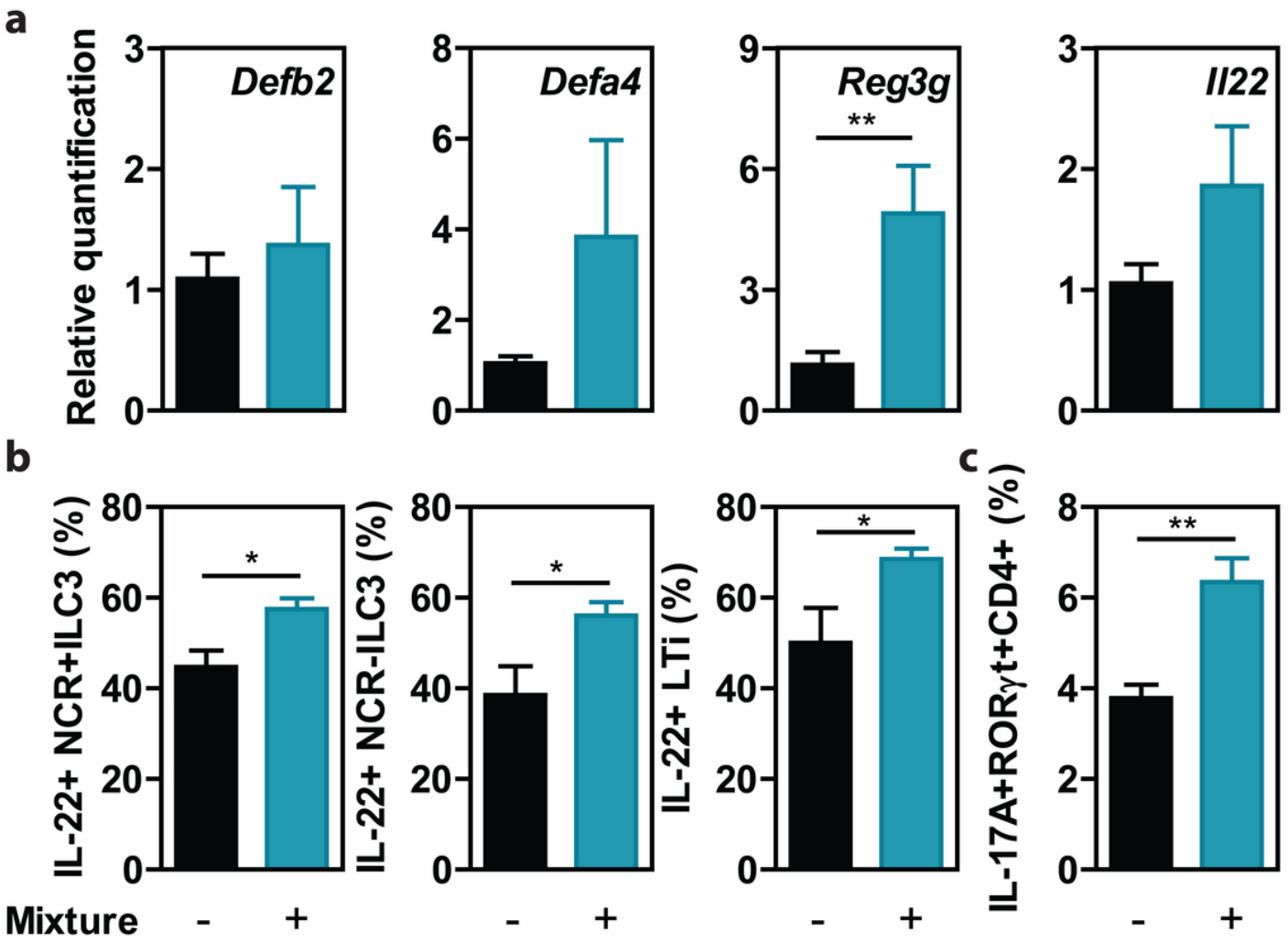

Figure 6

Effect of L. acidophilus BIO5768 and the mixture on antimicrobial peptides gene expression and impact of the mixture on IL-22 production by innate lymphoid cells type 3 (ILC3) and expansion of CD4+ CD25+ FoxP3+ regulatory T cells. L. acidophilus BIO5768 or the mixture containing the three bacterial strains (5x108 CFU/ day/ mice, all strains were present in equal amounts in the mixture) were administered by intragastric gavage for 5 days to naïve BALB/c mice. a) Gene expression of defb2, defb4 reg3g and Il22 evaluated by qRT-PCR. Impact of the mixture supplementation on b) IL-22 production by the different subsets of ILC3 expressed as % of IL-22+ cells; c) on IL-17 production by ROR+ CD4+ T cells expressed as % of IL-17A+ cells. Data represent means values of each group (n = 8 mice) ± SEM. * refers to the comparison of bacteria/mixture-treated groups versus untreated mice. NCR natural cytotoxicity receptor, LTi lymphoid tissue inducer; * $p< 0.05$; ** $p< 0.01$.

Supplementary Files

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