**Bifidobacterium lactis** attenuates onset of inflammation in a murine model of colitis

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AIM: To assess the anti-inflammatory effect of the probiotic **Bifidobacterium lactis** (*B. lactis*) in an adoptive transfer model of colitis.

METHODS: Donor and recipient mice received either *B. lactis* or bacterial culture medium as control (deMan Rogosa Sharpe) in drinking water for one week prior to transfer of a mix of naive and regulatory T cells until sacrifice.

RESULTS: All recipient mice developed signs of colonic inflammation, but a significant reduction of weight loss was observed in *B. lactis*-fed recipient mice compared to control mice. Moreover, a trend toward a diminution of mucosal thickness and attenuated epithelial damage was revealed. Colonic expression of pro-inflammatory and T cell markers was significantly reduced in *B. lactis*-fed recipient mice compared to controls. Concomitantly, forkhead box protein 3, a marker of regulatory T cells, was significantly up-regulated by *B. lactis*.

CONCLUSION: Daily oral administration of *B. lactis* was able to reduce inflammatory and T cell mediators and to promote regulatory T cells specific markers in a mouse model of colitis.

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**Key words:** Probiotics; **Bifidobacterium;** Colitis; Adoptive transfer model; Regulatory T cells; Inflammation; Mice

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INTRODUCTION

Crohn’s disease (CD) and ulcerative colitis (UC), referred to as inflammatory bowel diseases (IBDs), are chronic relapsing and remitting inflammatory diseases of the gastrointestinal tract. IBD affects people in the prime of their lives, with first diagnosis usually between the ages of 15 and 25 years, and due to the chronic nature of IBD, patients usually require lifelong treatment. More than one million people in the United States and more than four million people worldwide suffer from IBD. According to the current increase in affected people, IBD medical costs will reach approximately 2 billion euros by the end of 2010. The high cost and limited response to current thera-
pies prompt research and development of new treatment options.

IBD is thought to result from an inappropriate, overwhelming and ongoing activation of the mucosal immune system in genetically susceptible individuals driven by antigens originating from the microbiota of the gastrointestinal tract[1]. Some of the immune-related characteristics of IBD include over-activity of effector lymphocytes, induction of pro-inflammatory cytokines, and failure of regulatory T cells (Tregs) to control inflammation[2,3].

The crucial role of the intestinal microbiota in the induction and progress of disease has been validated in patients and in animal models of intestinal inflammation resembling IBD[4,5]. Indeed, it was demonstrated that intestinal inflammation could not be induced under germ-free conditions[6] and that antibiotic treatment attenuated the disease severity[7]. Interestingly, the equilibrium of the intestinal microbiota appears to be perturbed in IBD as decreased levels of Bifidobacterium and Lactobacillus strains have been described in fecal samples, whereas raised counts of Enterococcus and Bacteroides species are found in inflamed mucosa of patients[8]. Thus, as probiotics are known to have a strong homeostatic impact on the intestinal flora[9], it is valuable to consider probiotics as alternative therapies for IBD. Hence, although conflicting reports exist, a number of studies report the benefits of probiotic therapy in the alleviation of IBD[10-13]. For example, VSL#3, a mixture of four lactobacilli, three bifidobacteria and a streptococcus species, proved to be effective in inducing and maintaining remission in UC patients or in preventing pouchitis.[14-17]

While most clinical studies have relied on mixes of probiotic strains or on synbiotics to act on IBD, a few preclinical or ex-vivo studies have begun to reveal that the use of single probiotic strains, mainly consisting of Lactobacillus strains, can also be efficient in dampening inflammation[18-21].

The present study reports on the beneficial effects of the use of a single Bifidobacterium strain, Bifidobacterium lactis (B. lactis), on intestinal inflammation in a murine model of T cell-mediated colitis. This well established Th1-type cytokine-mediated hyper-response model relies on the adoptive transfer of CD4+CD45RBhigh naive T cells in immunodeficient mice[22], such as RAG2-/- mice which lack adaptive immunity[23]. This cell transfer initiates colitis pathology akin to that of humans[24], which develops as a result of the absence of suppressive regulatory cells in the recipient mice. Indeed, co-transfer of mature CD4+CD45RB+ T cells, a source of Tregs, reduces or even prevents colitis[25]. In the present work, naive T cells were adoptively transferred together with a low proportion of Tregs. This particular setting, still permitting the induction of inflammation in recipient mice, provided an interesting tool allowing the simultaneous analysis of the impact of prophylactic B. lactis administration on both T cell partners involved in the induction (naive T cells) or regulation (Tregs) of IBD pathology.

**MATERIALS AND METHODS**

**Animals**

C57BL/6j mice (8- to 12-wk-old) were purchased from Harlan (Oxon, UK). Immunodeficient RAG2-/- mice (8- to 12-wk-old[26]) were used from a colony of RAG2-/- mice maintained at the Institute for Labortierkunde, University of Zurich, Switzerland. The RAG2-/- mouse colony was derived from a colony from Bern (Switzerland) by embryo transfer under gnotobiotic conditions and recolonized with an Altered Schaedler Flora[27].

**Preparation of B. lactis and experimental design**

A freshly prepared solution containing Bifidobacteria lactis (B. animalis subsp. lactis NCC 2818) from the Nestlé Culture Collection (Nestlé Research Center, Lausanne, Switzerland) was used for this study. This strain was chosen according to in vitro anti-inflammatory properties (data not shown). Bacteria were grown for two passages under strictly anaerobic conditions in deMan Rogosa Sharpe (MRS) broth containing 0.05% cysteine (BD, Switzerland). After quantification of bacteria by serial dilution as described for fecal microbiota, 10% glycerol was added to the bacteria stock; aliquots were made and stored at -80℃ until use. Frozen bacteria were added to drinking water each day of the study at 1 × 10^9 colony-forming units (CFU)/mL leading to a dose approximating 3 × 10^8 CFU/d per mouse. Donor and recipient mice were supplemented with either probiotic or MRS control solution (both containing 10% glycerol) according to the study design (Figure 1). Note that as the settings of the present adoptive transfer model permit a focus on Tregs, probiotics were also given to donor mice in order to potentially stimulate these cells as soon as possible. Each group of recipient mice consisted of 5 mice.

**Induction of colitis in RAG2-/- mice by adoptive transfer**

CD4+ T cells from mesenteric lymph nodes (MLNs) were isolated from C57BL/6j donor mice by negative depletion using MACS technology (Miltenyi Biotec, Germany). The negative fraction, enriched for CD4+ T cells, was stained using Fluorescein isothiocyanate-conjugated CD45RB antibody (mAb) (eBioscience, San Diego, USA) and Phycoerythrin-conjugated CD4 mAb (eBioscience, San Diego, USA). Subsequently, CD4+ T cells were sorted according to the expression of CD45RB on a FACS Aria (BD; Allschwil, Switzerland). Sorted CD4+CD45RBhigh and CD4+CD45RBlow T cells were washed, resuspended at 1 × 10^6 cells/mL in sterile phosphate buffered saline and injected i.p. at the ratio of 9:1 respectively (2 × 10^6 total cells) into each of the 8- to 12-wk-old syngeneic RAG2-/- recipient mice. This 9:1 ratio was chosen because it still allowed development of colitis despite the co-injection of regulatory cells. Body weight of recipient mice was measured every three days from day 0 to day 21, and then every other day until sacrifice (day 27). Body weights were recorded as percentage of initial body weight.
Sample collection
Fresh fecal samples (approximately 0.05 g) were collected at three time-points during the study: before initiation of *B. lactis* supplementation (day -7), at the day of T cell transfer (day 0), and at the end of the study (day 27). They were collected into 0.5 ml Ringer’s solution, immediately homogenized and snap-frozen, then stored at -80°C until analysis. Blood samples were collected at the same time points as fecal samples and frozen at -80°C until needed. At the end of the experimental period, mice were sacrificed and colon was divided into three parts. The proximal and the distal parts were snap-frozen in liquid nitrogen for protein and mRNA expression analysis, respectively. A smaller sample of the middle colon was used for histopathological analysis.

Fecal microbiota
Fecal extracts were diluted in pre-reduced Ringer’s solution (0.5% cysteine) to perform serial dilutions (10^-7 to 10^-1) and then plated. Portions (100 μL) of appropriate dilutions were plated onto selective or semi-selective media. *Bifidobacteria* were counted on tomato juice agar medium. Plates for the enumeration of *Bifidobacteria* colonies were incubated at 37°C in anaerobic conditions, in a jar containing Anaerocult A tablets (Merck, Germany), for 48 h. After incubation, each plate was examined for bacterial colonies. The detection-limit for the assessed bacteria dilutions was 10^3. Bacterial counts were expressed as means log10 CFU/g feces ± SE. Detection of *B. lactis* was performed by polymerase chain reaction (PCR) using *B. lactis*-specific primers.

Histological assessment
Paraffin-embedded colonic tissue sections were scored as previously described[28], with minor modifications. Briefly, specimens of the transverse colon of each animal were collected and fixed immediately in 4% buffered paraformaldehyde for 16-24 h for subsequent preparation of paraffin-embedded tissue blocks. Tissue sections were stained with hematoxylin/cosin for subsequent histopathological assessment. Each tissue section was independently evaluated by at least two trained pathologists according to a standard evaluation sheet in a blinded fashion. Histological assessment was performed using the scoring criteria displayed in Table 1. The range of histopathological scores was from 1 (no alteration) to 16 (most severe signs of colitis).
B. lactis reduces experimental colitis

(30 V for 60 min). Western blot analysis was performed with antibodies against murine cyclooxygenase 2 (COX-2) (Cayman Chemicals, USA), p38 and phospho p38 (Cell Signaling Technology, USA), signal transducer and activator of transcription 3 (STAT-3) and phospho STAT-3 (Cell Signaling Technology, USA) and β-actin (Sigma, St. Louis, MO, USA). Secondary antibodies were from Molecular Probes (USA) or Jackson ImmunoResearch Laboratories (USA). Relative quantitation of bands was determined using the Scion Image Densitometry System (Scion Corp., USA), with all quantities normalized to expression levels of β-actin.

Immunohistochemistry for Ki-67 on formalin-fixed tissue sections: After dewaxing, tissue sections were pretreated in 10 mmol/L citrate buffer, pH 6.0, for 7 min at 1 bar/121°C. Pretreated tissue sections were subsequently incubated for 60 min with a primary rat-anti-mouse Ki-67 mAb (Clone: Tec-3; isotype: rat IgG2α; DakoCytomation, USA). After washing, sections were incubated with the biotinylated secondary reagent (rabbit-anti-rat Ig; absorbed with mouse Ig (DakoCytomation). Streptavidin-HRP complexes with 3,3’-diaminobenzidine as the chromogen were used for detection.

mRNA expression

RNA extraction of the homogenized tissues was performed using the NucleoSpin RNA II Kit (Macherey-Nagel, Germany). Total RNA was quantified using the Ribogreen RNA Quantitation Kit (Molecular Probes, USA), and the RNA quality was assessed by Agilent RNA 6000 Nano LabChip Kit (Agilent Technologies, USA). Total RNA (2 μg) was reverse transcribed using MultiScribe reverse transcriptase (Applied Biosystems, USA). Real-time PCR was carried out using custom design Low Density Array (Applied Biosystems, X, USA). The quantitative PCR (qPCR) reactions were performed with 2 ng of cDNA on ABI PRISM 7900HT qPCR machine (Applied Biosystems, USA) piloted by SDS 2.2 software (Applied Biosystems, USA). Results of target mRNA are expressed as the number of specific copies for 10^6 GAPDH mRNA molecules in the same sample.

Statistical analysis

Statistical analysis was performed using the two-tailed Mann-Whitney U test. Differences were considered statistically significant when P < 0.05. All data are shown as median ± interquartile-range (IQR) except for the body weight analysis.

RESULTS

Detection of B. lactis in fecal samples

In order to control the probiotic intake, detection of B. lactis in fecal samples was performed using PCR detection. No B. lactis was identified in the feces prior to administration of this strain in donor C57BL/6J and recipient RAG2-/- mice. All donor animals receiving the treatment were positive for B. lactis on the day of cell transfer (day 0, data not shown). In the recipient animals, supplemented with the probiotic strain, approximately 1 × 10^8 CFU of B. Lactis/g fecal content could be measured at day 0 and day 27 (sacrifice day).

Effect of B. lactis on body weight loss

As already described in other studies[29,30], control recipients of CD4+ T cells started to lose body weight from day 9 after adoptive cell transfer. Thereafter they continuously lost weight and on the day of sacrifice (day 27), their body weight was lower than on the day of initial T cell transfer (Figure 2). In contrast, B. lactis-fed recipient mice showed a marked delay of onset of body weight loss, starting only at day 18. Thereafter B. lactis-fed recipients also continued to lose weight until the day of sacrifice (day 27). At the end of the experimental period, body weight of B. lactis-fed recipients of CD4+ T cells was comparable to the initial body weight at the time of colitis induction by adoptive T cell transfer (Figure 2).

Effect of B. lactis on colon histopathology

Histopathology scores, ranging from 0 (no colitis) to 16 (most severe colitis) (Table 1), were based on the analysis of 6 criteria as described in the methodology section. At sacrifice, the total colitis scores of B. lactis-treated and -untreated recipient groups were not significantly different (data not shown). When each of the histopathology criteria was assessed individually, differences between the two groups of recipients were nonetheless observed at the level of mucosal thickness; B. lactis-treated recipient mice showing a tendency for an attenuated mucosal thickening compared to control recipient mice (Figure 3B), resulting in a lower colitis score as shown in Figure 3A.

Beyond criteria used for histopathology scoring, epithelial proliferation is considered to be strongly associated with a gastrointestinal inflammatory status[38]. In order to evaluate these criteria, paraffin-embedded colon sections were stained for Ki67 expression by immunohistochemical analysis.
B. lactis feeding on the expression of well known proinflammatory markers, such as COX-2, IL-6 or TNF-α, in the colon of recipient mice was assessed. COX-2 is an enzyme known to be strongly induced in intestinal epithelial cells upon inflammatory conditions. Upon disease induction and in absence of treatment (control recipient group), the protein expression of COX-2 was strongly detected in two out of five samples and slightly in a third one (Figure 3C). Conversely, in the B. lactis-fed group, only one out of five samples had slight COX-2 protein expression (Figure 3C). Relative densitometric quantification of the bands clearly revealed a significant decrease of COX-2 expression in the B. lactis-fed group compared to control mice (Figure 3C).

Feeding with B. lactis also resulted in a significant decrease of IL-6 and TNF-α protein production in the colon of recipient mice compared to the non-treated recipient mice (Figure 4A and C). Accordingly, phosphorylation of the transcription factors STAT-3 and p38, associated with the signaling pathways of these two cytokines, was also diminished by B. lactis feeding (Figure 4B and D).

Effect of B. lactis on dendritic cell markers
It is now well established that co-stimulatory interactions between antigen-presenting cells and cells of the adaptive immune system, such as the CD40/CD40 ligand (CD40L) and OX40/OX40 ligand (OX40L) (CD134/C134L) pathways, play a crucial role in colitis induction and severity of disease. mRNA expressions of these four molecules were thus assessed in colon tissue samples of recipient mice. Whereas they were all induced in recipient mice in comparison to healthy controls (Figure 5A-D), B. lactis feeding significantly down-regulated the expression levels of CD40L and OX40/OX40L when compared to non-treated colitic controls (Figure 5B-D). Expression of CD40 only showed a tendency to be down-regulated by B. lactis feeding.

Effect of B. lactis on colonic T cell markers
Colitis induction in the transfer model of colitis critically depends on the expansion and preferential differentiation of transferred T cells into Th1 T cells. Hence, T cell-related gene transcripts interferon (IFN)-γ, CD3γ and T-bet were measured in colon tissue samples in both groups of recipients. All of these T-cell transcripts, highly expressed in colitic animals when compared to healthy control mice, were significantly decreased in B. lactis-fed mice (Figure 6A-C). In order to gain insight into regulatory functions of T cells, mRNA expression of forkhead transcription factors STAT-3 and p38, associated with the signaling pathways of these two cytokines, was also diminished by B. lactis feeding.
box protein 3 (Foxp3) in colon samples was also quantified and showed that Treg cells were significantly potenti-
ated by feeding with the probiotics, as a 9.2-fold increase of the expression of Foxp3 was observed in B. lactis-fed mice when compared to control colitic mice (Figure 6D).

DISCUSSION

The exact etiology of chronic IBD is still unknown but seems complex and multifactorial. From human studies and animal models of experimental colitis, increasing evidence has been generated that the resident intestinal flora plays a critical role in the development of the intestinal inflammation in genetically susceptible individuals. Indeed, on given genetic backgrounds, such as in IL-2- and IL-10-knockout mice or in HLA-B27 transgenic rats, as well as in the present colitis model, colitis develops when animals are raised under specific pathogen-free conditions, but almost no disease can be observed under germfree conditions[28-30]. With regard to the importance of the quality of the microflora in colitis, clinical and preclinical observations have demonstrated beneficial effects of probiotic microorganisms in the treatment of IBD in humans[31,32,34,35] and in experimental models of colitis[36-39].

While most reports in the literature describe effects of mixes of probiotic strains or of synbiotics, only a few papers have investigated the beneficial potential of the use of single probiotic strains, and moreover of Bifidobacterium probiotic strains, to alleviate intestinal inflammation. As a preclinical model of IBD, the CD4+CD45RBhigh-naive T cell transfer model of colitis[39,40] was exploited in this study. This widely used model of colitis allows the study of the Th1 cell-mediated immune events leading, without treatment, to an irreversible colonic inflammation characterized by a massive influx of mononuclear cells into the colonic mucosa, an elevated level of pro-inflammatory cytokines, appearance of crypt abscesses and epithelial cell erosions[41]. Noteworthy, as for experimental models using genetically modified animals, the composition of the intestinal flora is also clearly known to affect the kinetics and the severity of the colitis in this particular model[41]. Moreover, in the present study, the fact that CD4+CD45RBhigh T cells were also transferred to recipient mice permitted us to also study the role that Tregs play in suppressing or limiting the onset and/or regulation of inflammation. Thus, for all the above mentioned reasons, this is to our knowledge one of the few studies that shows the preventive effects of a Bifidobacterium strain on the development of IBD in the CD4+CD45RBhigh T cell reconstituted RAG-2 deficient mouse model with insights on Tregs.

Hence, it was demonstrated in the present study that B. lactis actually possesses in vivo immuno-modulatory properties and was able to improve the inflammatory status of treated mice. Indeed, it was revealed that supplementation of mice with B. lactis, one week before and during onset of colitis, resulted in a diminished colitis-induced weight loss (Figure 2), a reduction of mucosal thickness (Figure 3), a decreased expression of pro-inflammatory cytokines and related transcription factors (Figure 4), a diminution of T cell infiltration (Figure 5) and an increase of regulatory T cell markers (Figure 6) when compared to non-supplemented control animals.
Follow-up analysis of the weight of mice showed that *B. lactis* treatment significantly delayed the body weight loss observed in control recipient mice (Figure 2), revealing a major impact of the *B. lactis* supplementation on the overall metabolism of treated mice. After some gain of weight due to normal growth of the mice, weight loss was already visible at day 9 in the control recipient mice whereas this only started at day 18 in the *B. lactis*-fed mice. In this latter group, no real weight loss was even observed at the end of the study, the mice having reached their starting weight. Whether this weight loss would continue under the starting weight should be tested in future studies.

**Figure 5** *Bifidobacterium lactis* feeding diminished the expression of mRNA coding for antigen-presenting cells and T cell costimulatory molecules in colon of recipient mice following adoptive T cell transfer. The expressions of mRNA coding for CD40 (A), CD40 ligand (CD40L) (B), OX40 (C) and OX40 ligand (OX40L) (D) were assessed in colon samples of colitic and healthy mice by real-time polymerase chain reaction using the low density array technology. Results are expressed as the mean ± interquartile-range (*n* = 5 mice per group) and statistical significance is indicated. *B. lactis*: *Bifidobacterium lactis*.

**Figure 6** *Bifidobacterium lactis* feeding significantly diminished mRNA expression of Th1 cell markers and significantly increased mRNA expression of a Treg marker in colon of recipient mice following adoptive T cell transfer. The expressions of mRNA coding for interferon-γ (IFN-γ) (A), CD3γ (B), T-bet (C) and FoxP3 (D) were assessed in colon samples of recipient mice by real-time polymerase chain reaction using the low density array technology. Results are expressed as the mean ± interquartile-range (*n* = 5 mice per group) and statistical significance is indicated. *B. lactis*: *Bifidobacterium lactis*. 
Concerning histopathological analyses of the colons at sacrifice, even if the benefit observed regarding weight loss was not visible on total score, among all criteria investigated mucosal thickness was particularly improved by probiotic supplementation, suggesting a diminution of cellular infiltration in the mucosa (Figure 3A).

In line with this reduction of colonic thickness, a strong decrease of the reparative proliferative activity of colonic epithelial cells, assessed by immunostaining using the proliferative marker Ki-67, was observed in the mice fed with *B. lactis*. Indeed, colonic inflammation induces strong epithelial erosion due to uncontrolled apoptosis[43]. This increased level of apoptosis, which contributes significantly to the IBD pathology[8], is presumably due in part to a strong increase of inflammatory cytokines such as TNF and IFN-γ as observed in our study; cytokines known to be able to induce apoptosis directly by suppressing anti-apoptotic signals in the epithelium[46-48]. Hence, since in our experimental setting mice were sacrificed at a late time point relative to onset of inflammation, reparative proliferation was the more relevant parameter to be investigated here rather than apoptosis.

The role of pro-inflammatory cytokines in IBD pathology has been firmly established[43]. Dysregulation of the intestinal immune system both at humoral and cellular level constitutes an important element in the multifactorial pathogenesis of IBD. A strongly elevated expression of pro-inflammatory mediators, most notably IL-6 and TNF-α, has been identified in IBD patients and experimental models of colitis in mice[49]. In cases of acute inflammation, IL-6 synthesized mainly by macrophages first binds to the IL-6 receptor (IL-6R); this complex then associates with gp130, inducing dimerization and the initiation of signaling through STAT-3. In the present study, the decrease of colonic IL-6 expression and STAT-3 phosphorylation induced by *B. lactis* strongly suggest that the probiotic could act, at least in part, on the IL-6 trans-signaling pathway and could by this process dampen the inflammation. Indeed, previous studies performed in both animal models and humans clearly described that abrogation of IL-6 pathway activation was associated with an improvement of colonic inflammation[47-49].

Moreover, analysis of p38 phosphorylation has confirmed the beneficial impact of *B. lactis* feeding on inflammation. Indeed, p38 kinase regulates the production of key inflammatory mediators, including TNF-α or IL-1β. In addition, p38 also acts downstream of cytokines such as TNF-α, mediating some of their effects[48]. Hence, the significant diminution of p38 phosphorylation observed in this study upon *B. lactis* feeding (Figure 4) revealed the broad range of potential effects of the probiotic treatment on colitis.

*B. lactis* feeding also modulated COX-2 protein expression in the colon of mice (Figure 3C). COX-2, an enzyme responsible for formation of important biological mediators called prostanoids (including prostaglandins, prostacyclin and thromboxane), has been shown to be specifically induced in epithelial cells under IBD conditions[50]. Among the prostanoids related to COX-2 activity, prostaglandins represent one of the most important components of mucosal defence in the small intestine and colon. The weight of evidence collected so far suggests that prostaglandins derived from COX-2 are important in promoting the healing of mucosal injury, in protecting against bacterial invasion, and in down-regulating the mucosal immune system. Suppression of COX-2 in a setting of gastrointestinal inflammation and ulceration has been shown in experimental models to result in impaired healing and exacerbation of inflammation-mediated injury[52-55]. Hence, at the intestinal level, expression of COX-2 is a natural response of the organism to prevent tissue damage due to inflammation and is sustained by this inflammation. In this way, COX-2 expression represents a good marker of the actual disease activity[56]. In the present study, the observed attenuated COX-2 expression upon *B. lactis* feeding strongly indicates an anti-inflammatory effect of this probiotic bacterium.

Colitis, in the T-cell adoptive transfer model, is accompanied by the accumulation of dendritic cells (DCs) in the MLN as well as locally in the colon[57]. DCs in the MLN express an activated phenotype with increased expression of CD40 and the TNF-like molecule OX40L[58]. Indeed, activated T cells express the cell-surface costimulatory molecules CD40L and OX40. CD40L binds to CD40 on antigen-presenting cells (APCs) inducing OX40L expression, and leading to the transmission of further activatory signals to both the T cell and the APC[59,60]. The CD40-CD40L and OX40L-OX40 pathways play functional roles in the inflammatory response in this model, as blockade of either pathway inhibits colitis[61,62]. The overall decrease in these four costimulatory partners observed upon *B. lactis* feeding (Figure 5) thus revealed a diminished activity of the immune system in sustaining the adaptive inflammatory reactions. The diminution in the number of T cells present in the colonic mucosa of *B. lactis*-treated mice compared to control animals, as revealed by the measurement of the relative expression of CD3 and IL-6 mRNA (Figure 6B), might be considered as a partial consequence of this dampening of the costimulatory activity.

Beyond the intensity of the DC-T cell interaction, *B. lactis* feeding could interfere with the outcome of the interaction in the mucosa. Indeed, *B. lactis* was also able to significantly decrease T-bet and IFN-γ mRNA expression compared to untreated animals (Figure 6A and C). IFN-γ is the hallmark Th1 cytokine and T-bet is a critical factor for both the initiation and perpetuation of Th1-mediated colitis. Indeed, in another type of adoptive transfer model, T-bet-deficient CD4+ CD62L- T cells failed to induce Th1-mediated colitis in immunodeficient hosts, whereas T-bet-overexpressing CD4+ CD62L+ T cells induced a more rapid onset of colitis[60]. The observed diminution of the IFN-γ mRNA expression is fully in line with the decrease of T-bet, revealing a functional impact on activated T cells by *B. lactis*.

Development of colitis following transfer of CD4+ CD45RBhi T cells into immunodeficient recipients can be modulated or even abrogated by cotransfer of cells from the antigen-experienced CD4+ CD45RBlo population[64], a potential source of Tregs. In the present study, as a small
proportion of such cells have been actually cotransferred, we performed a quantification of mRNA expression for Foxp3 in colon samples of recipient mice. This marker has been identified as being necessary for both the development and function of Tregs\(^{34,46}\). It appeared that \textit{B. lactis} feeding significantly potentiated the presence of these particular cells in the inflamed mucosa as treated mice showed a strong augmentation of Foxp3 mRNA expression compared to control mice (Figure 6D) whereas total number of T cells decreased in a converse fashion (Figure 6B). Moreover, while the dampened expression of all analyzed T cell-related proinflammatory markers might be a direct consequence of the diminution in T cell number in the colonic mucosa of \textit{B. lactis}-fed animals, the fact that Foxp3 mRNA expression was up-regulated indicates a strongly increased prevalence of Tregs in the inflamed tissues. This increase of Treg cells may be one of the key players in the alleviation of the colitis observed in this study.

Despite the fact that mechanisms of action remain unclear, two different options could be hypothesized to explain the effect of probiotics on the Tregs. The classical one will be that probiotics in the colon generated antigenic peptides or molecules able to inhibit or modulate DC activation responsible for uncontrolled T cell proliferation which leads to colitis development, as supported by the diminished expression of the CD40-CD40L and OX40L-OX40 costimulatory partners. More recently, a new hypothesis could be proposed to explain the probiotic effect, involving the toll-like receptor (TLR) pathway. Indeed, it has been revealed that TLR molecules that recognize a vast array of microbial products, thought to be only restricted to cells of innate immunity, are also expressed by Tregs\(^{37}\). Interestingly, a recent study demonstrated a role of the TLR2 pathway in the control of expansion and function of Tregs\(^{38}\). Such a direct effect of TLR ligands on Tregs thus opens a new way to consider the impact of probiotics in the regulation of colitis. Future investigations on the impact of \textit{B. lactis} on Tregs will provide interesting pieces of information regarding how and when probiotics exert their effect, as the present study does not allow discrimination as to whether Tregs are potentiated already in donor mice or only in recipient mice upon induction of inflammation.

In conclusion, it was shown in the present study that \textit{B. lactis} is a \textit{Bifidobacterium} probiotic strain able to provide anti-inflammatory properties in an adoptive cell transfer model of IBD. Mechanisms of actions are not completely elucidated and need further investigations, but a clear effect on Tregs may be suggested as a key influence.

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**COMMENTS**

**Background**

Ulcerative colitis and Crohn’s disease, commonly known as inflammatory bowel disease (IBD), affect 0.5%-1% of the Western world’s population and are increasing in the developing countries. IBD is a chronic disease that requires lifelong treatment. Many patients do not respond or do not comply well with the recent medications. This, in addition to the high cost of these approaches, urges the scientific community to develop new therapeutic approaches.

**Research frontiers**

Many probiotics have been identified as a powerful strategy to reduce intestinal inflammation in preclinical and human trials. However, despite a high number of publications there are very few explanations concerning their mechanism of action.

**Innovations and breakthroughs**

Using one of the most valuable murine models of colitis, we have highlighted the fact that a single \textit{Bifidobacterium} probiotic strain was able to prevent intestinal inflammation by increasing the number of regulatory T cells in the gut.

**Applications**

Through this publication we would like to emphasize that \textit{Bifidobacterium lactis} (NCC 2816) could be considered as a good auxiliary to current treatment used to reduce intestinal inflammation in IBD patients.

**Terminology**

According to the World Health Organization definition, probiotics are live microorganisms which when consumed in adequate amounts confer health benefits to the host.

**Peer review**

In the manuscript, the authors evaluated the anti-inflammatory properties of a \textit{B. lactis} strain in a colitis mouse model. The experiments were carefully designed and the manuscript is easy to read.
Philippe D et al. *B. lactis* reduces experimental colitis

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