Consumption of Probiotics Increases the Effect of Regulatory T Cells in Transfer Colitis

Emil Rathsach Petersen, BSc,* Mogens Helweg Claesson, DMSc,* Esben Gjerløff Wedebye Schmidt, MSc,* Simon Skjøde Jensen, MSc, PhD,† Peter Ravn, MSc, PhD,† Jørgen Olsen, DMSc,‡ Arthur C. Ouwehand, MSc, PhD,§ and Nanna Ny Kristensen, MSc, PhD*

Background: Probiotics may alter immune regulation. Recently, we showed that the probiotic bacteria Lactobacillus acidophilus NCFMTM influenced the activity of regulatory T cells (Tregs) in vitro. The aim of the present work was to demonstrate if L. acidophilus NCFMTM also affects the function of Tregs in vivo.

Methods: Development of colitis after transfer of CD4⁺CD25⁻ T cells and protection from colitis by Tregs was studied in immunodeficient SCID mice which were simultaneously tube-fed with L. acidophilus NCFMTM or L. salivarius Ls-33 for 5 weeks.

Results: Probiotic-fed SCID mice transplanted with low numbers of Tregs in addition to the disease-inducing T cells were completely protected from colitis. This was in contrast to the control group, which showed intermediate levels of inflammation. In addition, feeding with probiotics lowered serum levels of inflammatory cytokines in both colitic mice and in mice protected from colitis by Tregs. Gene expression patterns of rectum samples of protected mice that receive either one of the probiotics showed a closer resemblance to naive SCID mice than did patterns of the control group. The mechanism of action of the probiotics appears to be an indirect effect by inducing a Treg-favorable environment rather than a direct effect on the Tregs.

Conclusions: L. acidophilus NCFMTM and L. salivarius Ls-33 feeding of SCID mice increases the in vivo effect of Tregs, resulting in a gene expression pattern in the rectum resembling that of the naive SCID mouse.

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Key Words: regulatory T cells, colitis, inflammation, probiotics

Probiotics have been defined as: “live microorganisms which when administered in adequate amounts confer a health benefit on the host, beyond their inherent basic nutrition.”¹,² Much research has been done on probiotics, especially with a focus on the immune system, and the potential health benefits to people suffering from autoimmune diseases. The most common probiotics studied are the lactic acid bacteria strains from the genera Bifidobacterium or Lactobacillus.

The functions of probiotics comprise several mechanisms, both direct and indirect, which might influence the gut microbiota and gastrointestinal and immune function. Directly, probiotics may adhere to intestinal cells, thus modulating the composition and activity of the intestinal microbiota, and compete with pathogenic bacteria for nutrients and space.³ Indirectly, probiotics may induce immunoregulatory cytokines in antigen-presenting cells (APCs).⁴ In addition, probiotics may have beneficial effects in the production of antimicrobial substances that are harmful to pathogenic bacteria, and in production of short-chain fatty acids, which provide nutrition to colonoocytes, including lowering of pH in colon that is harmful to pathogenic bacteria.³,⁶,⁷ Human inflammatory bowel disease (IBD) is associated with changes in the intestinal barrier function, including decreased tight junction protein expression and increased apoptotic ratio, causing luminal antigens derived from nonpathogenic bacteria flora to come in contact with mucosal immune cells, leading to an immune response.⁸–¹⁰ Recent data indicate that improved integrity of the intestinal epithelial barrier under certain pathological circumstances might be an effect of probiotics.¹¹

Only relatively few experiments in this field have been carried out on the two probiotic strains L. acidophilus

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*Department of International Health, Immunology and Microbiology, University of Copenhagen, Panum Institute, Denmark. †Bioneer A/S, Hørsholm, Denmark. ‡Department of Cellular and Molecular Medicine, University of Copenhagen, Panum Institute, Denmark. §Danisco A/S, R&D Group Leader, Danisco Sweeteners, Health & Nutrition, Kantvik, Finland.

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Reprints: Nanna Ny Kristensen, Department of International Health, Immunology and Microbiology, Panum Institute, Building 18.3.52, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark (e-mail: nannak@sund.ku.dk)

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NCFM™ and L. salivarius Ls-33. However, we have recently shown that L. acidophilus NCFM™ in vitro inhibits the immunosuppressive effect of regulatory T cells (Tregs) on enterobacterial stimulated effector T cells, while L. salivarius Ls-33 seemed to have no significant effect on Tregs in this system. Other experiments have shown that L. salivarius Ls-33 has a significant preventive effect on trinitrobenzene sulfonic acid (TNBS)-induced acute colitis.

Recently, it has been shown by several groups that different probiotics have the ability to induce Tregs in an interleukin (IL)-10-dependent manner under acute inflammatory conditions toward foreign antigen (TNBS-induced colitis, CHS and EAE)16–19

In the present work we examine the physiological, immunological, and genetic effects of oral-administered probiotics in chronic intestinal inflammation triggered by natural enteroinfectious in SCID mice transplanted with colitogenic T cells alone or mixed with natural Tregs.

**MATERIALS AND METHODS**

**Mice**

Female type BALB/c and SCID mice were purchased from Taconic Farms (Lille Skensved, Denmark). The mice were allowed to rest for at least 1 week before entering experiments, at which time they were 6–8 weeks old. All animals were housed under controlled microbial environment conditions, which included testing of sentinels for unwanted infections according to the Federation of European Laboratory Animal Science Association standards; no such infections were detected.

**Isolation of CD4+ T-cell Subsets**

CD4+ T cells were positively selected from mesenteric lymph node single-cell suspensions using a mouse anti-CD4 mAb coated Dynabead and detach-a-bead system (Dynal, Norway) according to the manufacturer’s description. For transplantation of SCID mice the CD4+ T cells (>98% pure assessed by flow cytometry) were then separated into CD25+ and CD25− T-cell populations by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany), using PE-labeled anti-CD25 mAb followed by addition of anti-PE microbeads, according to the manufacturer’s description.

**Induction of and Protection from Colitis**

Colitis was induced in SCID mice by intraperitoneal (i.p.) transplantation of 3 × 10⁷ CD4+CD25− T cells in a volume of 0.2 mL. Protection from colitis was obtained by cotransplantation of 3 × 10⁷ CD4+CD25− T cells and 3 × 10⁷ CD4+CD25+ T cells in a volume of 0.2 mL. All mice were then monitored two times weekly for weight loss, loose stools, bloody diarrhea, and rectal prolapse.

**Preparation of Probiotics**

The following probiotic strains were included in the study: L. acidophilus NCFM™ ATCC 700396 (Danisco, Cultures Division, Paris, France) and L. salivarius Ls-33 ATCC SD5208 (Danisco). The cells were grown anaerobically in MRS broth (Oxoid, Nepean, Canada) at 37°C to stationary phase. Cells were harvested and washed twice in phosphate-buffered saline (PBS), and pelleted by centrifugation before freeze-drying.

To determine the amount of bacteria, the number of colony-forming units (cfu) was determined on a sample of the grown culture by dilution, plating on MRS, and incubation anaerobically at 37°C until formation of colonies.

**Tube Feeding**

The freeze-dried bacteria were resuspended in PBS each day and mice were tube-fed with 1 × 10⁹ cfu probiotic bacteria in a volume of 200 μL PBS. Control mice were tube-fed with PBS without bacteria. All mice were tube-fed for 5 weeks.

**Preparation of Total RNA**

At the end of the experimental period mice were killed by cervical dislocation. The rectums were removed immediately and feces were then removed. The upper 0.5 cm was used for histology and the distal 1.5 cm was used for RNA purification. The sample was snap-frozen for 2 minutes in dry ice-cooled methylbutan and then stored at −80°C until total RNA isolation was initiated.

The total RNA was extracted from lysed homogenized samples using the mirVana miRNA isolation kit, including phenol chloroform extraction (Applied Biosystems, Foster City, CA; AM1560) according to the manufacturer’s description. The amount, integrity, and purity were evaluated using a lab-on-a-chip Bioanalyzer (Agilent Technologies, Palo Alto, CA).

**Real-time Polymerase Chain Reaction (PCR) Array**

The Mouse Inflammatory Response and Autoimmunity RT² Profiler PCR Array (SABioscience) was used to profile the expression of 84 key genes involved in autoimmune and inflammatory immune responses (PAMM-077) in rectum samples. The PCR arrays were prepared according to the manufacturer’s description. Only RNA suitable (free of RNase, DNase, and DNA) equipment was used, including RNase-free gloves. All samples and tubes were kept on ice during the preparation of the array. Briefly, total RNA were converted to cDNA using the RT2 First Strand Kit, and the cDNA were added to the RT2 qPCR Master Mix according to the manufacturer’s description. Then 25 μL of the mixture was added to each well in the PCR array and the PCR array was sealed carefully with optical thin-wall 8-cap strips. To ensure that no
bubbles were present in the wells, the PCR array was centrifuged — 1 minute at 1000g. The plate was run on mx3000p.

The data from the mx3000p were further examined using the following software: “RT2 profiler PCR array data analysis,” from the SABioscience website (http://www.sabiosciences.com/pcr/arrayanalysis.php). This program converts the Ct values to a value that expresses the degree of gene expression, and calculates the fold down-/upregulation of the genes. The following housekeeping genes were used to normalize the data: Hprt1, Hsp90ab1, Gapdh, and actb. The level of significance was set to P < 0.05.

Quantitative PCR
RNA samples were treated with DNase, and mRNA was reversibly transcribed to cDNA using RevertAid H Minus First Strand cDNA synthesis kit (Fermentas Life Science, Burlington, ON, Canada). Real-time PCR reaction was performed using Brilliant SYBR Green QPCR (Agilent Technology, Palo Alto, CA) in combination with the following primers: TGF-β forward: 5’-CCA AGG AGA CGG AAT ACA GG-3’, TGF-β reverse: 5’-TTTGAATTCCTGGGTGAGAA-3’, IL-10 forward: 5’-GG AGAAAATCGATGACAGCGC-3’, GAPDH forward: 5’-CAAG GTCAATTCATGACACAGCGC-3’, GAPDH reverse: 5’-GTCC ACCACCCCTTGGCTTGAG-3’. Quantitative (q)PCR was performed on Stratagene (La Jolla, CA) Mx3000P. Data analysis was performed using Mx Pro.

Colitis Inflammation Scores
For histological examinations, the upper 0.5 cm of rectum was snap-frozen for 2 minutes in dry ice-cooled methylbutan and embedded at optimal cutting temperature medium (Tissue Tech; Sakura Fine Tek, Torrance, CA). Frozen tissue sections 6 μm were cut (Thermo Scientific, Pittsburgh, PA; Microm HM 520 / HM 525 routine cryostat) and stained with hematoxylin/eosin. The specimens were all examined in a blinded fashion by a pathologist (M.H.C.). Histopathological changes were evaluated as described previously.20 Score 0, no signs of inflammation; score 1, slight infiltration of lamina propria by mononuclear cells; scores 0 to 1 are present in non-transplanted SCID mice; score 2, moderate mononuclear cell infiltration of lamina propria, and 2–3-fold crypt elongation; score 3, as in 2, but more heavy mononuclear cell infiltration, patchy cell infiltration in the submucosal layer, and 3–10-fold elongation of crypts with depletion of goblet cells; score 4, as in 3, but including crypt destruction; score 5, as in 4, but increased crypt destruction and microabscesses, polymorphonuclear infiltration of the lamina propria, circumscript mononuclear cell infiltration in the submucosal layer, and epithelial ulcerative lesions.

Preparation of Fecal Extract
Extract was prepared by removing the colon and cecum from normal Balb/c mice and placing the content in PBS. This was sonicated three times (30 seconds) on ice, followed by centrifugation at 10,000g for 10 minutes to remove insoluble material. The supernatant was collected, sterile-filtered, and stored at −80°C. The protein concentration in the supernatants was typically 1–1.5 mg/mL as determined by the BCA method.

Preparation and Pulse of Splenocytes and Proliferation Assay
For in vitro proliferation assays, spleen cells from BALB/c mice were used as APCs. The splenocytes were cultured in 24-well plates as 8 × 10⁶ cells/well in a final volume of 2 mL and 400 μg/mL of fecal extract protein was added. The splenocytes were incubated for 24 hours at 37°C. The splenocytes were washed in culture medium and irradiated (2000 rad) and 1.0 × 10⁶ cells were cocultured in four replicates with CD4⁺ T cells (1.0 × 10⁵) from colitic or Treg-protected mice. After 4 days of culture, proliferation was measured by adding 0.5 Ci ³[H]-thymidine to each well, incubating for further 18 hours, and harvesting the cells to count the incorporated thymidine.

Cytokine Measurements
Cytokine content in serum samples or supernatants from proliferation cultures was measured using the Luminex multiplex system. A cytokine/chemokine 14-Plex (from Invitrogen, La Jolla, CA) was used according to the manufacturer’s protocol. Measurements were carried out using the Luminex 100 IS (Luminexcorp, Austin, TX). More than 100 events were acquired per bead set and all samples were run in duplicate. The following cytokines were measured in the supernatants: IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40), GM-CSF, IFNγ, TNFα, CXCL9 (MIG), CXCL1 (kc), CXCL10 (IP10), and CCL5 (Rantes).

Statistics
The Wilcoxon nonparametric test was employed to compare discontinuous variables and the t-test was used when comparing continuous variables. All tests were two-tailed and P < 0.05 was considered significant. Principal component analysis (PCA) was conducted using prcomp in the R statistical environment. The first two components were used and explained 73% of the variation in the dataset.

RESULTS
Feeding L. acidophilus NCFM™ or L. salivarius Ls-33 Increases the Effect of Tregs In Vivo
We have previously shown that the probiotic strain L. acidophilus NCFM™ in vitro can modify APCs to cause reduced Treg activity.14 We have also shown that transfer colitis in immunodeficient mice can be hindered by cotransfer of Tregs in a ratio of 1:1 with the disease-inducing CD4⁺CD25⁻ T cells.21 We therefore found it of
importance to investigate if \textit{L. acidophilus} NCFMTM feeding could reduce the activity of adoptively transferred Tregs in vivo. To address this question, 10 groups of SCID mice were studied (Table 1). The study also included the probiotic strain \textit{L. salivarius} Ls-33, which has previously shown to have no effect on Tregs in vitro,\textsuperscript{14} but shows a strong protective effect in TNBS-induced colitis.\textsuperscript{12}

Three groups of mice were transplanted with CD4$^+$CD25$^-$ colitogenic effector T cells from mesenteric lymph nodes (MLNs) of normal mice, three groups were transplanted simultaneously with effector and Tregs at a ratio of 6:1, and three groups were transplanted simultaneously with effector and Tregs at a ratio of 1:1 (Table 1). The mice in each group were tube-fed every day for 5 weeks either with PBS (control), \textit{L. acidophilus} NCFMTM, or \textit{L. salivarius} Ls-33. The clinical status of the mice was monitored twice a week; in Figure 1A,C,E the weight of the mice is shown in percent of their starting weight.

As expected, the weight of the naïve 6-week-old SCID mice increased during the 5 weeks experimental period, resulting in a 10% weight gain (Fig. 1A). In contrast, the three groups of mice transplanted with CD4$^+$CD25$^-$ T cells lost about 10% weight (Fig. 1A); these latter mice also suffered from diarrhea, pointy fur, and some mice had rectal prolapse, indicating that all mice suffered from colitis. Histological examinations of the rectum confirmed these observations (Fig. 1B) as most mice had a colitis score between 3 and 5. There was a trend that the colitic mice fed \textit{L. salivarius} Ls-33 had a slightly lower colitis score.

The three groups of mice cotransplanted with only 50,000 Tregs gained weight, although not to the same extent as naïve mice (Fig. 1C). Histological examinations of rectum samples (Fig. 1D) from these groups showed that the PBS-fed control group do in fact suffer from intermediate colitis (scores around 2–3) even though they do not lose weight. In contrast to this, the two groups of mice that had been tube-fed with either \textit{L. acidophilus} NCFMTM or \textit{L. salivarius} Ls-33 were completely protected from colitis (scores around 0), indicating that the two probiotic strains increase the activity of the transplanted Tregs.

The three groups of mice cotransplanted with 300,000 Tregs gained weight to the same extent as naïve mice (Fig. 1E), suggesting that they are completely protected from colitis development by the cotransplanted Tregs. Histological examinations of rectum samples (Fig. 1F) also confirmed that the protected mice tube-fed with either \textit{L. acidophilus} NCFMTM or \textit{L. salivarius} Ls-33 were just as protected as control mice with colitis scores around 0.

### Table 1. Experimental Design

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<thead>
<tr>
<th>Phenotype</th>
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<th>Regulatory T Cells CD4$^+$CD25$^+$</th>
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Feeding with \textit{L. acidophilus} NCFMTM and \textit{L. salivarius} Ls-33 Changes Serum Cytokine Levels in Both Colitic and Protected Mice

At the end of the experimental period serum samples from all mice were obtained and analyzed for cytokine content by multiplex analysis. In general, the serum levels of cytokines were low and 11 of the included 14 cytokines were below the detectable limits. However, data for the inflammatory cyto- and chemokines IL-12, IFN$\gamma$, and CXCL9 (MIG) were obtained and are shown in Figure 2. As expected, the cytokine levels in the protected mice were lower than in the colitic mice and the more Tregs transplanted the less cytokine was found in serum. IFN$\gamma$ and CXCL9 were decreased in the colitic groups fed probiotics compared to the control group. Similarly, mice in both the 50,000 and the 300,000 Treg protected groups fed with either \textit{L. acidophilus} NCFMTM or \textit{L. salivarius} Ls-33 were just as protected as control mice with colitis scores around 0.
below detection levels in the protected groups that had been fed with probiotics; in contrast, the control group protected by 50,000 Tregs had around 100 pg/mL of CXCL9. Thus, for both colitic and protected mice feeding with probiotics appeared to lower serum levels of inflammatory cyto- and chemokines.

**PCA of the Gene Expression in Rectum Samples from Protected Mice Fed Probiotics Compared to Protected Control and Naïve Mice**

We next examined the rectum for changes in gene expression related to inflammation and autoimmunity using the mouse inflammatory response and autoimmunity RT² Profiler PCR Array (PAMM-077, SABioscience), which includes detection of 84 genes. Total RNA was purified from rectum samples of mice protected by 300,000 or 50,000 Tregs, tube-fed with either *L. acidophilus* NCFMTM or *L. salivarius* Ls-33, and from protected control mice and naïve mice. To see if the mice fed with either of the two probiotics could be separated from the control mice based on their changes in gene expression, a PCA was carried out on the gene expression data.

The resulting PCA score plot in Figure 3, including the two components (PC1 and PC2), showed that the rectum samples from protected control mice was distributed differently than the other groups included in the analysis. Thus, control mice protected by 50,000 Tregs (open circles) are separated in a positive direction on the PC2...
axis and in a negative direction on the PC1 plot. The control mice protected by 300,000 Tregs (gray circles) are also all located in a positive direction on the PC2 axis. In contrast to this, the four groups of probiotic-fed protected mice all are located more closely to the naïve SCID mice, which are located in the negative direction on the PC2 axis.

Gene expression in rectum of protected SCID mice tube-fed with either \textit{L. acidophilus} NCFMTM or \textit{L. salivarius} Ls-33 resembles the gene profile of naïve SCID mice.

To study the gene expression in the protected groups of mice more closely, we next compared the significant altered genes ($P < 0.05$) of mice fed with probiotics and control, protected with either 50,000 Tregs (Fig. 4) or 300,000 Tregs (Fig. 5).

The mice that were protected by 50,000 Tregs and tube-fed with \textit{L. acidophilus} NCFMTM only differ from the protected controls in 18 genes (Fig. 4A). Most strikingly is that the genes for IL8rb and CXCL5 are found 9.5- and 15.0-
fold downregulated, respectively, compared to the unfed controls, whereas the genes *Tollip*, *Tlr5*, *Tlr1*, *Nr3c1*, *Nfkb1*, *Nfatc3*, *Il17*, *Il22ra2*, *Il1r1*, *Il18*, *Hdac4*, *Flt3l*, *Ccl25*, and *Bcl6* were around 2-fold significantly upregulated.

In the mice that were protected with 50,000 Tregs and fed *L. salivarius* Ls-33 the genes for IL8rb and CXCL5 are also found markedly downregulated, 18.9- and 51.3-fold, respectively, compared to the protected unfed control group (Fig. 4B). Also, the genes *Tnf*, *Cxc11*, *Ccl8*, *Ccl4*, and *Ccl3* were significantly downregulated between 3- and 6-fold compared to the unfed controls. As in the protected *L. acidophilus* NCFMTM-fed group, the genes *Tlr5*, *Nr3c1*, *Nfatc3*, *Il17*, *Hdac4*, *Flt3l*, and *Ccl25* were found around 2-fold upregulated compared to the protected unfed controls. In addition, the genes *Il6ra* and *Ccl11* were found ≈2-fold induced compared to the protected control.

When comparing the groups of mice that were protected by 300,000 Tregs (Fig. 5), the gene profile in the rectum samples is more diverse. Many genes were found significantly downregulated after feeding with both strains of probiotics compared to the unfed controls.

In general, probiotic feeding together with protection from colitis by large numbers of Tregs significantly downregulate many of the inflammatory genes. Only *Ccl25*, *Ccl11*, and *Tlr1* are slightly upregulated in the mice fed with *L. salivarius* Ls-33 compared to the unfed controls (Fig. 5B). In the Treg-protected *L. acidophilus* NCFMTM-fed mice 18 genes were found downregulated compared to the unfed controls (Fig. 5A). The genes *Il8rb*, *IFNγ*, *Cxc12*, *Ccl8*, *Ccl5*, and *Ccl20* were more than 4-fold downregulated. The rest of the significantly changed genes (*Tnfsf14*, *Tlr2*, *Il1a*, *Il18rap*, *Fasl*, *Cxc4*, *Cd40lg*, *Ccr3*, *Ccr2*, *Ccr1*, and *C3ar1*) were around 2-fold downregulated (Fig. 5A).
In the *L. salivarius* Ls-33 fed group that were protected by 300,000 Tregs, 28 genes were found significantly downregulated compared to the control (Fig. 5B). Thus, gene expression of *Ccl1*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl8*, *Ccx11*, *Ccx12*, *Ccx13*, *Ccx19*, IFNγ, and IL-10 were more than 4-fold downregulated compared to the unfed controls. The genes *Tnfsf14*, *Tlr2*, *Myd88*, *Il18rap*, *Flt3l*, *Fasl*, *Cxc11*, *Cd40lg*, *Cd40*, *Ccr4*, *Ccr3*, *Ccr2*, *Ccl7*, *Ccl19*, and *C3* are all found between 2- and 4-fold downregulated compared to the unfed control.

### Probiotics Do Not Affect Tregs Directly in BALB/c Mice

To measure if the probiotic strains affect Treg activity directly, normal BALB/c mice were tube-fed every day for 5 weeks either with PBS (control), *L. acidophilus NCFM™*, or *L. salivarius* Ls-33 corresponding to the experiments presented above. During the experimental period the weight and clinical status of the mice were monitored; as expected, no changes were detected due to the tube feeding (data not shown). At the end of the experimental period Tregs from MLNs were purified from all groups of mice and their function were tested ex vivo in a standard proliferation assay with enteroantigen exposed splenocytes from normal mice21 (Fig. 6). There were no differences in the ability of these Tregs to hinder proliferation of normal enteroantigen-specific CD4+CD25− effector T cells, indicating that the probiotic strains do not affect the Tregs directly in vivo.

### IL-10 and TGF-β Levels in Rectum Samples Are Affected by Probiotic Feeding

Probiotics may induce a Treg-favorable environment rather than affecting the Tregs directly. To investigate this, we prepared a qPCR on the Treg-related cytokines IL-10 and TGF-β in rectum samples from SCID mice that were protected by 50,000 Tregs and tube-fed with probiotics for 5 weeks (Fig. 7). The relative expression levels of IL-10 were significantly decreased in the protected group of mice that had been fed *L. salivarius* Ls-33 compared to the unfed controls; the expression level was even lower than in the naïve group of mice. In contrast, the expression level of IL-10 was not significantly decreased in the protected group of mice that had been tube-fed with *L. acidophilus NCFM™* compared to the unfed controls (Fig. 7A). The expression levels of Tgf-β were significantly lower in the probiotic-fed protected and in naïve mice when compared to the unfed controls. The Tgf-β levels in the *L. acidophilus NCFM™*-fed group were only slightly decreased, whereas the level in the *L. salivarius* Ls-33-fed group were even lower than in the naïve mice. Thus, the probiotic strains apparently do not, as might have been expected, increase the level of Treg-favorable cytokine but rather induce a “naïve” environment in a less well-defined manner.

![Figure 6. Probiotic feeding of BALB/c mice does not alter the ex vivo function of Tregs.](image)

**FIGURE 6.** Probiotic feeding of BALB/c mice does not alter the ex vivo function of Tregs. Splenocytes from normal BALB/c mice were pulsed for 24 hours with fecal extract and cocultured with CD4+CD25− T cells from normal BALB/c mice and with CD4+CD25− Tregs from pools of MLNs from mice that were tube-fed with either *L. acidophilus NCFM™*, *L. salivarius* Ls-33, or with PBS (control) daily for 5 weeks. Results are representative of two similar experiments. Proliferation was measured by [thymidine incorporation in the last 18 hours of culture. Error bars: SD.**

![Figure 7. Decreased IL-10 and Tgf-β levels in rectum of L. salivarius Ls-33-fed protected mice.](image)

**FIGURE 7.** Decreased IL-10 and Tgf-β levels in rectum of *L. salivarius* Ls-33-fed protected mice. mRNA was extracted from the rectum of mice that were protected from colitis by 50,000 Tregs and tube-fed with either *L. acidophilus NCFM™* or *L. salivarius* Ls-33; rectum from mice tube-fed with PBS served as controls. The expression of mRNA for IL-10 (A) and Tgf-β (B) was analyzed by qPCR. Average of three mice/group is presented. Error bars: SD. *Significant difference between cytokine expression levels of indicated group compared to control, *P* < 0.05 (t-test).
High Enteroantigen Specific Proliferation of CD4+ T Cells from Protected Mice Fed *L. acidophilus* NCFM™

Since the protected mice fed with the probiotics resemble naïve SCID mice more than the protected unfed control group, we found it important to test if the effector functions of CD4+ T cells are influenced by the probiotic treatment.

CD4+ T cells were purified from MLN of colitic and protected mice and stimulated ex vivo in a standard proliferation assay with enteroantigen exposed splenocytes from normal mice21 (Fig. 8).

In agreement with previous experiments,21 proliferation of CD4+ T cells from the protected control group was significantly decreased compared to proliferation obtained in the colitic control group. This was also the case when looking at CD4+ T cell proliferation from the mice fed *L. salivarius* Ls-33. There were no significant differences between these two groups of protected mice, even though they differ in gene expression. In contrast, the enteroantigen-specific proliferation of CD4+ T cells from protected mice fed *L. acidophilus* NCFM™ is at the same high level as that of colitic mice. So, despite the fact that the protected animals were clinically healthy and had a more “naïve” gene expression profile in rectum, their T cells show a high responsivity towards enteroantigen ex vivo. These latter data are thus in accordance with our previous in vitro data14 and suggest that *L. acidophilus* NCFM™ exposure both in vitro and ex vivo reduces the antiproliferative effect of Tregs on effector T cells.

**Protected Mice Which Have Been Tube-fed with *L. acidophilus* NCFM™ Display a Tₘ2 Cytokine Phenotype**

Supernatants from the proliferation cultures (Fig. 8) were analyzed for cytokine content in a Luminex assay.

**FIGURE 8.** High enteroantigen-specific proliferation of CD4+ T cells from protected SCID mice fed *L. acidophilus* NCFM™. Splenocytes from normal BABL/c mice were pulsed for 24 hours with fecal extract and cocultured with CD4+ T cells from pools of MLNs from the indicated mice for 5 days (Protected by 300,000 Tregs). Proliferation was measured by [³H]-thymidine incorporation the last 18 hours of culture. Error bars: SD. *Significant difference between CD4+ T cell proliferation from colitic and protected mice, *P* < 0.0001 (t-test).

**FIGURE 9.** *L. acidophilus* NCFM™ feeding increases the cytokine production of CD4+ T cells from protected mice. Supernatants were harvested from 5-day proliferation cultures (Fig. 6) and cytokine content was measured using cytokine/chemokine 14-plex for Luminex analysis. << Below detection level.
including 14 cytokines (Fig. 9). The culture from the colitic control mice produced high levels of IFNγ and GM-CSF and also IL-2, IL-1β, TNFα, IL-6, IL-4, CCL5, and CXCL1. As expected, proliferation cultures from mice protected from colitis by Tregs showed reduced cytokine levels and IL-4 was undetectable.

The CD4+ T cell culture from the colitic mice tube-fed with *L. acidophilus* NCFMTM produced the same panel of cytokines and at comparable levels with the unfed control group, although with slightly reduced IL-4 levels. However, proliferation cultures from protected mice tube-fed with *L. acidophilus* NCFMTM showed higher levels of IL-4 and IL-5 than the protected *L. salivarius* Ls-33 and the control groups, suggesting that the *L. acidophilus* NCFMTM in particular induces a Th2 skewed phenotype.

IL-10, IL-12, CXCL9, and CXCL10 were below the detectable levels in all samples.

**DISCUSSION**

In the present study we analyzed the effect of feeding with two different probiotic strains, *L. acidophilus* NCFMTM and *L. salivarius* Ls-33 on physiology, immunology, and gene expression of adoptive transfer colitis and the probiotic effect on Treg-induced protection from colitis. The study was inspired by a previous in vitro study on the same bacterial strains, where we showed that *L. acidophilus* NCFMTM, in contrast to *L. salivarius* Ls-33, had the ability to decrease the activity of natural Tregs in vitro, although it had no effect on the enteroantigen-specific proliferation of CD4+CD25− T cells.

More recently, a number of studies have demonstrated that consumption of some probiotics can increase the number and activity of Tregs in acute inflammation towards a foreign antigen in an IL-10-dependent manner and that these Tregs ameliorate or even hinder acute inflammation. Whether this effect of probiotics is mediated by direct effects on the Tregs or indirect via dendritic cells or other parts of the innate immune system is not yet clear. In the present study we examined if probiotics can affect the function in vivo of Tregs during chronic inflammation towards natural enteroantigen, which resembles human IBD closely. The adoptive transfer model of colitis has the advantage that it is possible to hinder disease development by simultaneous transplantation of effector T cells and Tregs. Thus, any effect of probiotics in this model is more likely to involve Tregs directly. We tube-fed both colitic and protected animals for 5 weeks with either one of the two probiotic strains. The weight development from all groups indicates that the tube feeding with probiotics do not affect disease development (all three colitic groups lost weight to the same extent) nor the ability of Tregs to function in vivo (the protected mice gain weight to the same extent). However, histological examinations of rectum samples revealed that the two probiotic strains increased the effect of Tregs in vivo as the mice that had been protected by low numbers of Tregs (50,000 Tregs per animal) and were tube-fed with probiotics were completely protected from colon inflammation. In contrast, the Treg-injected control group without probiotics showed an intermediate colon inflammation. Analyzing the level of cytokines in serum of the mice showed that there is a tendency that the inflammatory cytokine IFNγ and the chemokine CXCL9 are reduced in colitic mice that were tube-fed with either one of the two probiotics, perhaps most apparent in the colitic mice that were tube-fed with *L. salivarius* Ls-33. In line with this, the colan inflammation of probiotic-fed colitic animals appeared less severe than that of unfed mice. It is also interesting to find that the mice protected by 50,000 Tregs, which were tube-fed with either one of the probiotics, had decreased levels of IL-12, IFNγ, and CXCL9 in serum compared to the unfed controls. Being a Th1-inducing cytokine in autoimmunity, the lower level of IL-12 indicates a lower level of immune activation in Treg-protected mice that were tube-fed with probiotics. These data confirm a study on TNBS-induced colitis, where a mixture of two probiotic strains reduced the production of IL-12 and IFNγ in serum.17

The fact that both *L. acidophilus* NCFMTM and *L. salivarius* Ls-33 had the ability to affect the inflammatory response in mice protected from colitis by Tregs was also confirmed by the changes in gene expression related to inflammation and autoimmunity in rectum of the mice. In the mice that had been protected by low numbers of Tregs only relatively few genes were significantly changed between the probiotic-fed groups and the control. Most strikingly, we found the expression of *cxcl5* and its receptor *il8rb* (CXCR2) to be extremely low in both of the probiotic-fed groups compared to the unfed controls (Fig. 4). This is in agreement with a previous study where we found the expression of *cxcl5* to be upregulated more than 50-fold in colitic animals, indicating that this chemokine does play a very important role in induction and maintenance of colitis. The cytokine CXCL5 released by intestinal epithelial cells is known to recruit CXCR2-expressing neutrophils to the site of inflammation both in mouse and man. Feeding *L. acidophilus* NCFMTM or *L. salivarius* Ls-33 seems to hinder the upregulation of *Cxcl5* and its receptor, which in turn correlates with the animals being healthy. Likewise, the receptor CXCR2 has also been shown to play a key role in DSS-induced experimental colitis. Together these, results suggest that CXCR2 and CXCL5 could be an interesting therapeutic target when designing new drugs for treatment of human IBD patients, perhaps in combination with probiotic strains downregulating this chemokine and receptor pair.

In the *L. acidophilus* NCFMTM-fed group, *Il8rb* and *Cxcl5* were the only two genes being expressed at a lower
level compared to the unfed controls, confirming that these molecules may play a crucial role in the onset of colitis; other genes like Thr1, Thr5, and Thr6 were found upregulated around 2-fold. In the L. salivarius Ls-33-fed group Tnf, Cxcl1, Ccl8, Ccl4, and Ccl3 were found around 4-fold downregulated compared to the unfed controls. All these inflammatory chemokines play important roles in recruiting cells of the immune system to the site of inflammation. Even though the mice that had been fed with either one of the two probiotics undergo similar clinical and histological changes, their gene profile show that feeding L. salivarius Ls-33 induces stronger downregulation of inflammation related genes than does L. acidophilus NCFM™.

In the groups of mice that had been protected by high numbers of Tregs we found several chemokines and receptors playing a role in recruiting cells of the immune system to sites of inflammation, to be expressed at lower levels in the probiotic-fed groups compared to the unfed controls. Again, the L. salivarius Ls-33-fed group showed downregulation of several additional genes related to inflammation than the L. acidophilus NCFM™-fed group. One gene that is found downregulated in the L. salivarius Ls-33 group is Cxcl9. The chemokine CXCL9 plays an important role in recruiting T cells to sites of inflammation and we have previously shown that this chemokine play a very important role in the development of adoptive transfer colitis. In the present study we found that the serum level of CXCL9 was below the detectable limit in protected mice tube-fed with either of the two probiotic strains investigated, but detectable, although at low levels, in Treg-protected unfed control mice. In agreement with this, we also found that the level of Cxcl9 gene expression was decreased by more than 6-fold in the group that had been protected by high numbers of Tregs and fed L. salivarius Ls-33 compared to the unfed controls.

Ccl8 was expressed significantly lower in the two probiotic-fed protected groups compared to the protected unfed controls. CCL8 recognizes several different receptors, including CCR1, CCR2b, and CCR5 expressed by monocytes, eosinophils, basophils, T cells, NK cells, and mast cells. Thus, CCL8, like CXCL9, is an important inflammatory chemokine.

In the inflammatory response, IFNγ is initially produced by cells of the innate immune system, such as NK cells, DCs, and macrophages, and later by Th1 cells, favoring further differentiation of CD4+ T cells to Th1 cells. Aberrant expression of IFNγ is associated with a number of autoimmune and autoinflammatory diseases. The serum levels of IFNγ in the three Treg-protected groups of mice were almost the same; however, the gene expression in rectum showed a significantly lower expression level in the protected mice which had also been tube-fed with probiotics compared to the unfed controls, again confirming that the probiotics strengthen the in vivo effect of Tregs perhaps by skewing the immune homeostasis towards Th2 (see below). Thus, taken together, the PCA analysis, the gene expression, and the histological data suggest that the protected mice fed with L. acidophilus NCFM™ or L. salivarius Ls-33 more closely resemble naïve SCID mice than do the Treg-protected unfed control mice. However, feeding L. acidophilus NCFM™ is not as effective as feeding L. salivarius Ls-33, as it does not induce downregulation of inflammatory genes to the same extent. The probiotics-induced regulatory effects are probably caused by interactions between Toll-like receptors expressed on the surface of Tregs and PAMPs or other components of the probiotics, somehow causing modulation of the cytokine profile, as demonstrated previously in other probiotic exposed systems both in vitro and in vivo. The discrepancy between our previous study demonstrating a Treg-reducing effect of L. acidophilus NCFM™ in vitro and the present data showing the opposite effect most probably reflects significant differences between the in vitro and in vivo settings. Thus, in the former experiments the negative effects on Tregs were mediated indirectly by APCs exposed to L. acidophilus NCFM™ for 18 hours, whereas the Tregs of the present study are both indirectly via APC and directly exposed to L. acidophilus NCFM™ for 5 weeks.

Our data thus show that feeding probiotics strengthens the effect of Tregs; however, whether this is a direct or indirect effect or both of the probiotics on the Tregs is not clear. Feeding normal BALB/c mice with either of the two probiotics did not affect the Tregs ability to function ex vivo. This could indicate that the strengthening effect of Treg is mediated by a more indirect mechanism probably by inducing a Treg-favorable environment. This, however, is not caused by an upregulation of Treg-related cytokines like IL-10 and TGF-β.

We also found that CD4+ T cells purified from the MLNs of Treg-protected mice fed L. acidophilus NCFM™ have a high enteroantigen-specific reactivity ex vivo, which is dominated by a Th2 cytokine profile through induction of IL-4 and IL-5. Apparently, this enteroantigen-reactive population of CD4+ T cells is not colitogenic. Whether Treg exposed to L. acidophilus NCFM™ in vivo are involved in this process of Th2 skewing is an open question.

In conclusion, our results show that probiotic feeding increases the in vivo effect of Tregs in SCID mice protected from colitis by Tregs, and results in a gene expression pattern resembling that of the naïve SCID mouse.

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