

Gene Expression Profiling Identifies Mechanisms of Protection to Recurrent Trinitrobenzene Sulfonic Acid Colitis Mediated by Probiotics

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Background: Host–microbiota interactions in the intestinal mucosa play a major role in intestinal immune homeostasis and control the threshold of local inflammation. The aim of this study was to evaluate the efficacy of probiotics in the recurrent trinitrobenzene sulfonic acid (TNBS)-induced colitis model and gain more insight into protective mechanisms.

Methods: Moderate chronic inflammation of the colon was induced in BALB/c mice by repetitive intrarectal challenges with TNBS. Administration of probiotics started 2 weeks before colitis induction and was continued throughout colitis development.

Results: Long-term administration of *Lactobacillus plantarum* NCIMB8826 or the probiotic mixture VSL#3 reduced intestinal inflammation induced by TNBS, evident from improved colon morphology and less influx of innate (CD11b⁺) and adaptive (CD4⁺/CD8⁺) immune cells in the intestinal mucosa and decreased proinflammatory serum cytokines (interferon-gamma [IFN- γ], interleukin [IL]-17, IL-1 β , monocyte chemoattractant protein [MCP]-1) in probiotic-treated mice. Genomewide expression analysis of colonic tissues using microarrays revealed differences in expression of genes related to inflammation and immune processes between untreated and probiotic treated mice. Principal component analysis revealed that probiotic treatment resulted in a shift of gene expression profiles toward those of healthy controls. Effects of probiotics on colonic gene expression were most profound during active inflammation, in particular on gene clusters related to mast cells and antimicrobial peptides. The results were substantiated by suppression of chemokine gene expression.

Conclusions: Our data are in favor of a model in which probiotics downregulate expression of chemokines in the colon, thereby decreasing influx of inflammatory cells and rendering mice resistant to the induction of colitis.

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Key Words: TNBS colitis, probiotic bacteria, genome wide screening, immune modulation

Inflammatory bowel disease (IBD) refers to chronic, spontaneously relapsing inflammation of the gastrointestinal tract, including Crohn's disease (CD) and ulcerative colitis (UC) as two major entities. The exact etiology and pathogenesis of IBD is still unclear, but it includes genetic, immunological, and environmental factors.^{1,2} Standard therapies comprise salicylates, corticosteroids, immunomodulating, and immunosuppressive drugs.³ However, these

strategies are not effective in many patients and put the patients at risk to develop opportunistic infections or treatment-related cancers. Alternative strategies such as altering the composition of intestinal microbiota with selected prebiotics, probiotics, and/or synbiotics have been shown effective in inducing sustained remission of UC patients.⁴

Interactions between host and microbiota play a key role in the susceptibility for experimental colitis, as germ-free mice do not develop colitis.⁵ The intestine is home to a complex population of trillions of commensal bacteria that participate in the digestive process and interact with the mucosal immune system of the host.⁶ The interaction of commensal bacteria with epithelial and immune cells in the intestine may cause both proinflammatory and regulatory immune responses.⁷ Therefore, altering the composition of the intestinal microbiota with probiotics has been suggested as an alternative approach in either maintaining or establishing intestinal homeostasis in IBD patients.

Probiotics are defined as viable, nonpathogenic microorganisms that if ingested in sufficient numbers have a beneficial effect on the host. Different mechanisms have

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been suggested to explain this beneficial effect, such as colonization resistance, enhancing immune function by interacting with innate immune cells in the mucosa, and regulation of the intestinal barrier integrity.⁸ Indeed, clinical efficacy of probiotics has been proven in several studies in IBD patients and animal models of IBD.

In this study we examined the effect of two probiotics, *Lactobacillus plantarum* NCIMB8826 and VSL#3, on intestinal inflammation in the recurrent trinitrobenzene sulfonic acid (TNBS) colitis model. VSL#3 was selected as a clinically validated product to identify pathways leading to a decreased inflammatory state. In this respect, we had a particular interest in the modulation of Toll-like receptors (TLR) and pathways involved in the recruitment of inflammatory cells. We are the first to perform genomewide screening of colonic tissue in this recurrent model of colitis in order to identify processes and pathways that are modulated by probiotics. This screening revealed that effects of VSL#3 treatment were most abundant during active inflammation, especially on gene clusters related to mast cells and antimicrobial peptides, and these effects were reflected by decreased influx of inflammatory cells.

MATERIALS AND METHODS

Mice

Eight-week-old female BALB/c mice (18–21 g) were obtained from Janvier (St. Berthevin, France) and maintained in the animal facility of TNO under specific pathogen-free conditions with a 12-hour light/dark cycle. Mice had free access to standard mouse chow (SSNIFF R/M-H, Bioservices, Uden, The Netherlands) and water. Animal experiments were approved by the institutional Ethical Committee on animal care and experimentation.

Probiotic Strains

VSL#3 (Ferring Pharmaceuticals, Berkshire, UK) was purchased as a commercially available probiotic mixture containing freeze-dried bacteria (*Bifidobacterium longum*, *B. breve*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. casei*, *L. bulgaricus*, and *Streptococcus thermophilus*).

L. plantarum NCIMB8826 was grown at 37°C in Mann-Rogosa Sharpe broth (Scharlau Chemie, Barcelona, Spain) until mid-exponential growth phase ($OD_{600nm} = 1$). Bacteria were washed twice in phosphate-buffered saline (PBS) at pH 7.4, suspended at 2×10^9 cells/mL in PBS containing 25% glycerol, and stored at -80°C until use. Before use the bacterial suspensions were washed once with PBS. Mice were treated three times a week by oral administration of 3×10^8 CFU *L. plantarum* or VSL#3, suspended in 200 μL PBS, or 200 μL vehicle (PBS) only, starting 14 days before the first intrarectal challenge with TNBS.

Induction of Colitis

Colitis induction was performed as described previously,⁹ with slight modifications. Briefly, mice were sensitized by application of 3.75 mg of TNBS (Sigma-Aldrich, St. Louis, MO) in 48% (v/v) ethanol to the shaved dorsal skin on day 0. Experimental colitis was induced by administration of increasing doses (0.75, 1.0, and 2.5 mg/mouse) of TNBS in 40% ethanol on days 7, 14, and 21, respectively. Mice were anesthetized with 3% isoflurane and then administered TNBS in ethanol via a sterile 2.0 mm catheter (Unomedical A/S, Birkerød, Denmark) inserted 35 mm intrarectally. Mice were kept in a head-down position for an additional 30 seconds to ensure distribution of TNBS in the colon. On day 28, i.e., 7 days after the third TNBS challenge mice, were sacrificed and colons removed. After the removal of feces, colons were subjected to macroscopic evaluation before further processing for RNA isolation and histology.

Analysis of Serum Cytokines

Serum cytokine and chemokine protein levels were quantified using a Mouse Cytokine 23-PlexPanel (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The assay is based on multiplex technology and simultaneously measures interleukin (IL)-1 α , -1 β , -2, -3, -4, -5, -6, -9, -10, -12(p40), -12(p70), -13, -17, interferon-gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), RANTES, MIP-1 α , and -1 β , monocyte chemoattractant protein (MCP)-1, KC, G-CSF, GM-CSF, and eotaxin protein. The beads were read on a LiquiChip 200, (Qiagen, Hombrechtikon, Switzerland), and data were analyzed by the five parameter curve fitting in Luminex100 IS Software.

Transcriptome Analysis

Five mice per experimental group were selected for transcriptome analysis. The selection of animals was performed 1 day before sacrifice and based on weight loss only. The average weight loss of the mice selected for transcriptome analysis was representative for all mice in each of the experimental groups. Total RNA was isolated from frozen colon tissue using TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. RNA was treated with DNase and purified using a nucleospin RNAII Total RNA Isolation kit (Macherey-Nagel, Düren, Germany). Concentrations and purity of RNA samples were determined with a NanoDrop ND-1000 spectrophotometer (Isogen, The Netherlands). RNA integrity was checked employing an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) with 6000 Nano Chips according to the manufacturer's instructions. RNA integrity numbers (RIN) were above 8.

Biotinylated cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX) according to the manufacturer's specifications starting with 500 ng total RNA. Per sample, 750 ng of cRNA was used to hybridize to the Sentrix MouseRef-8 BeadChips (Illumina,

San Diego, CA). Each BeadChip contains eight arrays and each of the arrays harbors 25,697 probes. Hybridization and washing were performed according to the Illumina standard assay procedure. Scanning was performed on the Illumina iScanner. Image analysis and extraction of raw and background subtracted expression data were performed with Illumina Beadstudio v. 3 Gene Expression software using default settings.

GeneSpring GX 11.0 was used for quantile normalization of the probe level, background-subtracted expression values. After normalization, unexpressed probes were removed from the subsequent analyses. Probes were considered to be expressed if the probe was present (detection $P \geq 0.99$) in at least 3 out of 20 samples in the dataset.

After this filtering procedure, 13,551 probes remained in the analysis. All expression values below 5 (2.322 on log2 scale) were floored to 5. Differentially expressed probes were identified using the limma package of the R/Bioconductor project, applying linear models and moderated t -statistics that implement empirical Bayes regularization of standard errors.¹⁰ The statistical analyses were performed through the Remote Analysis Computation for gene Expression data (RACE) suite at <http://race.unil.ch>.¹¹ P -values below 0.05 were used as a threshold for significance of the differential expression. Pathway analysis software Metacore (v. 6.2), a highly curated Web-based application for identification of gene ontology processes in input genesets, was used to identify biological processes (GeneGo, St. Joseph, MO).

Reverse-transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Three genes encoding chemokines, which were upregulated during colitis, were selected for validation with RT-qPCR. 500 ng of RNA was used for single-stranded cDNA synthesis using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol. RT-qPCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR system. Primer sequences used were as follows: CCL2 5'-TAGGCTGGAGATCTACAAG AGG-3' (S) and 5'-AGTGCTTGAGGTGGTTGTGG-3' (AS), CCL11 5'-GGCTGACCTCAAACCTCACAGAAA-3' (S) and 5'-ACATTCTGGCTTGGCATGGT-3' (AS), CCL24 5'-GCAG CATCTGTCCCAAGG-3' (S) and 5'-GCAGCTTGGGGT CAGTACA-3' (AS). Each reaction contained 25 ng cDNA, 0.3 μ M sense and antisense primers, and Sybr Green Master Mix (Applied Biosystems) under the following thermal conditions: 50°C for 1 minute, 95°C for 10 minutes, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, followed by a dissociation stage. Relative mRNA expression was normalized to β -actin and was expressed using the $\Delta\Delta C_t$ method.

Immunohistochemistry

Colon fragments adjacent to those used for RNA isolation were fixed in 4% buffered formaldehyde and embedded in paraffin, or embedded in Tissue-Tek and snap-frozen in liquid nitrogen.

From paraffin-embedded tissue, 5- μ m sections were stained with either Toluidine blue and counterstained by hematoxylin-eosin in order to assess numbers of mast cells, or with hematoxylin-eosin-safran for microscopical evaluation.

Six- μ m cryosections from frozen colon fragments were fixed in ice-cold acetone for 10 minutes and air-dried. Sections were blocked for endogenous peroxidase (Peroxidase Blocking Agent, Dako Cytomation, Glostrup, Denmark), avidin/biotin (SP-2001, Vector Laboratories, Peterborough, UK), and 5% bovine serum albumin (BSA) for nonspecific antibody binding. Slides were incubated overnight at 4°C with the following rat antimouse monoclonal antibodies (BD Biosciences, San Diego, CA) in appropriate dilutions: CD4 (clone H129.19) and CD8-bio (clone 53-6.7), IgG2a isotype (clone R35-95); CD11b-bio (clone M1/70), FcR ϵ I-bio, (clone MAR-1), IgG2b-bio isotype (clone A95-1). Bound primary antibody was detected via goat antirat-biotin and/or peroxidase-labeled streptavidin (SA-5004, Vector Laboratories). Slides were developed with Novared substrate (SK-4800, Vector Laboratories) and counterstained with hematoxylin. Three nonserial colon sections per mice were used for quantification by counting immunopositive cells at 400 \times magnification, which were normalized against the mucosal area.

RESULTS

Treatment with Probiotics Reduce Primary Outcome Parameters of Recurrent TNBS Colitis

Mice were subjected to oral treatment with probiotics in PBS or with PBS (vehicle), starting 2 weeks before the first rectal TNBS challenge. During the experimental period, healthy control mice that were left untreated showed gain of bodyweight as compared to baseline (109.0 \pm 3.7%). In contrast, mice subjected to colitis induction showed a transient weight loss for 3 days immediately following each TNBS instillation (Fig. 1A). At endpoint (day 28), mice subjected to colitis induction showed less gain in bodyweight (104 \pm 4.0%; $P < 0.01$) as compared to the healthy controls. Although also the mice treated with *L. plantarum* or VSL#3 showed transient weight loss after each TNBS challenge, they showed less weight loss over the entire follow-up period compared to vehicle-treated mice challenged with TNBS (Fig. 1A; repeated measurements analysis of variance [ANOVA], $P < 0.001$ and $P < 0.01$ for *L. plantarum* and VSL#3, respectively).

Seven days after the third TNBS challenge (i.e., on day 28), mice were sacrificed and colons were evaluated macroscopically. Colons of vehicle-treated mice showed an increased weight/length ratio compared to healthy mice (Fig. 1B; $P < 0.001$). Colitis was also evident from thickening of the rectal part of the colon ($P < 0.01$; data not shown). Although hemorrhages (as shown by histological evaluation) and the absence of solid stool are a hallmark during the acute phase following each rectal TNBS

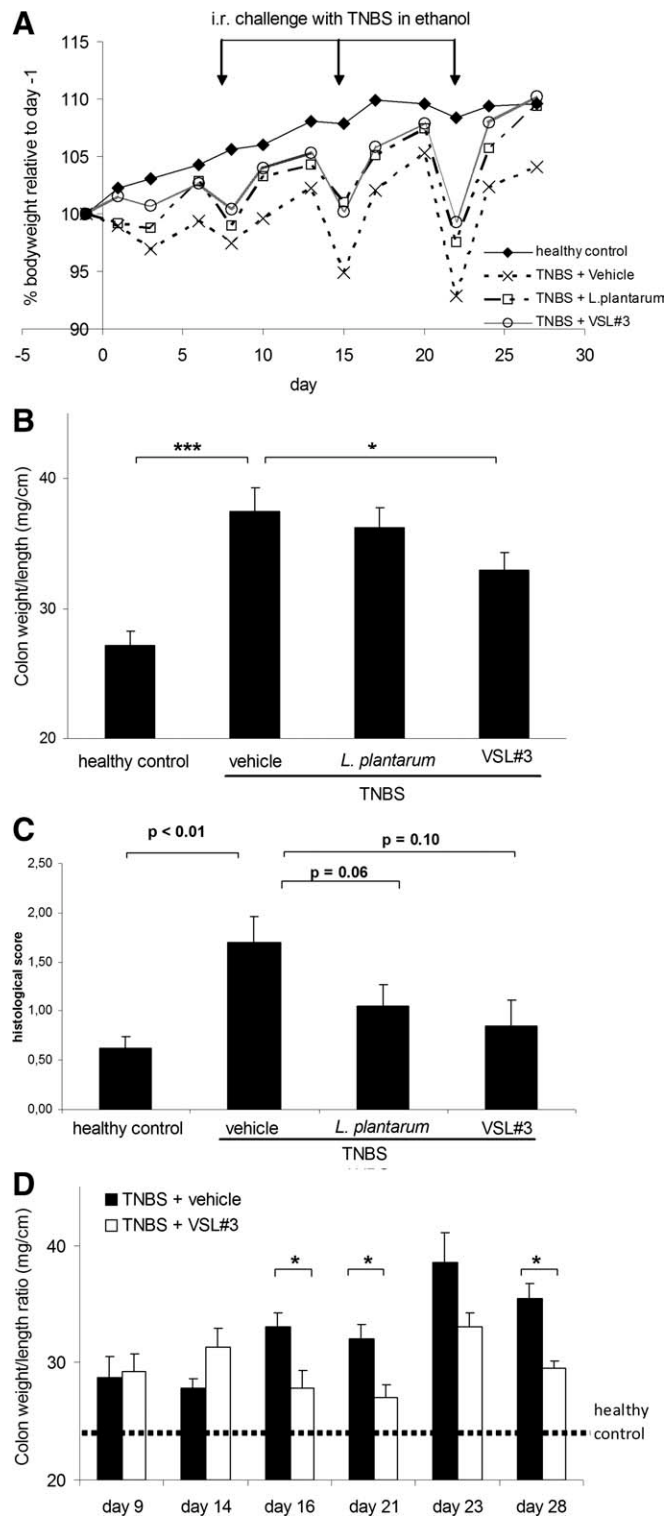


FIGURE 1. (A) Body weight development in the recurrent TNBS colitis model. Body weights were normalized against the body weight on day -1. Colon weight/length (B) is a parameter reflecting changes in colon morphology ($n = 10-12$ mice/group). (C) Histological score of the colon. (D) Development of colon weight/length ratio in time ($n = 8$ mice per group). (A,B,C) Representative of two independent experiments. Mann-Whitney U -test; * $P < 0.05$, *** $P < 0.001$.

instillation, these features were less pronounced on day 28 and are therefore not included in the evaluation of treatment effects. Adhesions of the colon were only sporadically observed.

VSL#3-treated mice showed a less pronounced increase in the colon weight/length ratio as compared to vehicle-treated mice (Fig. 1B; $P < 0.05$) without having a significant effect on the colon thickness (data not shown). *L. plantarum* did not show effects on the macroscopic score. In a separate time-course experiment, VSL#3 was shown to be most effective after the second and third challenge (Fig. 1D).

Histopathological evaluation of the rectal part of the colons was performed to establish a composite score as described before,¹² based on mucosal architecture, cellular infiltration, muscle thickening, goblet cell depletion, and crypt abscess formation. On day 28, in particular, cellular infiltration and loss of mucosal architecture contributed to the score in mice with colitis (Fig. 1C). Both VSL#3 and *L. plantarum* showed a modest inhibitory effect on this semiquantitative score. As will be discussed below, significant inhibitory effects of probiotics on cellular infiltration were demonstrated after quantification of discrete subsets of cells.

Genomewide Gene Expression Analysis

To gain insight into colitis-associated processes modulated by *L. plantarum* and VSL#3, RNA from colonic tissue was isolated 7 days after the last TNBS challenge and subjected to genomewide screening. Microarray analysis identified 831 probes that were differentially expressed between healthy mice and mice with colitis. Postgenomic validation of microarray data with RT-qPCR for six genes showed a strong correlation between both techniques (data not shown). The complete list of differentially expressed genes is provided in Supporting Table 1. The effect of probiotic treatment on TNBS colitis was visualized by principal component analysis (PCA) on the set of 831 probes that differed between healthy mice and mice with colitis (Fig. 2). PCA analysis allows grouping of individual mice with overall similar gene expression profiles. Although all 831 genes contributed to the differentiation between healthy control mice and colitis animals, dominant clusters of genes encoding mast cell enzymes, defensin-related peptides, as well as other immune-related transcripts. This plot showed that long-term probiotic administration to mice subjected to colitis induction exhibited a profile between healthy and diseased mice, although still closer to diseased mice. To gain insight into disease-related processes that were modulated by probiotics, we next focused on genes and gene clusters affected by probiotic intervention during colitis. Gene ontology (GO) classification using Metacore pathway analysis was applied on genes that were

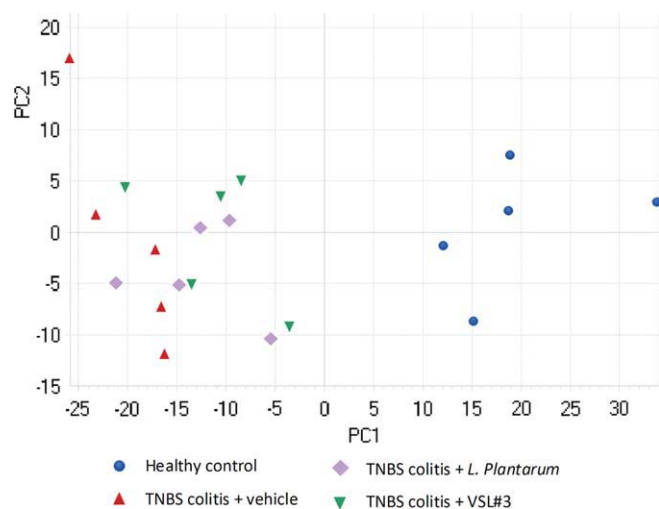


FIGURE 2. Two-dimensional visualization of PCA constructed with the set of 831 differentially expressed genes in the colon upon colitis induction, and position of colitis mice treated with *L. plantarum* or VSL#3 in this space. Each dot represents the expression profile of an individual mouse. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

normalized in probiotic-treated mice. Cellular processes that were normalized by VSL#3 treatment were mainly related to DNA replication and regulation of immune functioning. Each intrarectal TNBS challenge was associated with upregulation of gene clusters involved in remodeling of the extracellular matrix and the cytoskeleton; upregulation of these genes was suppressed by VSL#3 treatment in particular 2 days after the first TNBS challenge.

Another set of genes that was upregulated in the colons of mice with colitis comprised α -defensins and related antimicrobial peptides. Table 1 shows a list, including the fold-change and *P*-values, to illustrate the inhibitory effect of VSL#3 on upregulation of these defensin-related cryptidins. *L. plantarum* was ineffective in this respect.

At several timepoints dedicated evaluation of genes encoding TLR or associated pathways did not reveal major changes in response to colitis induction and/or probiotics. Yet, as will be discussed below, major effects of colitis induction and probiotic treatment concerned inflammatory mechanisms.

Probiotics Inhibit the Expression of Gene Transcripts Associated with Mast Cell Recruitment and Activity

Detailed analysis revealed increased expression of mast cell protease-1, mast cell protease-4, chymase-1, chymase-2, carboxypeptidase A3, and Fc ϵ RI in mice with colitis. Figure 3 shows a heat map of expression profiles of these mast cell-related genes in mice with colitis compared to healthy controls, as well as expression patterns in mice treated with probiotics. Although not all of these genes were comparably induced by colitis induction, the overall pattern strongly suggested an increase of mast cells in the tissues, which was not observed in mice subjected to treatment with VSL#3 or *L. plantarum*. As will be discussed in the next section, these observations were partly reflected by lower numbers of mast cells.

Because local chemokine production is key to the infiltration of the intestine by immune cells, we addressed the expression of a set of chemokines during the course of colitis in this model. We assessed gene expression profiles of mice treated with VSL#3, 2 and 7 days after each TNBS challenge, and compared these profiles with vehicle-treated mice. As shown in Figure 4A, genes encoding several chemokines involved in the recruitment of mast cell progenitors and other inflammatory cells to the intestinal mucosa were upregulated 2 days after each TNBS challenge, but not at 7 days postchallenge. RT-qPCR confirmed the enhanced expression of chemokines after each TNBS

TABLE 1. Effect of *L. plantarum* and VSL#3 Treatment on TNBS-induced Upregulation of α -defensins

α -defensin	TNBS VSL#3 vs. TNBS vehicle		TNBS <i>L. plantarum</i> vs. TNBS vehicle	
	Fold change	P	Fold change	P
defensin related cryptdin 20 (Defcr20))	-9.7	0.006	-1.9	0.38
defensin related cryptdin 26 (Defcr26)	-5.0	0.009	-1.8	0.48
defensin related cryptdin 4 (Defcr4)	-5.4	0.026	-1.6	0.62
defensin related cryptdin 5 (Defcr5)	-4.8	0.031	-1.8	0.44
defensin related cryptdin 6 (Defcr6)	-11.2	0.002	-2.7	0.21
defensin related cryptdin, related sequence 10 (Defcr-rs10)	-4.8	0.069	-1.4	0.77
defensin related cryptdin, related sequence 7 (Defcr-rs7)	-5.0	0.031	-1.3	0.74
defensin related sequence cryptdin peptide (Defcr-rs1)	-13.8	0.004	-2.4	0.28
defensin, alpha 1 (Defa1)	-12.5	0.006	-2.3	0.34

Fold change is defined as the ratio of the signal of mice treated with TNBS and probiotic to that of mice treated with TNBS + vehicle.

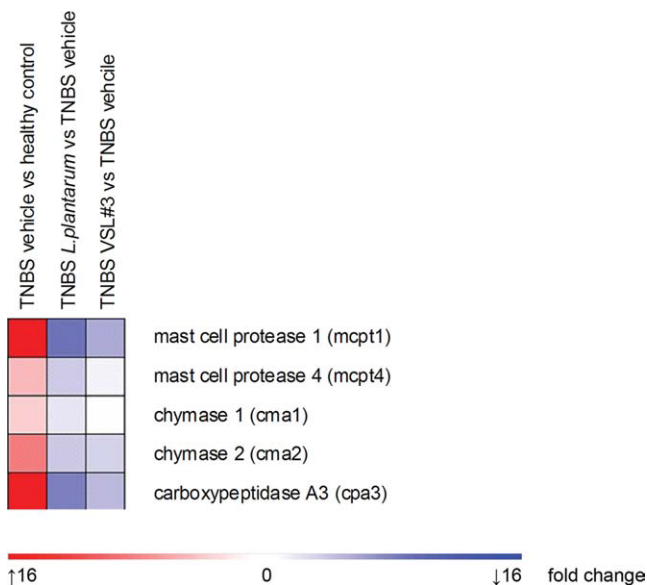


FIGURE 3. Heat map of gene expression of mast cell-associated enzymes (red indicates upregulation, blue indicates downregulation). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

challenge; this analysis also showed that VSL#3 treatment suppressed upregulation of these chemokines during the first TNBS challenge (Fig. 4B), but not at later timepoints (data not shown).

Probiotics Prevent Colitis-associated Influx of Inflammatory Cells into Colonic (sub)Mucosa

As shown in Figure 5A, TNBS-induced colitis was associated with increased infiltration of the lamina propria and the submucosa by mast cells. Although treatment with probiotics resulted in a lower expression of mast cell-associated enzymes, this was only in part reflected by lower numbers of mast cells, visualized by Toluidine blue or specific staining with anti-FcεRI.

Also, numbers of other inflammatory cells including CD4⁺ T cells, CD8⁺ T cells, and CD11b⁺ innate immune cells confirmed as macrophages by F4/80 staining were significantly increased after TNBS colitis induction. Mice treated with *L. plantarum* or VSL#3 showed significantly lower numbers of these cells (Fig. 5B).

To study whether these inflammatory processes were reflected in serum at endpoint, we performed a multiplex analysis and evaluated a panel of 23 cytokines and chemokines. Table 2 displays only those cytokines and chemokines that were modulated in mice treated with probiotics. Colitis induction was associated with increased levels of several (pro)-inflammatory cytokines and chemokines (GM-CSF, IFN-γ, IL-10, IL-17, IL-1β, and MCP-1). Mice treated with VSL#3 showed

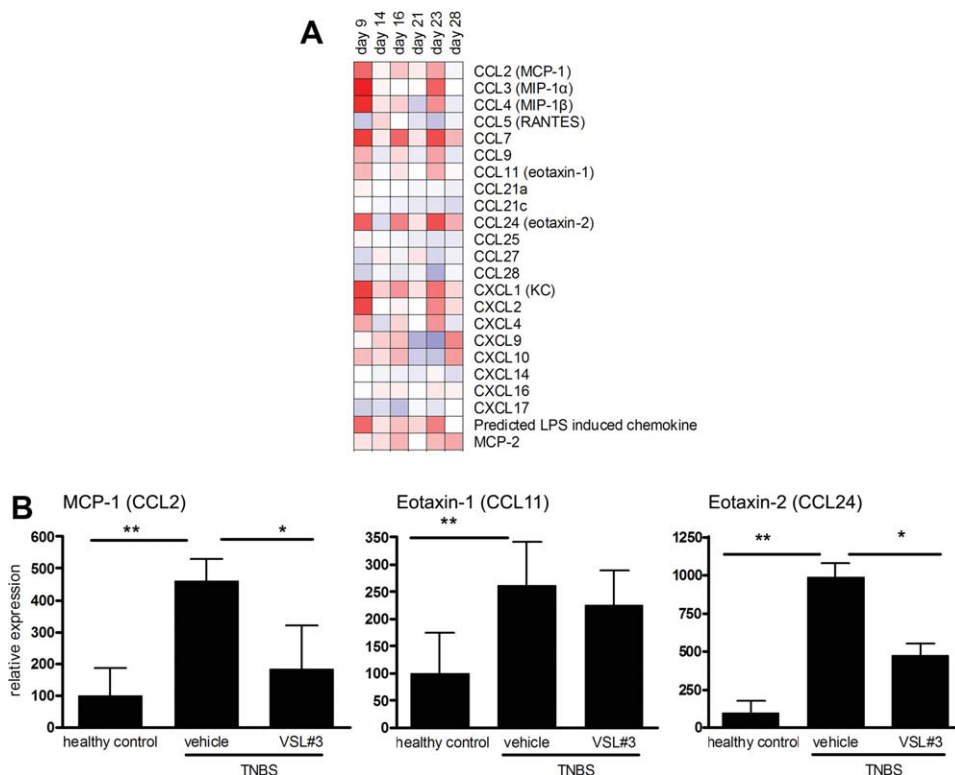


FIGURE 4. (A) Chemokine expression in the TNBS colitis model at various timepoints. (B) Effect of VSL#3 treatment on gene expression profiles of chemokines involved in the recruitment of mast cells on day 9. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

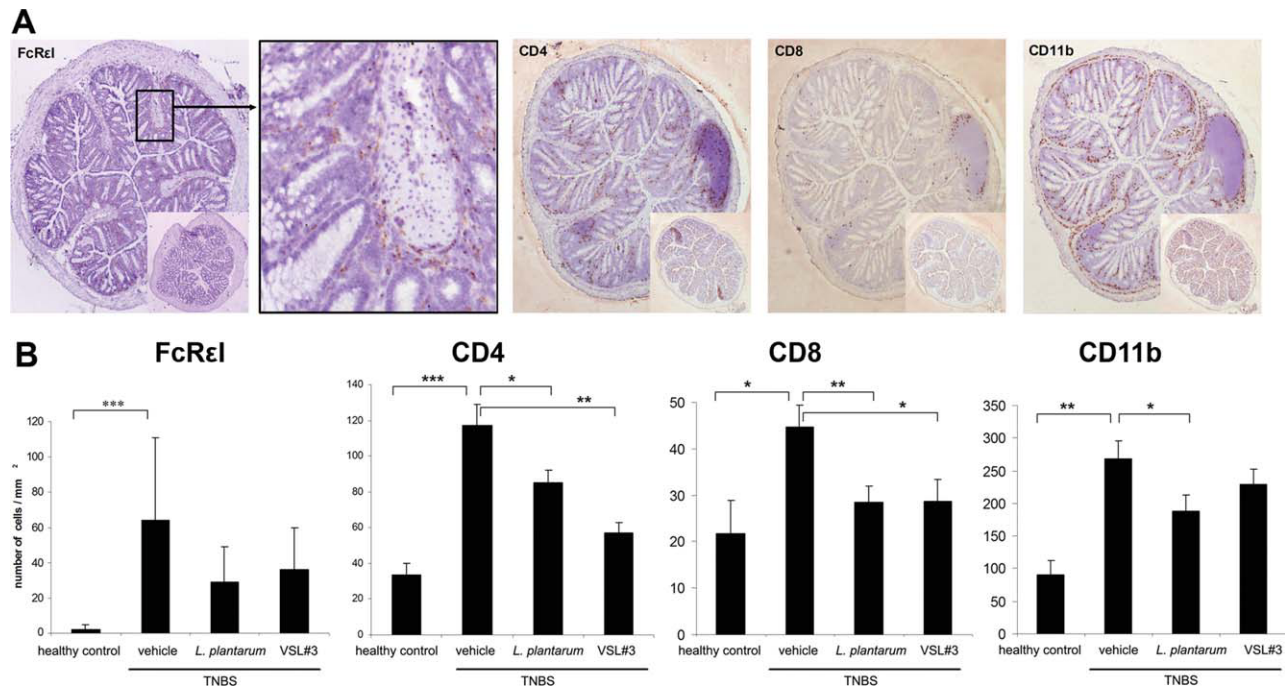


FIGURE 5. Probiotics decrease infiltration of lamina propria and submucosa by inflammatory cells. (A) Representative section of inflamed colon tissue collected on day 28 and immunostained with anti-FcRεI anti-CD4, anti-CD8, or anti-CD11b The inset shows positive cells in healthy control mice. (B) Quantification of positive cells was based on three nonserial colon sections per mice and normalized against the mucosal area. Bars represent group means for the number of cells on day 28. Mann-Whitney *U*-test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significantly decreased levels of IFN-γ, IL-17, GM-CSF, IL-1α, IL-1β, MCP-1, and MIP-1α as compared with vehicle controls. In this respect, *L. plantarum* treatment

was less effective, with a trend toward downregulation of several of these cytokines and only significant inhibition of IL-17 and IL-3.

TABLE 2. Probiotic Treatment Prevents Induction of Various Cytokines and Chemokines Associated with TNBS Colitis

	Concentration of Cytokine/Chemokine in Serum (Mean pg/mL ± SD)			
	Healthy vehicle	Vehicle ^a	<i>L. plantarum</i> ^b	VSL#3 ^b
G-CSF	53 ± 42	45 ± 19	42 ± 20	26 ± 10*
GM-CSF	185 ± 42	214 ± 43 [†]	182 ± 31 [†]	169 ± 29*
IFN-γ	530 ± 240	763 ± 184 [†]	643 ± 242	463 ± 170**
IL-10	63 ± 51	196 ± 101*	126 ± 49 [†]	121 ± 73
IL-17	77 ± 55	149 ± 68*	92 ± 54*	64 ± 30**
IL-1α	237 ± 91	273 ± 60	219 ± 69 [†]	179 ± 81*
IL-1β	1004 ± 379	1499 ± 547 [†]	1078 ± 521 [†]	887 ± 271*
IL-3	11 ± 5	19 ± 16	10 ± 3*	15 ± 4.3*
IL-9	193 ± 43	267 ± 185	187 ± 73	152 ± 66*
MCP-1	560 ± 243	878 ± 291*	712 ± 254	542 ± 185*
MIP-1α	1593 ± 434	1929 ± 399	1583 ± 346	1348 ± 268**

Serum of individual mice, collected at endpoint on day 28, was assayed by multiplex analysis as described in Materials and Methods. Results represent mean + SD of 6 to 10 mice per group.

^aMann-Whitney *U*-test as compared to healthy mice.

^bMann-Whitney *U*-test as compared to vehicle-treated mice with colitis.

[†]*P* < 0.10; **P* < 0.05; ***P* < 0.01.

DISCUSSION

Relapsing colitis in BALB/c mice was mimicked by weekly intrarectal administrations of low-dose TNBS in ethanol. The model is characterized by the initial production of Th1 cytokines followed by a Th17-like profile after 3 weeks.⁹ We confirmed increased levels of IFN- γ , IL-17, and several chemokines in serum samples collected 3 weeks after the first rectal TNBS challenge, in conjunction with a gradual increase of inflammatory cells in the colon.

It has been suggested that probiotics may modulate local immune responses by affecting regulatory T cells and/or T-cell differentiation.¹³ Therefore, we chose a relatively early timepoint with developing pathology to increase the likelihood to detect subtle effects of probiotics. Likewise, we selected a timepoint of 7 days after the third intrarectal challenge to avoid the acute phase directly following each rectal challenge. Altogether, our results reflect effects of probiotics on a gradually developing process of inflammation, characterized by the influx of T cells and macrophages with moderate pathology as an aspect of IBD, rather than effects on disease with high severity.

In the present study we have shown that this model is sensitive to prophylactic treatment with probiotics, evident from less intestinal inflammation and normalized colonic gene expression profiles. Beneficial effects of probiotics were previously reported for *L. plantarum* NCIBM8826 and VSL#3 in spontaneous^{14,15} and chemically induced¹⁶⁻¹⁸ models of experimental colitis. Here we confirm that probiotics significantly affect the host, rendering the mice largely resistant to the development of disease in response to multiple challenges with TNBS. Moreover, we substantiated these effects by genomewide mRNA profiling. In our experiments, both *L. plantarum* and VSL#3 attenuated the development of clinical features of colitis, with favorable effects on gain of bodyweight and a slight improvement of colon morphology.

Both in UC and CD, the colonic mucosa is infiltrated by neutrophils, macrophages, and lymphocytes. This feature of human IBD is reflected in the relapsing TNBS colitis model by the infiltration of CD4⁺, CD8⁺ T cells, macrophages, and mast cells in the intestinal mucosa after three repetitive colitis inductions. Influx of these inflammatory cells was reduced in mice treated with either *L. plantarum* or VSL#3, and this might be due to local downregulation of chemokines. Indeed, various chemokines key to the recruitment of inflammatory cells were less upregulated after the first TNBS challenge in mice treated with VSL#3. At endpoint, diminished levels of MIP-1 α , G-CSF, GM-CSF, and MCP-1 were demonstrated in serum. The beneficial effects of probiotics in this study were also evident from an overall reduction of inflammatory serum cytokines. Seven days after the last TNBS challenge untreated mice with colitis showed increased levels of sev-

eral cytokines, including IL-1 α , IL-1 β , IFN- γ , MIP-1 α , and IL-17. These cytokines are suggestive for an involvement of macrophages and a mixed Th1/Th17 population.¹⁹⁻²¹ These observations are of importance, since CD has recently been characterized as a Th1/Th17-driven inflammatory disease.²² Importantly, these cytokines also were significantly lower in serum of mice treated with VSL#3.

There is strong evidence for an involvement of mast cells in the pathogenesis of IBD.²³ This is of interest since the relapsing colitis model involves a mast cell component, evident from progressively elevated mRNA levels of transcripts encoding mast cells (Kremer et al., submitted). However, the exact role of mast cells in intestinal inflammation is a topic of debate. Mast cells were shown to protect from colitis by enhancing the barrier function of epithelial cells and by limiting spontaneous development of colitis in susceptible IL-10^{-/-} mice.²⁴ However, mast cells have also been shown to increase epithelial barrier permeability²⁵ and the recruitment of inflammatory cells to the site of infection by the release of cytokines and several proinflammatory mediators like trypases and chymases.^{26,27} In the TNBS colitis model the pathology is mast cell-dependent, since colitis could not be induced in mast cell-deficient mice.²⁸ Furthermore, mast cell stabilizers^{29,30} reduce symptoms of colitis. In our study, VSL#3 was able to reduce upregulation of mast cell-related genes in acute phases of colitis. During the first acute phase, following the first rectal challenge, this was accompanied by suppression of upregulation of MCP-1 (CCL2), Eotaxin-1 (CCL11), and Eotaxin-2 (CCL24). These chemokines have been implicated in the recruitment of mast cells^{31,32} and can be produced by intestinal epithelia cells or leukocytes in the colonic mucosa. Recently, studies in the dextran sodium sulfate (DSS) colitis model demonstrated that Ly6C^{high}CCR2⁺ inflammatory macrophages are the major source of CCL11.³³ It might therefore be that the antiinflammatory effects of VSL#3 are partly due to a direct effect on these cells, thereby limiting mast cell recruitment. Strategies to modulate mast cell infiltration may be a therapeutic strategy in human IBD, since increased numbers of mast cells are found in both inflamed and noninflamed tissue in UC and CD patients.³⁴

Although several genes downstream of TLR, including cytokines and chemokines, were modulated by probiotics, we did not observe differential expression of all 13 known mouse TLRs. Also, Metacore analysis did not reveal significant enrichment of genes involved in TLR signaling. Possibly such effects require a more dedicated analysis of specific cell type rather than a heterogeneous population of cells as present in the intestinal mucosa.

In view of the antiinflammatory effects of VSL#3 treatment, it was surprising to observe that VSL#3 reduced colitis associated upregulation of α -defensin mRNA levels

in the inflamed colon. α -Defensins are predominant antimicrobial factors involved in the host defense against bacteria, fungi, protean, and viruses.³⁵ In the intestines these antimicrobial peptide are mainly produced by Paneth cells. Until recently, these α -defensins were not considered to be produced in the colon of mice.^{36,37} However, this idea was based on normal colon in homeostatic conditions and more recently α -defensins producing Paneth cells were described to play a role under inflammatory conditions in the colon.³⁸ Our observations are of interest because an increased expression of human defensin A5 and A6 (DEFA5, DEFA6) was also observed in the colon of (pediatric) IBD patients,^{39,40} which was largely due to Paneth cell metaplasia. It has been suggested that the antimicrobial peptides are produced as a mucosal defense mechanism to counteract a bacterial attack, induced by tissue damage. Therefore, we speculate that reduced α -defensin gene expression in VSL#3-treated mice is due to less excessive inflammation and consequently less involvement of metaplastic Paneth cells.

The recurrent TNBS colitis model comprises several features of IBD and the cytokine profiles and histopathological features during the early stages suggest similarities with CD. In view of this idea, our results showing suppression by probiotics may be surprising, because in human clinical trials probiotics have been shown effective in prolonging the remission phase of UC but not in CD.⁴¹ However, it should be taken into account that the recurrent TNBS model is rather a more generalized model of colitis, with shifting cytokine profiles. Moreover, it should be taken into account that our studies reflect the prophylactic activity of probiotic treatment, whereas in patients treatment was initiated during ongoing disease. In view of our data it is tempting to speculate that the primary effect of probiotics is to downregulate chemokines and thereby control the severity of inflammation. Evaluation of probiotic treatment initiated after the induction of colitis may help to reveal which mechanisms are relevant to the efficacy of probiotics in this model.

Further studies addressing the effect of probiotics on local chemokine expression and underlying mechanisms in colitis may open new avenues for the treatment of IBD.

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