The Probiotic Mixture VSL#3 Has Differential Effects on Intestinal Immune Parameters in Healthy Female BALB/c and C57BL/6 Mice1–3

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Abstract

Background: Probiotic bacteria may render mice resistant to the development of various inflammatory and infectious diseases.

Objective: This study aimed to identify mechanisms by which probiotic bacteria may influence intestinal immune homeostasis in noninflammatory conditions.

Methods: The effect of VSL#3, a mixture of 8 probiotic bacteria, on intestinal gene expression was studied in healthy female BALB/c and C57BL/6 mice after prolonged oral treatment (28 d, triweekly) with $3 \times 10^8$ colony-forming units of VSL#3. In a separate experiment in BALB/c mice, the effects of prolonged administration of VSL#3 and of phosphate-buffered saline (PBS), followed by 1 single dose of VSL#3, on innate and adaptive immune cells were evaluated.

Results: Microarray analysis of the intestines of mice treated with PBS confirmed well-established differences in the expression of immune-related genes between C57BL/6 and BALB/c mice. Prolonged administration of VSL#3 was associated with downregulation of $\text{Il}13$ (fold change (FC) = 0.46) and Eosinophil peroxidase ($\text{Epx}$) (FC = 0.44) and upregulation of $\text{Il}12rb1$ (FC = 2.1), $\text{Ccr}5$ (FC = 2.6), $\text{Cxcr}3$ (FC = 1.6), and $\text{Cxcl}10$ (FC = 2.8) in BALB/c mice but not in C57BL/6 mice. In BALB/c mice, it was shown that 28 d of treatment with VSL#3 affected the Peyer’s patches (PPs) and mesenteric lymph nodes (MLNs), which was evident from an increase in B cells (26% and 8%, respectively), a decrease in T cells (21% and 8%, respectively), and an increase in cluster of differentiation (CD) 11c+ cells (57% in PPs) compared with PBS-treated mice. This treatment was also associated with increased frequencies of T helper 17 (13%) and regulatory T cells (11%) in the MLNs. Treatment with PBS followed by 1 single dose of VSL#3, 18 h before killing, was associated with a 2-fold increase in CD103+CD11c+ dendritic cells in MLNs and PPs.

Conclusion: VSL#3 treatment mediates mouse strain–specific alterations in immunologic phenotype in conditions of homeostasis, suggesting that the effects of probiotic bacteria depend on the genetic background of the host.


Keywords: probiotics, immune modulation, microarray, host genetics, flow cytometry

Introduction

Directly after birth, the (human) gastrointestinal tract is colonized by a complex community of microorganisms termed the “microbiota” (1). The community structure of this microbiota directly contributes to a variety of physiologic and metabolic processes that are important to host function (2, 3). Moreover, interactions between microbes and the host are of critical importance in orchestrating the (mucosal) immune system (4). For example, the absence of intestinal bacteria in germfree mice results in defects in the development of gut-associated lymphoid tissue (5) reflected by reduced IgA production by plasma cells (6), fewer Peyer’s patches (PPs) and mesenteric lymph nodes (MLNs) as well as impaired development of isolated lymphoid

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1 This study was performed in the framework of the Dutch Top Institute Pharma project D1-101.
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3 Supplemental Tables 1–3 and Supplemental Figures 1–3 are available from the ‘Online Supporting Material’ link in the online version of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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6 Abbreviations used: APC, Allophycocyanin; Ccl, CC chemokine ligand; CD, cluster of differentiation; Ccr5, C-C chemokine receptor type 5; Cldn, claudin; Cxcl10, C-X-C motif chemokine 10; Cxcr3, chemokine (C-X-C motif) receptor 3; DC, dendritic cell; Epx, Eosinophil peroxidase; FC, fold change; FITC, fluorescein isothiocyanate; FoxP3, Forkhead box P3; Gata3, GATA binding protein 3; IgA, immunoglobulin A; IL-12p70, interleukin 12; MLN, mesenteric lymph node; PB, Pacific Blue; PE, Phycoerythrin; PerCp, Peridinin chlorophyll; PS, Peyer’s patch; ROR, RAR-related orphan receptor; Tbet, T-box transcription factor; Th, T helper; TLR, Toll-like receptor; Treg, regulatory T.
follicles (7). Likewise, the induction of either regulatory T (Treg) or effector T cell responses in the mucosa depends on the bacterial species present (8). For example, segmented filamentous bacteria induce effector T helper (Th) 17 responses, which provide protection against gut pathogens (9, 10). In addition, certain Clostridium species and Bacteroides fragilis favor the induction of Foxp3+ Treg responses (11). These Treg cells can prevent systemic and tissue-specific autoimmunity and inflammatory lesions at mucosal interfaces and are thereby essential for the maintenance of immune homeostasis. In turn, the intestinal immune system shapes the composition of the gut microbiota (12–14). In view of the interplay between the host and the intestinal microbiota, probiotic bacteria may also have favorable effects and contribute to intestinal immune homeostasis (15, 16). Beneficial effects of probiotic bacteria have been observed in patients with ulcerative colitis in remission and in patients with atopic disease (17, 18). Several mechanisms of action have been proposed, including modulation of innate and adaptive immune responses, effects on the composition of the gut microbiota, strengthening of the mucosal barrier, and the prevention of microbiological translocation (19, 20). These mechanistic studies mostly relied on in vitro assays or animal models for inflammation (21–23). However, immunomodulation by probiotic bacteria in a simplified in vitro system or in an established inflammation model in vivo does not reflect effects in conditions of homeostasis. Only few studies have addressed the effects of probiotic bacteria in healthy subjects (24–27), and substantial variability in the response of human subjects to probiotic intervention was found (28, 29).

We previously showed that prophylactic treatment with VSL#3 renders BALB/c mice largely resistant to the induction of colitis (30), evident from improved colon morphology, less influx of innate and adaptive immune cells in the intestinal mucosa, and decreased proinflammatory serum cytokines in VSL#3-treated mice.

Therefore, probiotic bacteria may represent important nutritional ingredients that contribute to intestinal immune homeostasis and protection against inflammatory disease. To gain insight into the underlying mechanism of protection, we evaluated the effect of a mixture of probiotic bacteria in healthy mice. To simulate the genetic variation in the human population, we evaluated the effect of a mixture of probiotic bacteria in a simplified in vitro model (19, 20). These mechanistic studies mostly relied on in vitro assays or animal models for inflammation (21–23). However, immunomodulation by probiotic bacteria in a simplified in vitro system or in an established inflammation model in vivo does not reflect effects in conditions of homeostasis. Only few studies have addressed the effects of probiotic bacteria in healthy subjects (24–27), and substantial variability in the response of human subjects to probiotic intervention was found (28, 29).

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Methods

Mice. Specific pathogen-free 9-wk-old female C57BL/6 mice (Charles River) and female BALB/c mice (Janvier) were used in this study. Female mice were chosen to avoid the potential for aggressive behavior, which can be an uncontrolled variable in group-housed male mice. Upon arrival, mice were allowed a 1-wk acclimation period at a housing density of 6 mice per cage. At the end of the acclimation period, mice were randomly assigned to the experimental groups. Mice were conventionally housed with free access to standard mouse feed pellets and acidified tap water. All caging and equipment were routinely mechanically sanitized in an automated cage/rack washer and sterilized before use. Sentinel mice were kept in each room to evaluate their microbial status on a regular basis. The SSNIFF R/M-H feed pellet diet (BioServices B.V.) contains (g/kg dry matter) protein, 216; nitrogen-free extract, 617; fat, 38; fiber, 56; and ash, 73; and is supplemented with vitamins and minerals and 16.0 MJ/kg metabolizable energy. More detailed information about the diet composition can be found in Supplementary Table 1. Animal experiments were approved by the Institutional Animal Welfare Committee of The Netherlands Organization for Applied Scientific Research (TNO; approval numbers DEC2981 and DEC3288), in compliance with European Community regulations regarding the use of laboratory animals.

Study design. VSL#3 (Ferring Pharmaceuticals) was purchased as a commercially available probiotic mixture containing freeze-dried bacteria (Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus bulgaricus, and Streptococcus thermophiles). In this report, the results of 2 experiments are discussed in detail. A graphical representation of the experimental design is depicted in Supplemental Figure 1. In Expt. 1, mice were treated by oral gavage with PBS or 3 × 108 CFU VSL#3 suspended in 200 μL PBS, 3 times/wk, for a period of 28 d (n = 5/group). Both BALB/c and C57BL/6 mice were included in this experiment. After killing, the intestines were dissected and feces were removed by gentle pressure with a forceps. The distal portion (~30 mm) of the small intestines and entire colons were snap-frozen in liquid nitrogen and stored at −80°C for RNA isolation. Vehicle-treated mice were used to establish baseline differences between BALB/c and C57BL/6 mice.

In Expt. 2, we evaluated the effect of prolonged VSL#3 treatment and the immediate effects of 1 single dose of VSL#3 with respect to innate and adaptive immune cells in BALB/c mice. Again, mice were treated by oral gavage with PBS or 3 × 108 CFU VSL#3 suspended in 200 μL PBS, 3 times/wk, for a period of 28 d (Supplemental Figure 1; n = 8 mice/group). In addition, a group was included that received PBS for 27 d, followed by 1 single oral dose of 3 × 108 CFU VSL#3 18 h before killing. In this way, we controlled for the age and handling effects, which allowed us to use the same control group for both treatment arms in this experiment. Spleen, MLNs, and PPs were removed and stored in Roswell Park Memorial Institute medium at 4°C.

Our previous studies (32) in colitis showed that 5 mice/group were sufficient to demonstrate modulation of gene expression and immune variables in the intestines. For Expt. 2, we anticipated on low (or insufficient) cell yields related to the isolation of cells from the different tissues and therefore increased the number of mice per experimental group at the start of the experiment.

Flow cytometry analysis. Spleen, MLNs, and PPs were isolated and passed through a 70-μm nylon cell strainer (BD Biosciences) to prepare single-cell suspensions. Cells were stained with different rat anti–mouse antibodies obtained from Biologen/BD Biosciences: cluster of differentiation (CD) 3–fluorescein isothiocyanate (FITC) (145–2c11), CD4–Peridinin chlorophyll (PerCp) (DM4–5), CD8–PerCp-Cy7 (53–6.7), CD25–allophycocyanin (APC) (PC61.5), CD45–PerCp-Cy5 (30-F11), CD19–V450 (ID3), IFN-γ–FITC (XM1G1.2), IL-17–Phycoerythrin (PE) (TC11–18H10), CD11c–PE-Cy7 (N418), CD103–Pacific Blue (PB) (2E7), F4/80–APC (BM8), Forkhead box P3 (FoxP3) (FJK–16s), and CD11b–FITC (M1/70). Where needed, isotype-matched controls were included and intracellular cytokine and intranuclear FoxP3 staining was performed as described previously (33). Flow cytometry analyses were performed on a fluorescence-activated cell sorter (FACSCanto II; BD Biosciences) and results were analyzed with FACSDiva software 6.1.2.

Transcriptome analysis. Total RNA was isolated from frozen intestinal tissue by using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was treated with DNase and purified by using a Nucleospin RNAII kit (Macherey-Nagel), according to the manufacturer’s instructions.

Quality control of RNA samples, RNA labeling, and hybridization to the Sentrix MouseRef-8 BeadChips (Illumina) were performed at ServiceXS (Leiden, The Netherlands), as described previously (32). Image analysis and extraction of raw and background subtracted expression data were performed with Illumina BeadStudio, version 3.0, following the instructions. RNA was treated with DNAse and purified according to the manufacturer’s instructions.

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cutoff value to eliminate unexpressed probes. All expression values <5 (2.322 on a log2 scale) were set to this minimal threshold. RT-qPCR for a set of genes [CC chemokine ligand (Ccl2, Ccl11, and Ccl25) as well as various and transcripts related to tight junctions [claudin (Cldn1, Cldn4, and Cldn11)] showed a high correlation with data obtained by microarrays and thereby confirmed the relative gene expression presented in Figure 1 (data not shown).

**Statistical analysis.** In Expt. 1, differentially expressed probes were identified by using the limma package of the R/Bioconductor project, applying linear models and moderated t-statistics that implement empirical Bayes regularization of SEs (35). A P-Bayes value <0.05 was used as the threshold for significance of the differential expression. To establish and confirm differences between C57BL/6 and BALB/c mice, gene expression profiles between both mouse strains in the small intestine and colon were compared after 28 d of treatment with PBS. In view of well-established differences between these mouse strains, effects of prolonged VSL#3 treatment were only made within each of the strains.

In Expt. 2, we evaluated the effects of 28 d of treatment with VSL#3 vs. PBS on the phenotype of cells using the Mann-Whitney U test, with the use of SPSS software. Likewise, mice treated with PBS were compared with mice that were treated with PBS followed by 1 single dose of VSL#3 18 h before killing. P values <0.05 were considered significant.

**Results**

**Differences in intestinal gene expression between C57BL/6 and BALB/c mice.** To establish differences between C57BL/6 and BALB/c mice, we compared gene expression profiles between both mouse strains in the small intestine and colon. Figure 1A shows for individual untreated mice an unbiased analysis approach using all 25,697 genes on the array for principal components analysis. This principal components analysis plot reveals 2 distinct clusters of colon- and small intestine–related genes (component 1), whereas component 2 reveals differences in gene expression between the 2 mouse strains. We next identified differentially expressed genes [fold-change (FC) >1.5 and P-Bayes <0.05] between BALB/c and C57BL/6 mice for both tissues. A complete list of all differentially expressed genes can be found in Supplemental Table 2.

Metacore analysis revealed that overlapping genes were predominantly related to the immune response and include major histocompatibility complex haplotype variation. Moreover, the expression of a variety of antimicrobial peptides differed between the 2 mouse strains, especially in the small intestine. These antimicrobial proteins include lactopeptidases, lysozymes, and a set α-defensins. We also analyzed the expression of key regulators involved in the differentiation of CD4+ T cells. As shown in Figure 1B, the expression of the Th2 regulator GATA binding protein 3 (Gata3) was greater in the small intestine of BALB/c mice than in that of C57BL/6 mice. In contrast, the expression of RAR-related orphan receptor γ (Rorc) (key regulator of Th17 cells) was greater in the colon and small intestine of C57BL/6 mice (Figure 1B, C).

**VSL#3 modulates gene expression in the intestinal mucosa in conditions of homeostasis.** We next determined the effect of prolonged administration of VSL#3 to mice, significantly overrepresented pathway maps were created with MetaCore software (Thomson Reuters). This unbiased approach was applied to map the differentially expressed genes into clusters with common biological functions. Table 2 shows all of the significant pathway maps, including the number of genes with the corresponding P values. This table shows profound effects of VSL#3 on genes related to the immune response. To visualize expression patterns of these immune-related genes, a heat map was constructed including a set of genes [CC chemokine ligand (Ccl2, Ccl11, and Ccl25) as well as various and transcripts related to tight junctions [claudin (Cldn1, Cldn4, and Cldn11)] showed a high correlation with data obtained by microarrays and thereby confirmed the relative gene expression presented in Figure 1 (data not shown).

To gain insight into the processes that are associated with these differently expressed genes after prolonged administration of VSL#3 to mice, significantly overrepresented pathway maps were created with MetaCore software (Thomson Reuters). This unbiased approach was applied to map the differentially expressed genes into clusters with common biological functions. Table 2 shows all of the significant pathway maps, including the number of genes with the corresponding P values. This table shows profound effects of VSL#3 on genes related to the immune response. To visualize expression patterns of these immune-related genes, a heat map was constructed including all of these modulated genes in the small intestine and colon (Supplemental Figure 2A, B, respectively). For the small intestine in BALB/c mice, these genes were further subdivided into genes related to the innate and adaptive arm of the immune system. Supplemental Figure 3 shows a protein–protein interaction chart of these genes, in which upregulated genes are indicated in red and downregulated genes in blue. Innate immune transcripts upregulated upon VSL#3 administration include Nos2 and Nod2. In BALB/c mice we also observed
TABLE 1 Effects of prolonged administration of VSL#3 on intestinal gene expression in female BALB/c and C57BL/6 mice

<table>
<thead>
<tr>
<th>Pathway map folders</th>
<th>BALB/c COLON</th>
<th>BALB/c SMALL INTESTINE</th>
<th>C57BL/6 COLON</th>
<th>C57BL/6 SMALL INTESTINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune system response</td>
<td>31 Genes, 1 n = 0.01</td>
<td>29 Genes, 1 n = 0.01</td>
<td>24 Genes, 1 n = 0.01</td>
<td>39 Genes, 1 n = 0.01</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>27 Genes, 1 n = 0.01</td>
<td>16 Genes, 1 n = 0.01</td>
<td>12 Genes, 1 n = 0.01</td>
<td>15 Genes, 1 n = 0.01</td>
</tr>
<tr>
<td>Obesity</td>
<td>16 Genes, 1 n = 0.01</td>
<td>16 Genes, 1 n = 0.01</td>
<td>12 Genes, 1 n = 0.01</td>
<td>15 Genes, 1 n = 0.01</td>
</tr>
<tr>
<td>Cardiac hypertrophy</td>
<td>16 Genes, 1 n = 0.01</td>
<td>16 Genes, 1 n = 0.01</td>
<td>12 Genes, 1 n = 0.01</td>
<td>15 Genes, 1 n = 0.01</td>
</tr>
<tr>
<td>Tissue remodeling and wound repair</td>
<td>16 Genes, 1 n = 0.01</td>
<td>16 Genes, 1 n = 0.01</td>
<td>12 Genes, 1 n = 0.01</td>
<td>15 Genes, 1 n = 0.01</td>
</tr>
<tr>
<td>Calcium signaling</td>
<td>16 Genes, 1 n = 0.01</td>
<td>16 Genes, 1 n = 0.01</td>
<td>12 Genes, 1 n = 0.01</td>
<td>15 Genes, 1 n = 0.01</td>
</tr>
<tr>
<td>DNA-damage response</td>
<td>16 Genes, 1 n = 0.01</td>
<td>16 Genes, 1 n = 0.01</td>
<td>12 Genes, 1 n = 0.01</td>
<td>15 Genes, 1 n = 0.01</td>
</tr>
<tr>
<td>Mitotic signaling</td>
<td>16 Genes, 1 n = 0.01</td>
<td>16 Genes, 1 n = 0.01</td>
<td>12 Genes, 1 n = 0.01</td>
<td>15 Genes, 1 n = 0.01</td>
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<tr>
<td>Cell differentiation</td>
<td>16 Genes, 1 n = 0.01</td>
<td>16 Genes, 1 n = 0.01</td>
<td>12 Genes, 1 n = 0.01</td>
<td>15 Genes, 1 n = 0.01</td>
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<tr>
<td>Hematopoiesis</td>
<td>16 Genes, 1 n = 0.01</td>
<td>16 Genes, 1 n = 0.01</td>
<td>12 Genes, 1 n = 0.01</td>
<td>15 Genes, 1 n = 0.01</td>
</tr>
<tr>
<td>Cell cycle and regulation</td>
<td>16 Genes, 1 n = 0.01</td>
<td>16 Genes, 1 n = 0.01</td>
<td>12 Genes, 1 n = 0.01</td>
<td>15 Genes, 1 n = 0.01</td>
</tr>
</tbody>
</table>

1 Number of genes present in the pathway.
2 Level of significance for the enrichment of the pathway.
intestine, we focused on mRNA expression of the transcription factors T-box (Tbet), GATA-3 (Gata3), Foxp-3 (Foxp3), and RORα (Rorc), which are associated with Th1 cells, Th2 cells, Treg cells, and Th17 cells, respectively (45). In both the small intestine and the colon, C57BL/6 mice showed increased expression of RORα, which is suggestive for a more profound Th17-like phenotype. In contrast, the Th2-associated transcription factor Gata3 was higher in the small intestine of BALB/c mice than in C57BL/6 mice. These findings are consistent with the well-established polarized immune responses in these 2 mouse strains. Although it is widely assumed that this polarization is due to the genetic background of these 2 mouse strains, it cannot be excluded that a different composition of the intestinal microbiota of these mice plays an important role as well. The assessment of the exact contribution of each of these phenomena therefore requires further studies in germ-free animals.

To evaluate the effect of probiotics in healthy mice, we chose a dosage of $3 \times 10^8$ CFU probiotic bacteria 3 times/wk, an amount that is estimated to be 0.1% of the total cecal content (46, 47). Moreover, this dosage was shown to be optimal in previous mouse colitis studies (30, 48). For humans, a daily average dosage $4 \times 10^{10}$ to $9 \times 10^{11}$ CFU is recommended for both dietary management and ulcerative colitis (49, 50). This means that, corrected for body weight, the dosages are in the same order of magnitude.

Regardless of these differences in gene expression between the 2 mouse strains, we observed significant effects of treatment with VSL#3, with an emphasis on immune-related processes. Whereas these effects in C57BL/6 mice could not be linked to a defined immunologic process, treatment of BALB/c mice with VSL#3 showed upregulation of IL12rb1, Ccr5, Cxcr3, and Cxcl10 and downregulation of Il13 and Epx. This suggests a shift from a Th2 to a Th1 phenotype in BALB/c mice. These data therefore suggest that probiotic treatment may be favorable to control immune responses under polarized conditions, which is consistent with a previous study showing host-specific responses.
against probiotic bacteria in a model of allergic airway disease (51). It was suggested that this might be associated with differences in pattern recognition receptors, including Toll-like receptor (TLR), on innate immune cells in these mice. Indeed, we recently demonstrated that DCs from C57BL/6 and BALB/c mice respond differently to ultrapure TLR ligands with respect to cytokine and chemokine induction (40). However, it should be noted that the protective effect of the probiotic strain *Lactobacillus rhamnosus* against *Escherichia coli* O157:H7 infection was equally effective in BALB/c and C57BL/6 mice (52). However, it might be that the effect of this strain is not primarily due to immune modulation, but rather due to competition for adhesion sites, production of antimicrobial substances, or the competition for nutrients.

To verify the effects of probiotic treatment at the level of innate and adaptive immune cells, we evaluated such cells in PP, MLN, and spleen. Irrespective of the mouse strain studied, the numbers of CD19+ B lymphocytes in the PP and MLN were significantly increased after prolonged administration of VSL#3. Most B cells in the intestine are plasma cells that secrete IgA into the lumen (41, 53). IgA is a predominant immunoglobulin in mucosal secretions, which serves as a first line of humoral defense at all mucosal surfaces where binding of IgA to microorganisms reduces their motility and adhesive properties within the mucosal lumen and its surface (54). Previous studies linked beneficial effects of specific *Lactobacillus* species to increased CD19+ B lymphocytes in the ileal mucosa (55) and increased numbers of IgA-producing cells in the lamina propria (56, 57). Therefore, it is tempting to speculate that increased B cell frequencies observed in our study contribute to the beneficial effects of VSL#3 via IgA-mediated mechanisms.

Interestingly, prolonged treatment of BALB/c mice with VSL#3 also resulted in an increase in CD4+CD25+Foxp3+ Treg cells locally in MLNs but not in the spleen. It was shown that the induction of CD4+CD25+Foxp3+ Treg cells in MLNs depends on CD103+ DCs in the lamina propria (36, 37). Therefore, our observation that this CD103+CD11c− DC subset increased in the PP and MLNs of BALB/c mice, 18 h after 1 single dose of VSL#3, may explain the increased numbers of Treg cells we found at a later stage in the MLNs. On the other hand, the increase in Treg cells and Th17 cells in MLNs may in part be due to migration from the intestine, in view of the decreased expression of Foxp3 and Il17a in the colon.

Recently, we showed by transcriptome profiling of colons that inflammation in the recurrent trinitrobenzene sulfonic acid colitis model is mediated by a gradual involvement of mast cells (32) and T cell immune processes that are suppressed by treatment with VSL#3 (30). Collectively, our data suggest that VSL#3 treatment has favorable effects in this model by modulating local inflammation through the induction of Treg cells.

The variation between the 2 mouse strains under investigation (i.e., BALB/c vs. C57BL/6) was more profound than the effect of the intervention by VSL#3. This is in line with observations in human subjects, in whom duodenal biopsies were taken before and after intake of a probiotic supplement (58). In this study, transcriptome profiles clustered per person and not per intervention, showing that person-to-person variation in gene expression was the largest determinant of differences between the transcriptomes. Our present data in BALB/c and C57BL/6 mice substantiate these observations. Therefore, a full appreciation of the health benefits of probiotic bacteria with an emphasis on translational value should take both genetic and microbial factors into account.

Acknowledgments

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