VSL#3 probiotic upregulates intestinal mucosal alkaline sphingomyelinase and reduces inflammation

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BACKGROUND: Alkaline sphingomyelinase, an enzyme found exclusively in bile and the intestinal brush border, hydrolyzes sphingomyelin into ceramide, sphingosine and sphingosine-1-phosphate, thereby inducing epithelial apoptosis. Reduced levels of alkaline sphingomyelinase have been found in premalignant and malignant intestinal epithelia and in ulcerative colitis tissue. Probiotic bacteria can be a source of sphingomyelinase.

OBJECTIVE: To determine the effect of VSL#3 probiotic therapy on mucosal levels of alkaline sphingomyelinase, both in a mouse model of colitis and in patients with ulcerative colitis.

METHODS: Interleukin-10 gene-deficient (IL10KO) and wild type control mice were treated with VSL#3 (10⁹ colony-forming units per day) for three weeks, after which alkaline sphingomyelinase activity was measured in ileal and colonic tissue. As well, 15 patients with ulcerative colitis were treated with VSL#3 (900 billion bacteria two times per day for five weeks). Alkaline sphingomyelinase activity was measured through biopsies and comparison of ulcerative colitis disease activity index scores obtained before and after treatment.

RESULTS: Lowered alkaline sphingomyelinase levels were seen in the colon (P<0.02) and ileum (P<0.04) of IL10KO mice, as compared with controls. Treatment of these mice with VSL#3 resulted in upregulation of mucosal alkaline sphingomyelinase activity in both the colon (P<0.04) and the ileum (P=0.01). VSL#3 treatment of human patients who had ulcerative colitis decreased mean (± SEM) ulcerative colitis disease activity index scores from 5.3±1.8946 to 0.70±0.34 (P<0.02) and increased mucosal alkaline sphingomyelinase activity.

CONCLUSION: Mucosal alkaline sphingomyelinase activity is reduced in the intestine of IL10KO mice with colitis and in humans with ulcerative colitis. VSL#3 probiotic therapy upregulates mucosal alkaline sphingomyelinase activity.

Key Words: Alkaline sphingomyelinase; Inflammatory bowel disease; Probiotic; Ulcerative colitis; VSL#3

Sphingomyelin is hydrolyzed by sphingomyelinase, a sphingomyelin-specific form of phospholipase C; this hydrolysis appears to be a major source of ceramide. Ceramide is a lipid-signalling molecule that activates downstream caspases, which have antiproliferative and apoptotic effects on various cell types in the human body (1). Three types of sphingomyelinase have been identified in mammals. Neutral and acidic sphingomyelinases are found in various tissues throughout the body (2,3). In contrast, alkaline sphingomyelinase is found only in the intestinal tract (4,5) and biliary epithelium (6). Alkaline sphingomyelinase hydrolyzes sphingomyelin into ceramide, sphingosine and sphingosine-1-phosphate, which can induce apoptosis. It has been shown that reduced levels of alkaline sphingomyelinase can contribute to the development of intestinal inflammation. VSL#3 probiotic therapy has been shown to reduce inflammation in ulcerative colitis by upregulating alkaline sphingomyelinase activity.

HISTORIQUE : La sphingomyéline alcaline, une enzyme contenue exclusivement dans l'ile et la brousse intestinale, hydrolyse la sphingomyéline en céramide, en sphingosine et en sphingosine-1-phosphate, ce qui induit une apoptose épithéliale. On a trouvé des taux moins élevés de sphingomyéline alcaline dans l'épithélium intestinal pré-cancéreux et cancéreux et dans des tissus de colite ulcéreuse. Les bactéries probiotiques peuvent être une source de sphingomyéline alcaline.

OBJECTIF : Déterminer l’effet de la thérapie probiotique VSL#3 sur les taux de sphingomyéline alcaline, à la fois dans un modèle de souris atteinte de colite et chez des patients atteints de colite ulcéreuse.

MÉTHODOLOGIE : Les auteurs ont traité des souris sans gène d’interleukine-10 (IL10KO) et des souris témoins de type sauvage au VSL#3 (10⁹ unités formant des colonies par jour) pendant trois semaines, après lesquelles ils ont mesuré l’activité de la sphingomyéline alcaline dans les tissus ileaux et coliques. De plus, il ont traité 15 patients atteints de colite ulcéreuse au VSL#3 (900 milliards de bactéries deux fois par jour pendant cinq semaines). Ils ont mesuré l’activité de la sphingomyéline alcaline au moyen de biopsies et de comparaisons entre les indices d’activité de la maladie avant et après le traitement.

RÉSULTATS : Les auteurs ont observé des taux de sphingomyéline alcaline moins élevés dans le côlon (P<0.02) et l’iléon (P<0.04) de souris IL10KO que ceux des sujets témoins. Le traitement de ces souris au VSL#3 a donné lieu à une régularisation positive de l’activité de la sphingomyéline alcaline muqueuse à la fois dans le côlon (P<0.04) et l’iléon (P<0.01). Le traitement des patients humains atteints de colite ulcéreuse au VSL#3 a réduit les indices d’activité de la colite ulcéreuse moyens zéteur-type de 5.3±1.8946 à 0.70±0.34 (P<0.02) et accru l’activité de la sphingomyéline alcaline muqueuse.

CONCLUSION : L’activité de la sphingomyéline alcaline muqueuse est réduite dans l’intestin des souris IL10KO atteintes de colite et chez des humains atteints de colite ulcéreuse. La thérapie probiotique VSL#3 assure la régularisation positive de l’activité de la sphingomyéline alcaline muqueuse.
this process, stimulates the production of ceramide, which in turn, plays a significant role in apoptosis and in the prevention of intestinal epithelial dysplasia and tumourigenesis (9,10).

Numerous studies have revealed associations between decreases in alkaline sphingomyelinase levels and inflammatory, premalignant or malignant conditions of the colon. For example, chronic ulcerative colitis has been found to display a 60% decrease in alkaline sphingomyelinase in areas with histologically identified dysplasia (11). In patients with familial adenomatous polyposis, a 90% reduction in alkaline sphingomyelinase activity was seen in both familial adenomatous polyposis adenomas and in surrounding normal mucosa, compared with sphingomyelinase activity in controls (12). Finally, a 75% decrease in enzyme activity in colorectal carcinomas has been documented, compared with enzyme activity in surrounding normal tissue (13).

Upregulation of sphingomyelinas has been the focus of several experiments. For instance, exposure of sphingomyelinas to trypsin cleaves the carboxyl-terminal, releasing alkaline sphingomyelinas from the mucosa and enhancing its activity (14). Psyllium is a water-soluble fibre that acts as a food source for colonic bacteria and functions to increase stool bulk. Results from mice fed a diet that included psyllium showed a 57% increase in alkaline sphingomyelinas in activity over that of controls. The addition of psyllium to a high fat diet also partially reversed that diet’s negative effect on alkaline sphingomyelinase levels. However, the mechanism by which psyllium affects alkaline sphingomyelinase levels remains unclear, although a relationship to luminal bacterial growth has been suggested (15).

Gavage of Sprague-Dawley rats with an ursodeoxycholic acid suspension resulted in a dose-dependent increase in alkaline sphingomyelinas in activity, to a maximal increase of 250% (8). Ursodeoxycholate has been shown to stabilize the intestinal membrane against cytotoxic agents and enhance commensal bacterial colonization. This may explain, in part, the increase of alkaline sphingomyelinas associated with ursodeoxycholate supplementation (16).

One potential mechanism for upregulating intestinal sphingomyelinas activity would be to encourage colonization of the intestinal mucosa by bacterial organisms that produce sphingomyelinase (17). VSL#3 (VSL3 Pharmaceuticals, Inc, USA) is a probiotic product consisting of eight different strains of bacteria (Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus plantarum and Streptococcus salivarius subspecies thermophilus). It has been shown to increase the numbers of its component bacteria in the colon (18,19). Streptococcus thermophilus has been shown to engage in sphingomyelinas activity (20). When administered orally in the VSL#3 compound, S. thermophilus colonizes the colon of 100% of patients with inflammatory bowel disease (IBD) (21).

The objectives of the present study were to examine the levels of mucosal alkaline sphingomyelinas in both an animal model of colitis and in human ulcerative colitis, and then investigate the effects of VSL#3 probiotic therapy on the upregulation of mucosal alkaline sphingomyelinas levels.

METHODS

Animal studies

Animals: Homozygous interleukin-10 gene-deficient (IL10KO) mice (generated on a 129/SvEv background) and wild-type 129 Sv/Ev controls were housed behind a barrier under specific pathogen-free conditions. All provisions for the facility were sterilized using an autoclave. Nonautoclavable supplies were sprayed with disinfectant and introduced into the facility through a high-efficiency particulate air-filtered air lock. The facility’s air was high-efficiency particulate air-filtered. The animals were housed in microisolator cages covered with tight-fitting lids that contained a spun polyester fibre filter. The animals had ad libitum access to autoclaved (sterile) 9% fat diet and sterile filtered water. The facility’s sanitation was verified by the Health Sciences Laboratory Animal Services at the University of Alberta (Edmonton, Alberta). Sentinel BALB/c mice were housed in the animal room and examined for known bacterial, parasitic and viral pathogens. Bacterial cultures, parasitological examinations, serological tracking profiles and histological stais were negative for all known murine viral and bacterial pathogens, indicating an intact barrier.

Animal protocol: Mice were divided into three groups: wild type placebo-fed control mice (n=5); IL10KO placebo-fed mice (n=7); and IL10KO mice gavaged two times per day for three weeks with 10^{9} colony-forming units of VSL#3 dissolved in saline (n=8). All three groups were treated for 21 days. After 21 days, the mice were given an overdose of phenobarbital, after which the ileum and colon were removed, opened longitudinally and rinsed with ice-cold phosphate-buffered saline. A 1 cm segment of ileum and a similar segment of colon were extracted for histological injury assessment (see below). Ileal and colonic mucosal scrapings were obtained using a glass slide; the scrapings were immediately placed in liquid nitrogen and transferred to a −80°C freezer. They were then assayed for alkaline sphingomyelinas activity using the assay procedures described below.

Animal microscopic injury score: Mice in each group (see above) were analyzed for severity of colonic inflammation. Colonos were excised from sacrificed animals, and one-half of the 4 cm segment was fixed in 10% phosphate-buffered formalin. The samples were embedded in paraffin in toto, sectioned at 1 cm intervals, and stained with hematoxylin and eosin for light-microscopic examination. The slides were reviewed in a blinded fashion and were assigned a histological score for intestinal inflammation using a scheme modified from Saverymuttu et al (22). Briefly, the histological grades assigned (ranging from 0 [representing no injury] to 10 [representing maximal injury]) correspond to the numerical sum of four scoring criteria: mucosal ulceration, epithelial hyperplasia, lamina propria mononuclear infiltration and lamina propria neutrophilic infiltration.

Human studies

Human protocol: The present study was performed in June and July of 2005 and was approved by the Health Research Ethics Board at the University of Alberta. Fifteen patients with clinically, endoscopically and histologically diagnosed ulcerative colitis participated. Inclusion criteria included the following requirements: being 18 years of age or older; for female participants, having a negative pregnancy blood test for the duration of the study, as well as adhering to the requirement of not breastfeeding during the study; and having a diagnosis of active or quiescent ulcerative colitis. Before admittance into the study, written and oral informed consent were obtained from all study participants. The study was performed...
in accordance with the International Conference on Harmonisation’s Good Clinical Practice Guidelines, which are based on the Declaration of Helsinki. Each subject took one sachet of VSL#3 (containing 900 billion lyophilized bacteria) orally two times per day for five weeks. Immediately before starting the VSL#3 treatment (day 0) and again after five weeks, before stopping the VSL#3 treatment (day 35), subjects participated in ulcerative colitis disease activity index (UCDAI) determination (23) and mucosal biopsy sampling for alkaline sphingomyelinase activity. No change in ulcerative colitis medication or dosage was permitted during the study period.

**Human disease activity index**: Briefly, the UCDAI is the sum (maximum of 12 points) of the scores of four criteria (scores ranked from 0 to 3 for each criterion): stool frequency, rectal bleeding, mucosal appearance, and the investigator’s rating of disease activity. Disease remission is defined as two points or less. Severe disease is defined as more than nine points. All biopsies were taken in the usual fashion at 20 cm from the anal verge. Mucosal biopsies for alkaline sphingomyelinase determination were immediately placed in liquid nitrogen and then transferred to a -70°C freezer for storage. At baseline, the subjects completed a questionnaire that included questions on sex, ethnic background, year of diagnosis, frequency of disease flare-ups, surgical history and medical treatment history. At the end of the study, subjects completed a follow-up questionnaire that reported on adverse events and the initiation of new medications or surgical procedures.

**Alkaline sphingomyelinase activity assay**

Alkaline sphingomyelinase activity was determined using the methods previously described by Duan et al (24), with small modifications. Twenty-five microlitre samples (100 μg to 200 μg protein) were incubated for 30 min at 37°C in a buffer solution containing 50 mM of tris-hydrochloride, 0.15 M of sodium chloride, 2 mM EDTA (pH 9.0), and a 3 mM bile salt mixture of taurocholic acid, taurodeoxycholic acid, glycocholic acid and chloride, 2 mM EDTA (pH 9.0), and a 3 mM bile salt mixture. The reaction was started by the addition of 16 μL of radiolabelled sphingomyelin (5 nmole) in a total volume of 200 μL. B. longum, B. infantis and B. breve); four strains of Lactobacillus (L. acidophilus, L. casei, L. bulgaricus and L. plantarum); and one strain of Streptococcus (S. salivarius subspecies thermophilus). Each VSL#3 sachet contained 900 billion lyophilized bacteria and contained 50.2 micro units of alkaline sphingomyelinase (1 micro unit of alkaline sphingomyelinase is defined as the amount of enzyme that hydrolyzes 1 mmol of sphingomyelin/h at 37°C).

**Statistical analysis**

Data were analyzed using the statistical software program, Statistical Package for the Social Sciences 12.0 (SPSS Inc, USA). Unpaired T tests were performed on all comparisons of associations and of changes in enzyme activity before and after treatment. Reported P values were for two-tailed tests; P<0.05 was considered to be significant for all statistical tests.

**RESULTS**

**Effect of VSL#3 supplementation on colitis and mucosal alkaline sphingomyelinase activity in IL10KO and wild type control mice**

Previous studies have shown that VSL#3 treatment normalizes the colonic epithelial barrier function and reverses histological injury; these changes correlate with a reduction in mucosal levels of proinflammatory cytokines (25). In the current study, baseline mucosal levels of alkaline sphingomyelinase activity were decreased in IL10KO mice, compared with those of wild-type control mice, in both the ileum (P=0.04, Figure 1) and the colon (P=0.02, Figure 2). Treatment of IL10KO mice with the VSL#3 probiotic mixture significantly increased mucosal alkaline sphingomyelinase activity, compared with levels seen in placebo-fed IL10KO mice, for both the ileum (P=0.01,
IL10KO mice that were not treated with VSL#3 demonstrated a mean (± SEM) microscopic injury score of 6.7±0.6 units; in contrast, mice treated with VSL#3 demonstrated a marked reduction in mucosal injury, with an injury score of 2.5±0.4 units (P=0.02) (Figure 3).

To confirm that the VSL#3 itself was increasing alkaline sphingomyelinase levels, and that the increase was not simply due to improvement in inflammation, non-IBD control animals were supplemented with VSL#3. VSL#3 administration increased mucosal alkaline sphingomyelinase levels 4.5-fold (P<0.05) over those animals that were not supplemented with VSL#3 (Figure 4). Before alkaline sphingomyelinase assessment, the VSL#3 bacteria were removed by washing, and cultures demonstrated no residual bacteria remaining.

**Table 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>VSL#3* treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men (%):women (%)</td>
<td>9 (60):6 (40)</td>
</tr>
<tr>
<td>Mean ± SD age at entry, years</td>
<td>52±12</td>
</tr>
<tr>
<td>Maximum disease extent, n (%)</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Rectum, sigmoid</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Distal to midtransverse colon</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Pancolitis</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Concomitant medications, %†</td>
<td></td>
</tr>
<tr>
<td>Corticosteroids – oral</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Corticosteroid – enema</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Azathioprine or 6-mercaptopurine</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mesalamine – oral</td>
<td>14 (93)</td>
</tr>
<tr>
<td>Mesalamine – enema</td>
<td>0 (0)</td>
</tr>
<tr>
<td>None</td>
<td>1 (7)</td>
</tr>
</tbody>
</table>

*Manufactured by VSL Pharmaceuticals, Inc (USA); †Medications taken within two weeks of initiating study protocol.

**Effect of VSL#3 supplementation on colitis and mucosal alkaline sphingomyelinase activity in human ulcerative colitis subjects**

A total of 15 patients completed the study. The demographics of the study group are summarized in Table 1. Mean pooled data for alkaline sphingomyelinase activity demonstrate an increase in activity from 3658±1324 pmol to 9864±1189 pmol sphingomyelin hydrolyzed/h/mg protein at the end of five weeks (P<0.05, Figure 5). An overall decrease in UCDAI...
was evident with VSL#3 treatment, as depicted in Figure 6 (P=0.02). Adverse events were minimal, with three patients reporting minor abdominal bloating.

**DISCUSSION**

Numerous human studies have investigated the association between inflammatory, premalignant and malignant conditions of the colon, and decreased alkaline sphingomyelinase levels (26). Studies that have investigated the upregulation of alkaline sphingomyelinase activity have focused on healthy animal models (8,15). The current study, however, examined the use of VSL#3 probiotic therapy to upregulate alkaline sphingomyelinase activity in both an animal model of colitis and humans with ulcerative colitis.

Alkaline sphingomyelinase levels in human patients with ulcerative colitis are decreased (11); our study confirms this finding and also demonstrates that IL10KO mice with colitis also demonstrate decreases in enzyme activity. Furthermore, VSL#3 therapy upregulated both ileal and colonic mucosal levels of alkaline sphingomyelinase for these IL10KO mice to nearly normal levels. This finding was not surprising, because it was similar to results previously seen in animal models of IBD (8,15). Both ileal and colonic tissues in IL10KO mice are inflamed, with evidence of elevated proinflammatory cytokines. Associated with this upregulation of alkaline sphingomyelinase by VSL#3 administration was a statistically significant improvement in the histological injury score.

To determine whether VSL#3 itself upregulated mucosal alkaline sphingomyelinase or whether the alkaline sphingomyelinase upregulation was due to reduced inflammation levels, VSL#3 was administered to control (nonmucosally inflamed) animals. There was a fourfold increase in mucosal alkaline sphingomyelinase (Figure 4). Furthermore, this increase reflected pure mucosal alkaline sphingomyelinase and not residual bacterial alkaline sphingomyelinase because the VSL#3 bacteria had, themselves, been completely washed away before the mucosal alkaline sphingomyelinase assay.

The ability of VSL#3 treatment to upregulate mucosal alkaline sphingomyelinase activity has potential implications for acting as an anti-inflammatory and anticarcinogenic agent. Increases in alkaline sphingomyelinase have been found to correlate positively with increases in levels of caspase-3 (8), an IL-1-beta-converting enzyme that has the capacity to signal apoptosis (27). In contrast, the inhibition of caspase-3 has been shown to suppress ceramide-induced apoptosis (28). Interestingly, colon cancer HT-29 cultured cells have been found to possess an inactive deletion in alkaline sphingomyelinase (9). In addition, an accumulation of sphingomyelin and a reduction in sphingomyelinase activity have been found in both human colon cancers (29) and colonic adenomatous tissue (30). The upregulation of cellular messengers, including caspase-3, may provide a possible explanation for the ability of alkaline sphingomyelinase to both induce apoptosis in adenocarcinoma cells (31) and inhibit the process of carcinogenesis (32).

In the human component of the present study, where findings were similar to those seen in the IL10KO mice with colitis, VSL#3 administration resulted in a significant increase in mucosal alkaline sphingomyelinase activity. Sphingomyelinase activity has been described as varying in response to dietary content of fibre and fat (33), mesalazine use, changes in arachidonic acid levels (34,35,36) and differences in age (11). It is possible that such factors may have resulted in the increased level of sphingomyelinase observed in the human samples following VSL#3 therapy; however, it is unlikely, because patients served as their own controls and these external variables were similar both before and during the VSL#3 treatment. Furthermore, in the animal experiments, supplementing non-IBD controls demonstrated a 4.5-fold increase in mucosal sphingomyelinase activity.

**CONCLUSIONS**

Mucosal alkaline sphingomyelinase activity is reduced in the intestines of IL10KO mice with colitis and humans with ulcerative colitis. Probiotic therapy with VSL#3 upregulates alkaline sphingomyelinase activity.

![Figure 5](https://example.com/figure5.png)  
**Figure 5** Human colonic mucosal alkaline sphingomyelinase activity. Feeding VSL#3 (VSL Pharmaceuticals, Inc, USA) for five weeks to human patients with ulcerative colitis (solid bar) increased colonic mucosal alkaline sphingomyelinase activity relative to before therapy (grey bar). Values represent mean ± SEM for 15 subjects. *P=0.02 relative to before VSL#3 treatment.

![Figure 6](https://example.com/figure6.png)  
**Figure 6** Ulcerative colitis disease activity index (UCDAI). The mean UCDAI score was reduced in human patients fed VSL#3 (VSL Pharmaceuticals, Inc, USA) for five weeks (solid bar) compared with before VSL#3 therapy (grey bar). Values represent mean ± SEM for 15 subjects. *P=0.02 relative to before VSL#3 treatment.
REFERENCES


