

VSL#3 Probiotic-Mixture Induces Remission in Patients with Active Ulcerative Colitis

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- BACKGROUND AND AIMS:** Intestinal bacteria have been implicated in the initiation and perpetuation of IBD; in contrast, "probiotic bacteria" have properties possibly effective in treating and preventing relapse of IBD. We evaluated the safety and efficacy of VSL#3 and the components and the composition of the biopsy-associated microbiota in patients with active mild to moderate ulcerative colitis (UC).
- METHODS:** Thirty-four ambulatory patients with active UC received open label VSL#3, 36,000 billion bacteria daily in two divided doses for 6 wk. The presence of biopsy-associated bacteria was detected using a nucleic acid-based method and the presence of VSL#3 species confirmed by DNA sequencing of 16S rRNA.
- RESULTS:** Thirty-two patients completed 6 wk of VSL#3 treatment and 2 patients did not have the final endoscopic assessment. Intent to treat analysis demonstrated remission (UCDAI ≤ 2) in 53% (n = 18); response (decrease in UCDAI ≥ 3 , but final score ≥ 3) in 24% (n = 8); no response in 9% (n = 3); worsening in 9% (n = 3); and failure to complete the final sigmoidoscopy assessment in 5% (n = 2). There were no biochemical or clinical adverse events related to VSL#3. Two of the components of VSL#3 were detected by PCR/DGGE in biopsies collected from 3 patients in remission.
- CONCLUSION:** Treatment of patients with mild to moderate UC, not responding to conventional therapy, with VSL#3 resulted in a combined induction of remission/response rate of 77% with no adverse events. At least some of the bacterial species incorporated in the probiotic product reached the target site in amounts that could be detected.

(Am J Gastroenterol 2005;100:1-8)

INTRODUCTION

The intestinal microbiota has been implicated in the pathogenesis of chronic intestinal inflammation by experimental animal and human studies (1). Rodents with dysfunctional immune systems, which serve as models of inflammatory bowel diseases do not develop inflammation when kept under germ-free conditions. They develop colitis when associated with gut bacteria (2, 3). Such models have shown an increased adherence or invasion of bacteria, which precedes the development of inflammation (4), and exhibit antigen-specific CD4+ T lymphocyte responses directed at cecal bacteria (5). This implies that commensal enteric bacteria have a primary role in the development of colitis (6). Moreover, bacterial species have differential abilities to induce colitis (4-7), demonstrating that not all gut bacteria are equal in their abilities to induce disease. In humans, it is known that the anatomical sites of highest bacterial concentration are

the sites most frequently affected by ulcerative colitis (UC) and Crohn's disease (8). Furthermore, enteric bacteria and their products have been detected in the affected mucosa of inflammatory bowel disease patients and alterations in fecal flora of inflammatory bowel disease patients have been noted (9-12). Clinical interventions that target bacteria affect the progress of the UC and Crohn's disease in different ways, nevertheless there are few controlled studies with these interventions and the majority remain anecdotal. Antibiotics tend to be most effective in Crohn's colitis, ileocolitis, and pouchitis, but are less effective in UC and isolated Crohn's ileitis (13-15). Another method that has been suggested to alter the bacteriological milieu of the gut involves the use of probiotics (16, 17). Probiotics are preparations of living microbial cells that, when ingested, are believed to influence the composition of the gut microbiota and consequently to benefit the health of the consumer (18, 19). Probiotics have been shown to reduce colitis in animal models (6, 20, 21)

and to help to treat acute, and maintain remission of, UC in humans (22–28). A probiotic preparation consisting of a mixture of eight lactic acid bacterial species (VSL#3) has been reported to be effective in preventing flare-ups of refractory pouchitis (29, 30), as a prophylactic agent to prevent the development of pouchitis after ileo-anal pouch formation (31) and in maintenance of remission of UC (26). In contrast to the convincing controlled evidence for VSL#3 in prevention and maintenance of remission the role of VSL#3 in acute active disease remains to be determined. VSL#3 is a unique probiotic mixture that appears to have antiinflammatory activities both with the live organism (26, 29–32) as well as thorough its DNA (33).

The primary objective of this study was to study the proportion of patients achieving an improvement in UC activity from baseline following 6 wk of VSL#3 therapy as measured by the Ulcerative Colitis Disease Activity Index (UCDAI). Additional analyses were conducted to monitor the safety of VSL#3. Baseline and 6 wk UCDAI scores were determined by a single experienced IBD physician at each site. The secondary objective was the detection of bacterial species contained in the VSL#3 product in association with biopsies collected after treatment with the probiotic, and to determine whether their detection was associated with remission of disease.

MATERIALS AND METHODS

Participants

The study was performed between June 2002 and July 2003. Patients were eligible to participate if they were between 18 and 65 yr of age; had an endoscopic and histologic confirmed diagnosis of UC for at least 1 yr; and had at least a 2 wk history of active UC that had not responded to mesalamine therapy. The following patients were not eligible: disease less than 25 cm from the anal canal, active enteric infection, evidence of severe disease characterized by hemoglobin <8.0 g/dl, white blood cell count >20,000 cells/mm³, temperature >38.5°C, albumin <25 g/dl, active disease >2 months, UCDAI < 2 or >9 (see description of scoring system below). Patients with a history of dysplasia of the colon or any cancer within 5 yr, clinically significant hematologic values (see above) or biochemical values (serum creatinine concentrations >1.5 times the upper limit of normal or alkaline phosphatase, aspartate aminotransferase, or alanine aminotransferase concentrations >2.5 times the upper limit of normal) were also ineligible. Each patient provided written, informed consent before participation. Approval for the study protocol was granted by the appropriate institutional human subject review board at each participating site.

Concomitant Therapy

Continued treatment with oral or rectal mesalamine, oral or rectal corticosteroids was allowed, provided the dose had been stable for at least 2 wk. Patients receiving stable doses of

6-mercaptopurine, or azathioprine for at least 8 wk were eligible. Patients who had received infliximab or cyclosporine within 12 wk were not eligible. Treatment with antidiarrheal agents (loperamide, diphenoxylate, and opiates) were allowed, provided doses remained stable during the 6-wk evaluation period. No other medications for UC were permitted. No patient had received investigational therapies within 30 days preceding randomization.

Study Medication

VSL#3 (VSL Pharmaceuticals, Inc., Ft Lauderdale, FL) sachets contained 900 billion viable lyophilized bacteria consisting of four strains of *Lactobacillus* (*L. casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus*), three strains of *Bifidobacterium* (*B. longum*, *B. breve*, and *B. infantis*), and one strain of *Streptococcus salivarius* subsp. *thermophilus*. 3,600 billion bacteria (four sachets) of VSL#3 were administered orally each day for 6 wk in two divided doses (1,800 billion bacteria twice each day).

Interventions

The 6-wk study was an open label trial performed at three IBD referral centers (University of Alberta, Canada, University of Bologna, Italy, and University of North Carolina at Chapel Hill, USA). After a 1-wk screening period, eligible patients received open label VSL#3, 3,600 billion bacteria, in two divided doses, for 6 wk. At entry (screening visit), each patient's demographic characteristics, medical history, and current medications were recorded. Disease activity was assessed at the baseline visit and after 3 and 6 wk. At each visit a physical examination and history was performed. At baseline and at 6 wk a sigmoidoscopy with endoscopic and histologic assessment of the mucosa at the site of most severe disease activity was determined. All laboratory tests were performed at local laboratories. All adverse events were classified and graded according to the consort dictionary criteria.

Outcome

The intention-to-treat population included all patients who were evaluated at the screening and baseline visits and who received at least one dose of study medication. The sole primary outcome measure, as specified in the study protocol before initiating the study, was number of patients entering remission (UCDAI ≤ 2). The secondary outcome measures that were specified in the study protocol before initiating the study were as follows: (1) number of patients with a response, but not entering remission, (decrease in UCDAI ≥ 3 points, but final score ≥ 3), (2) mean decrease in UCDAI, (3) number of patients worsening (UCDAI increasing > 3 points), and (4) number of patients exhibiting toxic effects of the study medication. Disease activity was assessed using the previously described UCDAI (Table 1) (34). The UCDAI is the sum of the scores from four criteria, ranked zero to three for each criteria, including stool frequency, rectal bleeding, mucosal appearance, investigator's rating of disease of activity, with a maximum of 12 points. Disease in remission is defined as two

Table 1. Ulcerative Colitis Disease Activity Index (34)

Variable	Score
1. Stool frequency	
Normal	0
1–2 stools/day > normal	1
3–4 stools/day > normal	2
>4 stools/day > normal	3
2. Rectal bleeding	
None	0
Streaks of blood	1
Obvious blood	2
Mostly blood	3
3. Mucosal appearance	
Normal	0
Mild friability	1
Moderate friability	2
Exudation, spontaneous bleeding	3
4. Physician's rating of disease activity	
Normal	0
Mild	1
Moderate	2
Severe	3

or lower points. Severe disease is defined as greater than nine points. Patients were permitted enrollment with a UCDAI of 3–9.

Extraction of Bacterial DNA from Biopsies and VSL#3 Preparation

In a subset of 11 patients mucosal biopsies were obtained at the time of sigmoidoscopy (0 and 6 wk) from 40 cm proximal to the anal verge. Biopsies were immediately frozen in liquid nitrogen and then stored at -80°C prior to analysis. Of the 11 patients sampled, 8 showed remission of disease, and 3 remained with active disease after 6 wk of treatment.

DNA was extracted from biopsies, the VSL#3 powder and the individual bacterial strains that make up the VSL#3 product, as described elsewhere (35). Briefly, 15–30 mg of tissue specimens, or 35 mg of the VSL#3 powder were incubated with lysozyme (25 mg/ml) for 30 min at room temperature. In the case of the individual VSL#3 strains, overnight cultures in *Lactobacilli* MRS broth (Difco) were centrifuged and the pellets were washed twice before the lysozyme treatment. Samples were transferred to sterile tubes containing 0.3g of sterile zirconium beads, and disrupted in a Mini-bead beater. Then, samples were treated with a series of phenol-chloroform extractions. DNA was precipitated overnight at -20°C in isopropanol, washed with 80% ethanol and air-dried at room temperature. Finally, DNA pellets were dissolved in TE buffer (pH 7.5) and stored at -20°C .

Detection of Bacteria by PCR–DGGE

Amplification of the V3 region of the 16S rRNA gene of biopsy-associated bacterial DNA and of VSL#3 DNA was carried out using the universal bacterial primers HDA1-GC and HDA2, as described previously (36) and the Taq PCR Core Kit (Qiagen Inc., Ontario, Canada). PCR products were checked by electrophoresis in a 2% agarose gel stained with

ethidium bromide and viewed by UV transillumination. Amplicons were analyzed by DGGE performed with a DCode apparatus (Bio-Rad, Hercules, CA) as described previously (37). An identification ladder was prepared by extracting DNA from the individual components of VSL#3 and then by PCR amplification using the same pair of primers and amplification kit.

Cloning DNA Fragments Eluted from DGGE Gels to Identify Bacterial Origin

DNA fragments were cut from the polyacrylamide gel using a sterile scalpel blade, and DNA was eluted and purified using a commercial kit (Qiagen Inc., Ontario, Canada) according to the manufacturer's instructions. Purified fragments were digested with S1 nuclease (Roche) to remove single stranded DNA as described previously (37). S1-nuclease-treated DNAs were used as templates in PCR reactions with the HDA primers prior to ligation. The resulting PCR products were cloned in *Escherichia coli* TOP10 using the pCR[®]2.1-TOPO vector system (Invitrogen, Carlsbad, CA). Recombinant plasmids were purified from colonies grown in LB broth (Difco) supplemented with 100 μg ampicillin/ml, using the Wizard Plus SV minipreps DNA purification system (Promega, Madison, WI). The migration of the amplified inserts was checked using DGGE by comparison to the original profile from which the fragments had been obtained. Cloned inserts were amplified from the cloning vector using M13 primers for sequencing. Sequences were compared to those in the GenBank database using the BLASTn algorithm. Similarities between the DGGE profiles were determined by calculating similarity indices of the densitometric curves of the profiles using Dice's correlation with the aid of computer software (Bionumerics, Applied Maths, Texas, USA). An unweighted pair group method using an arithmetic averages (UPGMA) algorithm was performed.

Role of the Funding Source

The investigators designed the study, recruited the patients, managed the data, performed the statistical analysis, and wrote the manuscript detailing the results of the study. The completed case report forms were sent by the investigators to the principal investigator, where the statistical analysis was performed. The study was funded by research grants from the CCFC and CCFA and VSL Pharmaceuticals, Inc., Study drug was supplied by VSL Pharmaceuticals, Inc., Representative from VSL Pharmaceuticals, Inc., had the opportunity to review and comment on the study design and on the manuscript, but the principal investigator made the final decisions regarding the design of the study and the content of the manuscript.

RESULTS

Forty-six patients were screened for entry into the trial, from which 34 (16 female, 18 male) were randomized to 6 wk of open label VSL#3. The baseline characteristics of the patients

Table 2. Patient Demographic and Baseline Characteristics

Variable	VSL#3-Treated Group
Male/Female gender	16 (47) / 18(53)
Mean (SD, range) age at entry (yr)	35 ± 13
Maximum disease extent (no. of patients) (%)	
Rectum, sigmoid	4 (12)
Distal to the mid transverse colon	23 (67)
Pancolitis	7 (21)
Concomitant medications (%)	
Corticosteroids oral*	5 (15)
Corticosteroid enema*	4 (12)
Azathioprine or 6-mercaptopurine*	6 (18)
Antibiotics	None
Mesalamine oral*	25 (74)
Mesalamine enema*	4 (12)
None	1 (3)

*Duration on concomitant medication prior to study entry; oral corticosteroid (range 22–15 wk day), rectal corticosteroid (range 12–4 wk), azathioprine or 6-mercaptopurine (range 116–13 wk), oral mesalamine (range >104–27 wk), rectal mesalamine (range >52–5 wk).

are shown in Table 2. Patients screened but not randomized were excluded for not matching the inclusion criteria or for having exclusion criteria at the time of screening.

Clinical Effectiveness

Intent to treat analysis demonstrated that remission was achieved in 53% (n = 18) of patients; response in 24% (n = 8) of patients; no response in 9% (n = 3) of patients; (Fig. 1); worsening in 9% (n = 3) of patients; and 5% (n = 2) did not have the final sigmoidoscopy assessment. Of those patients with no response baseline UCDAI was 8, 6, and 8, and all patients were on baseline mesalamine 2 g/day. Of those patients with worsening of the disease baseline UCDAI was 7 in each patient and all were on baseline corticosteroids and mesalamine. Results of UCDAI scores at baseline study entry and following 6 wk of VSL#3 therapy are shown in Table 3. There was no difference in baseline mean UCDAI scores among those patients who achieved remission or response and

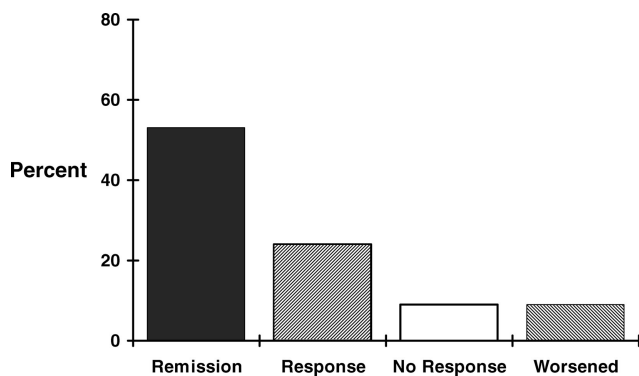


Figure 1. Intent to treat analysis following 6 wk of VSL#3 treatment in patients with active ulcerative colitis. Remission was achieved in 53% (n = 18), response in 24% (n = 8), no response in 9% (n = 3), and worsening of disease activity in 9% (n = 3). Two patients did not complete the final endoscopic assessment.

Table 3. Ulcerative Colitis Disease Activity Index Scores for Each Responding Group

Patient response to VSL#3	UCDAI Score		p-Value
	Baseline	Following 6 wk of VSL#3 therapy	
Remission	6.33 ± 0.41	1.06 ± 0.19	p < 0.001
Response	7.63 ± 0.42	4.25 ± 0.49	p < 0.001
No change	6.67 ± 0.33	6.00 ± 0.58	p = 0.188
Worsened	6.67 ± 0.33	9.67 ± 0.33	p < 0.001

Values are mean ± SEM relative to baseline.

those with no change or worsening of their disease activity. However, there were highly significant changes in UCDAI from baseline to study completion in those patients entering remission or responding (Table 3), implying not only a biologic but also a clinical effect of VSL#3 therapy. Eight patients continued their rectal therapy (Table 3) throughout the study period. Rectal therapy also did not appear affect the VSL#3-induced efficacy results as 5 of these patients demonstrated disease remission, while 2 had no change and 1 worsened, reflecting a similar proportion of response to those without rectal therapy.

Per-protocol analysis for the 32 patients that completed 6 wk of VSL#3 treatment demonstrated that remission was achieved in 56% (n = 18) of patients; response in 25% (n = 8) of patients; no response in 9% (n = 3) of patients; and worsening in 9% (n = 3) of patients.

Adverse Events

There were no biochemical adverse events related to VSL#3. Twenty-nine percent of patients (n = 10) reported increased bloating that they attributed to the VSL#3. In no cases was the bloating severe enough to stop the study medication.

BACTERIOLOGICAL ANALYSIS. All eight components of the VSL#3 preparation could be detected using a nucleic acid-based method to analyze the bacterial content of a sachet similar to that provided to the patients (Fig. 2). The bacterial components of VSL#3 could be differentiated, except that the 16S rRNA gene fragments from *L. acidophilus* and *L. delbrueckii* subspecies *bulgaricus* had the same migration properties in the DGGE gel. Similarly, *B. infantis* and *B. longum* DNA fragments could not be differentiated.

The presence of specific components of VSL#3 could be detected in DGGE profiles from patients by comparing the migration distances of their DNA fragments to the identification ladder. The bacterial origin of these fragments was subsequently determined by cloning and sequencing DNA fragments cut from the gel. Two bacterial species, *S. salivarius* subspecies *thermophilus*, and *B. infantis* were detected in association with biopsies collected after (but not before) the administration of VSL#3 in the case of 3 patients in remission. VSL#3 components were not detected in the profiles of the remaining 8 patients (Fig. 3).

Comparison of DGGE profiles generated from biopsies collected from patients before and after VSL#3 administra-

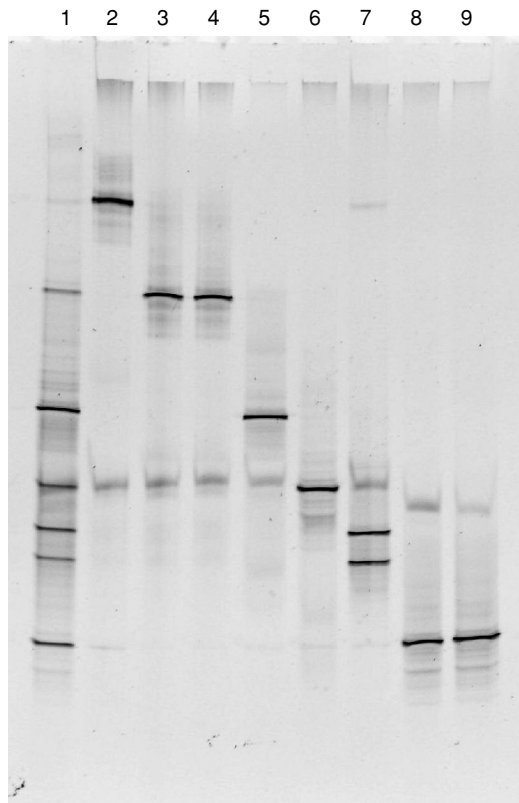


Figure 2. PCR–DGGE profiles generated from bacterial DNA extracted from the VSL#3 product and its eight individual components. Lane 1: VSL#3; lane 2: *Lactobacillus plantarum* MB452; lane 3: *Lactobacillus acidophilus* MB443; lane 4: *Lactobacillus delbrueckii* subsp. *bulgaricus* MB453; lane 5: *Streptococcus salivarius* subsp. *thermophilus* MB455; lane 6: *Bifidobacterium breve* Y8; lane 7: *Lactobacillus casei* MB451; and lane 8: *Bifidobacterium infantis* Y1; lane 9: *Bifidobacterium longum* Y10.

tion could be made for a subset of 7 patients. The DGGE profiles of 4 out of 5 patients in remission were considerably different after VSL#3 administration, whereas the 2 patients with continued active disease had high similarity before and after the consumption of VSL#3 (Fig. 3; Table 4). However, the study was not controlled with respect to temporal changes that might occur in biopsy profiles in the absence of probiotic treatment, or for the effect of concurrent medication, although the latter were administered in the same doses before and after therapy.

DISCUSSION

Treatment of patients with mild to moderate UC, not responding to conventional therapy, with the probiotic mixture VSL#3 results in a combined induction of remission/response rate of 94% in patients that completed the study; 77% of patients responded when analyzed in an intent to treat fashion. No adverse events were noted other than mild bloating.

Only a minority of patients were using concomitant mesalamine, corticosteroids, and purine anitmetabolites (Table 2) prior to VSL#3 treatment. It is unlikely that these

medications played a significant role in disease response since patients had been on oral therapies for a minimum of 13 wk and rectal therapies a minimum of 4 wk (both long enough for a therapeutic effect to the concomitant therapy to be identified before VSL#3 was started), indeed the majority had been on these agents much longer.

These results are relatively unique in that they examined the use of a highly concentrated mixture of probiotic bacteria to induce remission in patients with active UC. The only similar study in the management of active UC reported that uncontrolled administration of *Saccharomyces boulardii* for 4 wk induced clinical remission in 71% of 24 patients with mild to moderate UC (26). Previous studies have reported that *E. coli* 1917 Nissle was as effective as low dose mesalamine in preventing relapse of quiescent UC (22, 23). Similarly, an uncontrolled pilot study demonstrated that VSL#3 maintained remission over an interval of 12 months in 75% of UC patients (24). Bifidobacteria-fermented milk decreased the relapse rate of UC from 90 to 27% (28). These studies suggest that several probiotic preparations may have a role in treating and preventing relapse of UC. In contrast to the evidence for VSL#3 in prevention and maintenance of remission the role of VSL#3 in acute active disease remains to be determined.

Unlike most probiotics products that are composed of either single microbes or a combination of a few, VSL#3 is a mixture of eight probiotic lyophilized bacteria consisting of four strains of *L. casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus*, three strains of *B. longum*, *B. breve*, and *B. infantis*, and one strain of *S. salivarius* subsp. *thermophilus*. VSL#3 has been convincingly demonstrated, in randomized controlled clinical trials, to prevent the recurrence pouchitis following antibiotic-induced remission (29, 30) and postoperatively (31). In pouchitis, VSL#3 appears to exert several antiinflammatory mechanisms of action, including alteration in cytokine profile, and expression of nitric oxide and matrix metalloproteinases (32). Where the efficacy of VSL#3 is attributable to these antiinflammatory mechanisms and whether all eight bacteria are required remains under investigation.

PCR/DGGE provides a rapid screening method that is suitable for the analysis of bacterial communities (35–37), including the detection of altered microbiota composition. The method can be applied to biopsies collected from the human gut as well as feces (38). The method permits the detection of not only normal inhabitants of the gut, but also bacteria that have been consumed with food (39). PCR based methods have also been employed for the detection and quantification of the bifidobacterial strains in the VSL#3 product (40, 41); however, nucleic acid-based detection of the full bacterial mixture in the product has not been reported. We were able to detect all eight components of the product using a combination of PCR and DGGE.

Two of the components of VSL#3, *S. salivarius* subspecies *thermophilus* and *B. infantis*, were detected in 3 of the patients in remission, demonstrating that these bacteria reach the

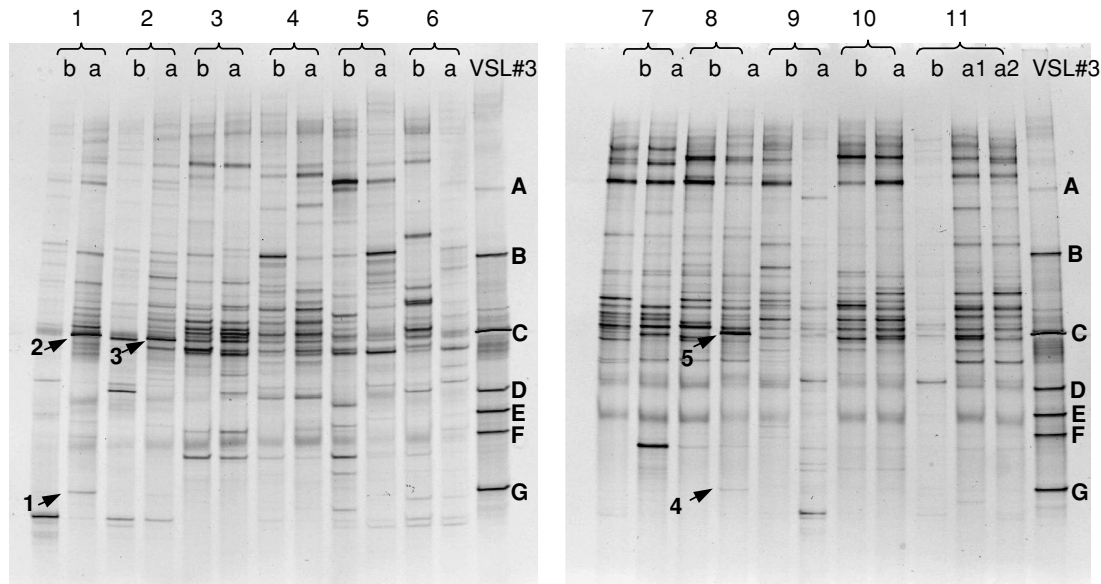


Figure 3. PCR–DGGE gels generated with DNA extracted from biopsies collected before (b) and after (a) the administration of VSL#3 in a subset of 11 patients. Ladder profile obtained with DNA extracted from the VSL#3 is shown in the last lane on the right of each gel. Numbered/lettered fragments were identified as described in the text. Fragment A: *Lactobacillus plantarum*; fragment B: *L. acidophilus*/*L. delbruekii* subsp. *bulgaricus*; fragment C: *Streptococcus salivarius* subsp. *thermophilus*; fragment D: *Bifidobacterium breve*; fragment E: *L. casei*; fragment F: *L. casei*; and fragment G: *B. infantis*/*B. longum*. Fragments 1 and 4: *Bifidobacterium infantis* Y1 (97.4% identity), fragment 2, 3, and 11: *Streptococcus thermophilus*/*Streptococcus salivarius* (99.5–100% identity).

diseased bowel site. Similarly, *B. infantis* Y1 and *B. breve* Y8 have been transiently detected previously by specific PCR in the feces of IBD patients and normal subjects who had consumed the product (40). Despite of the sensitivity of PCR, very low concentrations of the target gene (few bacteria) might not be detected in the DGGE gels. For instance, in the case of the VSL#3 preparation, the *L. plantarum* strain was poorly detected in the product and its association with biopsies might escape detection. However, it could be questioned whether such low numbers of bacteria in association with the gut mucosa would be of biological significance. We did not attempt to measure VSL#3 components in fecal samples,

since mucosal association may be more relevant to local biologic activity (6, 42). *S. salivarius* subspecies *thermophilus* and *B. infantis* may be of particular interest in future studies of probiotic treatment of active mild to moderate UC. They were the only two components of VSL#3 that were detected in association with biopsies and may therefore be the active ingredients of the bacterial mixture *in vivo*.

Our results suggest that oral administration of high doses of VSL#3 may effectively treat active UC and is associated with no serious adverse effects. A randomized placebo-controlled trial is currently underway to confirm the efficacy of VSL#3 in treating active UC.

Table 4. Comparison of the DGGE Profiles Generated from Biopsies from Ulcerative Colitis Patients Before and After the Administration of VSL#3. Concurrent Medication is also Indicated

Patient No.	Primary Outcome	Dice's Similarity Coefficient (%)	Concomitant Medication
2	R	57.9	Pentasa (4 g), betnesol (daily)
3	A	89.4	Salofalk (4 g), asacol (1.2 g)
4	R	85.2	Pentasa (4 g)
5	R	56.5	None
7	R	70.8	Pentasa (4 g), betnasol (daily)
8	R	79.1	Salofalk (4 g, daily)
10	A	95.2	Asacol (1.6 g), prednisone (10 mg)

Primary outcome; remission, R; active, A.

ACKNOWLEDGMENT

This work was partially supported by Crohn's and Colitis Foundation of Canada. VSL#3 provided by VSL#3 Inc.

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Queries

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