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Pretreatment with the probiotic VSL#3 delays transition from inflammation to dysplasia in a rat model of colitis-associated cancer

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Appleyard CB, Cruz ML, Isidro AA, Arthur JC, Jobin C, De Simone C. Pretreatment with the probiotic VSL#3 delays transition from inflammation to dysplasia in a rat model of colitis-associated cancer. *Am J Physiol Gastrointest Liver Physiol* 301: G1004–G1013, 2011. First published September 8, 2011; doi:10.1152/ajpgi.00167.2011.—Evidence supports involvement of microflora in the transition of chronic inflammation to neoplasia. We investigated the protective efficacy of the probiotic VSL#3 in a model of colitis-associated colorectal cancer. Chronic colitis was induced in Sprague-Dawley rats by administration of trinitrobenzene sulfonic acid (TNBS), followed 6 wk later by systemic reactivation. To induce colitis-associated dysplasia and cancer, the animals received TNBS (intravenously) twice a week for 10 wk. One group received VSL#3 in drinking water from 1 wk before colitis induction until death. The colons were examined for damage and presence of dysplasia or cancer. Samples were analyzed for cell proliferation and apoptosis, vitamin D receptor (VDR) expression, angiogenic factors, and presence of alkaline sphingomyelinase or phosphatase. Microbial community composition was evaluated by terminal restriction fragment-length polymorphism analysis of the bacterial 16S rRNA gene. None of the probiotic-treated animals developed carcinoma, and no high-grade dysplasia was found in either the proximal or mid colon. In contrast, 29% of the animals in the control group developed carcinoma in one or more regions of the colon. VSL#3-treated animals had significantly less damage than the vehicle treated-controls in all areas of the colon, and this correlated with decreased richness and diversity of the mucosally adherent microbiota. Treatment with the probiotic increased the antiangiogenic factor angiostatin, VDR expression, and alkaline sphingomyelinase. We concluded that pretreatment with the probiotic VSL#3 can attenuate various inflammatory-associated parameters, delaying transition to dysplasia and cancer, thus offering its potential therapeutic use in patients with long-standing colitis.

animal model; colon cancer

COLORECTAL CANCER (CRC) is the third most common type of cancer in the United States, causing thousands of deaths each year. Inflammatory bowel diseases (IBD) including ulcerative colitis (UC) and Crohn's disease have been linked to the pathogenesis of CRC (29). The incidence of IBD has risen significantly during the past several decades, possibly linked to nutrition and environmental factors (25). Several lines of evidence suggest that chronic inflammation is a key predisposing factor to CRC in IBD (23). The risk for developing CRC increases with both the duration and extent of the disease (4). Patients with UC are up to 30 times more likely to develop CRC than the general population, and are three times more likely to die from it (50).

Effective prevention of colitis-associated cancer is still far from being successfully achieved. Prophylactic colectomy is a nonattractive, efficacious method to prevent colon cancer in patients with dysplasia. Surveillance colonoscopy is burdened by the lack of understanding, even by gastrointestinal specialists, of the meaning of dysplasia (not preneoplasia, but neoplasia at the epithelial level) and by the need for a large number of biopsies to be taken from a single patient (as many as 65) to reduce the error linked to sampling (13, 47). Until now, reliable molecular markers predictive of early stages of cancer progression have not been available; therefore, to recommend patients with IBD a lifetime of annual colonoscopies may not be justified.

On these grounds, there is a definite need for safe agents that, by controlling the chronic inflammation affecting the colon of patients with IBD, may also reduce the risk of neoplasia. The role of 5-aminosalicylate (5-ASA) to prevent the progression from dysplasia to colon cancer has been supported by retrospective analysis. If patients with UC have an associated primary sclerosing cholangitis, then ursodeoxycholic acid has been suggested to be an effective prevention tool for colon cancer (1, 55).

In the past few years, multiple double-blind randomized placebo-controlled clinical trials have shown that the multistrain, high-potency probiotic preparation VSL#3 can control inflammation, and hence the clinical conditions of UC and pouchitis in adults and children (17, 21, 39). The anti-inflammatory properties associated with VSL#3 administration have been linked to changes in the microflora and local immune responses in both humans and animals (30, 53). Several animal studies and the use of CRC cell lines have demonstrated the potential effectiveness of administering probiotics alone, or, in combination with prebiotics, the potential to prevent neoplastic changes (35); however, knowledge of their effects and mechanisms of action in colitis-associated dysplasia is still sorely needed.

We have extended these findings by evaluating the potential efficacy of VSL#3 in our model of chronic inflammation that progresses to dysplasia and colon cancer. Our results suggest that treatment with VSL#3 can delay the transition to dysplasia and hence the development of cancer, offering a potential therapeutic use in patients with long-standing colitis.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (~6 wk of age) were singly housed in restricted-access rooms with controlled temperature (23°C)

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and 12-h:12-h light/dark cycle (Southern Veterinary Service, Ponce, Puerto Rico). Standard laboratory chow was provided ad libitum, and rats were weighed daily. Protocols were approved by the IACUC at Ponce School of Medicine.

Induction of colitis and development of dysplasia. Chronic colitis was induced via prolonged reactivation as described previously (3). Trinitrobenzene sulfonic acid (TNBS; 0.5 ml of 60 mg/ml in 50% ethanol; Sigma Aldrich; St. Louis, MO) was administered intracolonically. Six weeks later, rats were reactivated by intravenous administration of 5 mg/kg TNBS in 0.9% saline via a tail vein every 24 h for 3 consecutive days. Colitis-associated dysplasia was induced by repeating the TNBS twice a week for 10 wk (38). Euthanasia was by intraperitoneal pentobarbital (~1.5 ml of 65 mg/kg for rats >500 g). The experiments followed the *Guide for the Care and Use of Laboratory Animals* [publication no. DHHS (NIH) 86-23].

Probiotic treatment. Animals were randomly assigned to one of two treatment groups: VSL#3 ($n = 23$) or control (water, $n = 22$). VSL#3 (VSL Pharmaceuticals, Gaithersburg, MD) contains 333 billion colony-forming units (cfu) of lyophilized bacteria/g, including eight different strains (*Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus planatarum*, and *Streptococcus salivarus* subspecies *thermophilus*). VSL#3 was administered in drinking water from 1 wk before the initial colitis induction until death (Fig. 1*A*) at a dose adjusted to ensure consumption of 5 billion cfu bacteria per 100 g of body weight, based on a daily intake of 3,600 billion bacteria for an adult human with a mean weight of 70 kg (5).

Assessment of macroscopic injury. Each colon was scored for macroscopic damage based on a well-defined, four-criteria scoring system: presence of adhesions (0, 1, or 2 for none, minor or major, respectively), diarrhea (0 or 1 for absence or presence), colon thickness (in mm), and ulceration (0 for no damage, with increasing scores up to 10, depending on the extent of ulceration) (38). These variables were added to give a total macroscopic damage score.

Sample collection. Equal thirds (proximal, mid, and distal) of the colon were sectioned, and segments were cut longitudinally; ~100 mg from each segment was frozen in liquid nitrogen and stored at −80°C. The other half from each segment was fixed (10% buffered formalin) for histology. The Swiss-roll technique was used to assess the entire colonic tissue (38).

Microscopic damage and pathological assessment. Two blinded observers scored tissues for microscopic damage including loss of mucosal architecture (0–3, absent, mild to severe), cellular infiltration

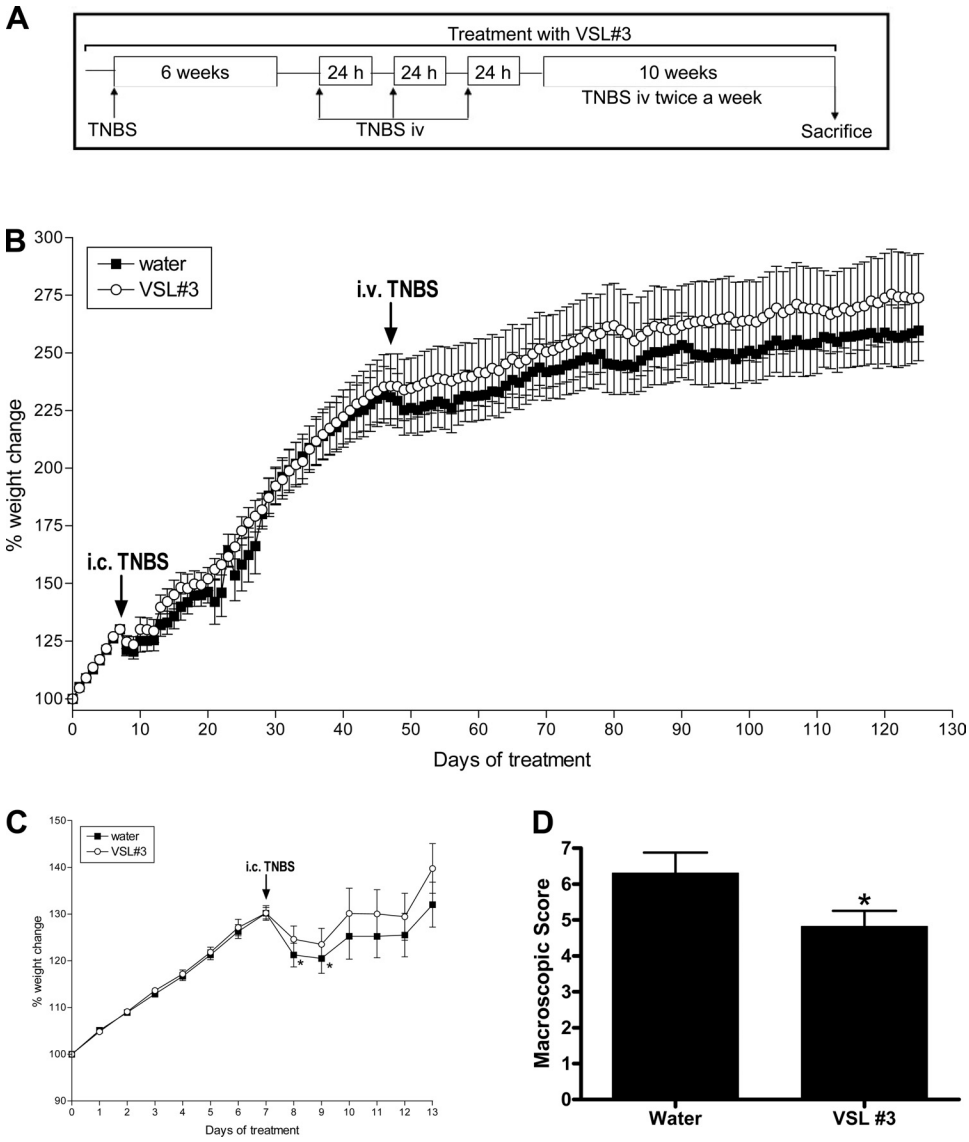

Fig. 1. Pretreatment with VSL#3 decreases macroscopic damage in colitis-associated dysplasia. *A*: experimental design and treatment protocol. *B*: effect of VSL#3 administration on weight change. *C*: weight change during first 13 days of treatment. *D*: macroscopic damage (*$P < 0.05$, $n = 22$–23/group ± SE). TNBS, trinitrobenzene sulfonic acid.

(0, none; 1, in muscularis mucosae; 2, in lamina propria/villi; 3, in serosa), muscle thickening (0, muscle <1/2 of mucosal thickness; 1, muscle = 1/2–3/4 of mucosal thickness; 2, muscle = mucosal thickness; 3, muscle > mucosal thickness; 4, all muscle), goblet cell depletion (0, absent; 1; present), and crypt abscess formation (0, absent; 1, present) (2). The score of each variable was added to give a total microscopic damage score (maximum of 12), and the average of the two assessments was calculated. A blinded pathologist classified tissues as dysplasia negative, dysplasia positive, or carcinoma (38). Tissues negative for dysplasia were subcategorized as normal (small nucleus, normal architecture), nonspecific inflammation, or active colitis/IBD (cryptitis, glandular invasion by neutrophils, crypt abscesses, microabscesses). Positive dysplasia was characterized by low-grade dysplasia, including hyperchromasia, increased nuclear/cytoplasmic ratio, irregular nuclear outline, and increased number of normal mitosis. High-grade dysplasia also included loss of mucosal architecture in the crypts. High-grade dysplasia with an increased number of atypical mitosis were classified as carcinoma (14, 6).

Assessment of cell proliferation. A random subset of animals (6 per treatment) received bromodeoxyuridine (BrdU; 50 mg ip/kg body wt) 1 h before euthanasia. The distal colon was analyzed by immunohistochemistry for BrdU. Representative crypts extending from the lumen to the muscularis mucosae were randomly analyzed. Two blinded observers counted a minimum 1,000 total crypt cells per slide. The average percentage of crypt cells expressing BrdU per slide was calculated.

Immunohistochemistry. Formalin-fixed 4-μm tissue sections were deparaffinized with xylene and then hydrated through descending grades of ethanol to distilled water. Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide for 15 min. Following antigen retrieval on a hot plate with the appropriate buffer (0.01 M citrate buffer, pH 6.0, 95–99°C for 40 min), slides were cooled at room temperature for 20 min, rinsed with distilled water (2 changes, 2 min each), and placed in PBS for 5 min. Slides were dried and blocked with normal serum for 15 min, followed by overnight incubation with the primary antibody, BrdU (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or vitamin D receptor (VDR; 1:2,000 dilution; Abcam, Cambridge, UK). Super-sensitive link-label immunohistochemistry detection system and liquid diaminobenzidine (BioGenex, Freemont, CA) were used.

TUNEL staining. Fragmented nuclear DNA (apoptosis) was detected by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method using a kit (GenScript, Piscataway, NJ) in 4-μm paraffin-embedded sections; 4′,6-diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain. Fluorescent images were captured on an Olympus BX51 microscope and analyzed with Image J software (NIH, Bethesda, MD). TUNEL (+) cells were quantified, and the percentage of positive cells was calculated from ×10 magnification photomicrographs of proximal colon.

Quantification of VEGF, endostatin, and angiostatin. Proteins were extracted using lysis buffer and analyzed by Western blots (9). Lysates (100 μg) were separated (15% SDS-PAGE) and blotted to polyvinylidene difluoride. Membranes were blocked (Tris/NaCl, 5% nonfat dry milk) before overnight incubation with primary antibodies: vascular endothelial growth factor (VEGF, Santa Cruz Biotechnology), angiostatin (Abcam), or endostatin (R&D Systems, Minneapolis, MN). Membranes were washed with Tris/NaCl and incubated with horseradish peroxidase-labeled secondary antibodies. Bands were detected using ECL-Plus reagent kit (GE Amersham, Piscataway, NJ) and densitometrically quantified.

Expression of the angiogenic factor, VEGF, was assessed in rat serum by a Quantikine Rat VEGF solid-phase ELISA (R&D Systems). The VEGF concentration in the serum samples was extrapolated from a standard curve (R^2 = 0.9997). A rat VEGF control (measured at 220.96 pg/ml) fell within the range specified (184–307 pg/ml).

Quantification of alkaline sphingomyelinase. The levels of alkaline sphingomyelinase (Alk-SMase) were assessed in rat serum or colon using a commercially available ELISA (E90801Ra; USCN; Life Science, Wuhan, China; sensitivity of 0.043 ng/ml). Tissue samples were homogenized in ice-cold PBS (pH 7.2) and then centrifuged at 5,000 *g* for 5 min. Duplicate samples were averaged, and Alk-SMase concentration was extrapolated using standard curves (R^2 = 0.9935 and R^2 = 0.9998 for serum and tissue, respectively).

Quantification of alkaline phosphatase. Levels of alkaline phosphatase were measured in fecal samples (43). In brief, fecal pellets were collected at euthanasia, frozen in liquid nitrogen, and stored at −80°C until assay. Fecal samples were homogenized in water (~1 g/ml) and centrifuged. Supernatants were diluted 125, 250, and 500 times in 0.05 M ammediol buffer containing 2 mM $MgCl_2$. Dilutions were analyzed in duplicate on 96-well plates. Fifty microliters of 10 mg/ml 4-nitrophenyl phosphate disodium salt were added to each well, and the plate was incubated at 37°C for 30 min. The reaction was stopped by adding 105 μl 1 N NaOH, and optical densities were measured at 405 nm. After a blank from each sample was subtracted, the average activity was calculated from the three dilutions.

T-RFLP analysis and quantification of S. thermophilus and microbiota analysis. DNA was extracted from 100 mg of proximal colon tissue for terminal restriction fragment-length polymorphism (T-RFLP) analysis (48). The relationships between dysplasia and luminal (fecal) and proximal colon tissue microbial community structure, species richness (number of species), and diversity (relative abundance) were investigated using a grading system where normal = 0, nonspecific inflammation = 1, IBD = 2, low-grade dysplasia = 3, high-grade dysplasia = 4, and cancer = 5. T-RFLP abundance was compiled into a Bray Curtis similarity matrix using PRIMER version 6 (Primer-e, Ivybridge, UK), and ANOSIM was used to test for differences in global microbial community composition between groups. Quantitative real-time PCR (using the ΔΔCt method) was performed on total DNA from fecal and colon samples to quantify the number of *S. thermophilus* (48).

Statistical analysis. Values are means ± SE, where *n* represents one sample from one animal for a single experimental replicate; α = 0.05. Biodiversity was measured by Margalef's test for richness and Shannon-Weiner diversity index. Differences in richness or diversity between groups were assessed by Student's *t*-test. Data sets were tested for normality using D'Agonstino and Pearson omnibus normality test. Pearson correlation was used to assess linear relationships between dysplasia score and species richness or diversity. Statistical analyses were by GraphPad Instat v3.0 and GraphPad Prism v3.0 (GraphPad Software, San Diego, CA).

RESULTS

Probiotic treatment attenuates macroscopic damage. All animals were observed daily throughout the study for general appearance and behavior. There were no significant differences in weight of the animals between the two treatment groups before induction of colitis (average body wt = 243.27 ± 5.08 g).

During the study (~18 wk), all rats gained weight overall (Fig. 1*B*). After colitis was initially induced (on *day 7*), the water-treated animals lost a significant amount of weight until recovery by *day 13* ($P < 0.05$; Fig. 1*C*). Interestingly, the VSL#3-treated animals did not lose a significant amount of weight during this time period. In addition, after TNBS was administered for colitis reactivation, the VSL#3-treated animals did not show a decrease in weight and gained more weight during the remainder of the study. All probiotic-treated animals tolerated the dosing regimen, with no notable differences observed in the general appearance of the animals or

occurrence of diarrhea between the two groups. There was no significant difference in absolute weight of the animals between the two groups at the time of euthanasia (459.81 ± 10.65 vs. 443.00 ± 6.67 g for VSL#3 and water, respectively). There were no differences in the volume of fluid consumed per day between the groups, and all VSL#3-treated animals consumed 5 billion cfu of bacteria per 100 g of body weight. Successful administration of the probiotic was confirmed by measuring *S. thermophilus* levels in stool and colon mucosal tissue samples by real-time PCR. *S. thermophilus* levels in the feces and colonic mucosa were 64 ± 3.63 and 36 ± 2.88 times higher, respectively, in animals that received VSL#3.

Treatment with VSL#3 had no effect on the mortality rate following the induction of colitis [7 out of 23 (30.4%) animals who received VSL#3 treatment died vs. 5 out of 22 (22.7%) animals receiving water]. In both groups, this usually occurred ~1 wk after colitis induction (6.6 ± 1.0 days in water-treated vs. 7.7 ± 1.8 days in VSL#3-treated). This is consistent with the effect of intracolonic TNBS in the acute model of colitis, where, in some animals, the initial inflammation (characterized by massive granulocyte infiltration, epithelial destruction, and transmural necrosis) does not resolve over time, either leading to intestinal perforation or obstruction of the intestine resulting in a "mega-colon" appearance with eventual death.

Treatment with VSL#3 significantly decreased the total macroscopic damage score compared with results in control animals ($P < 0.05$; Fig. 1*D*). Although each individual parameter was reduced by VSL#3 treatment, the most significant change was regarding the presence of adhesions ($P < 0.05$; Table 1).

VSL#3 administration decreases microscopic damage. In the water-treated animals, the amount of microscopic damage increased going down the colon, with significantly more damage found in the distal than in the proximal region ($P < 0.01$; Fig. 2, *A*, *B*, and *D*). A similar pattern was observed in VSL#3-treated animals, where both the mid and distal regions had significantly more damage than the proximal region ($P < 0.05$; Fig. 2, *A*, *C*, and *E*). The total microscopic damage was significantly lower in all regions of the colon in VSL#3-treated animals compared with the control group ($P < 0.05$).

VSL#3 administration prevents development of carcinoma. Pathological analysis was carried out to identify normal tissue, inflammation (IBD and nonspecific inflammation), dysplasia, and carcinoma. On analysis of any region of the colons, neither group received a normal diagnosis. In VSL#3-treated animals, 81.2% had dysplasia in some region of the colon compared with 76.5% of vehicle-treated animals. Notably, none of the VSL#3-treated animals developed cancer in any region of the colon; however, 29.4% (5/17) of control animals developed carcinoma (Fig. 3*A*; Table 2). The most severe pathology found in any of the VSL#3-treated animals was high-grade dysplasia. In general, VSL#3 treatment caused a shift toward a less severe diagnosis in the animals with less IBD but a more nonspecific inflammation (Table 2). Development of high-grade dysplasia in VSL#3-treated animals was also limited to the distal region of the colon.

Species richness and diversity correlate with pathology in VSL#3-treated animals. In VSL#3-treated animals, there was a positive correlation between proximal colon dysplasia score and proximal colon tissue microbial richness (Fig. 4*A*) or diversity (Fig. 4*B*). In these animals, a lower dysplasia score correlated with lower species richness and diversity in the tissue microbial community. This correlation was not found in control animals. Colons were considered neoplastic if they exhibited low-grade dysplasia, high-grade dysplasia, or cancer (scores 3–5). We found that colon tissue species richness and diversity did not differ between rats with neoplastic colons.

Administration of VSL#3 increases VDR expression. Because low levels of vitamin D and the VDR have been noted in CRC, VDR expression was examined and was found to be significantly higher in the proximal and distal colons of VSL#3-treated animals than in water-treated animals (Fig. 5*A*). Regardless of treatment, VDR expression was highest in areas with a normal diagnosis and lowest in those areas with carcinoma ($P < 0.05$) (Fig. 5, *B* and *C*).

Probiotic treatment decreased alkaline phosphatase levels. Proliferation of colon cells was examined by analyzing the incorporation of BrdU in the distal colon. BrdU incorporation in water-treated animals was 6.97 ± 0.85% (Fig. 6, *A* and *B*). Treatment with VSL#3 reduced crypt cell proliferation by 22%, but this did not reach significance (Fig. 6, *A* and *C*).

To assess cell apoptosis, DAPI was used to stain all nuclei followed by TUNEL-specific staining with merge to calculate the percentage of apoptotic cells (Fig. 6*D*). No significant differences were found in the percentage of apoptotic cells in the colon mucosa between the treatment groups (3.11 ± 0.46% in water-treated vs. 3.01 ± 0.61% in VSL#3-treated animals).

The concentration of Alk-SMase found in the rat serum of animals treated with VSL#3 was twice that found in the water-treated animals (0.12 ± 0.65 vs. 0.06 ± 0.05 ng/ml) but did not reach significance. Alk-SMase levels in the colon tissue were similar between the two groups (4.29 ± 0.78 for water-treated vs. 3.67 ± 0.65 ng/ml for VSL#3-treated animals). Fecal alkaline phosphatase levels in VSL#3-treated rats were ~50% of controls ($P = 0.057$).

VSL#3 increases expression of angiostatin in the colon. Expression levels of the angiogenic factor, VEGF, and antiangiogenic factors (angiostatin and endostatin) were measured in tissue samples taken from the proximal colon of animals with colitis-associated cancer treated with or without VSL#3. Angiostatin expression levels were significantly increased in the VSL#3-treated animals (Fig. 7, $P < 0.05$; $n = 10$/group). No differences were found in the expression of endostatin between the two treatment groups (1.09 ± 0.09 for water-treated vs. 1.03 ± 0.13 units for VSL#3-treated animals; $n = 14–15$).

Table 1. *Effect of VSL#3 on macroscopic damage (individual parameters)*

Treatment	Adhesions	Diarrhea	Ulceration	Thickness	Total Score
Water	1.29 ± 0.19	0.18 ± 0.09	3.53 ± 0.46	1.34 ± 0.08	6.31 ± 0.57
VSL#3	0.56 ± 0.16*	0.06 ± 0.06	2.88 ± 0.34	1.31 ± 0.09	4.82 ± 0.44*

Values are means ± SE. *$P < 0.05$ vs. water treatment for same parameter.

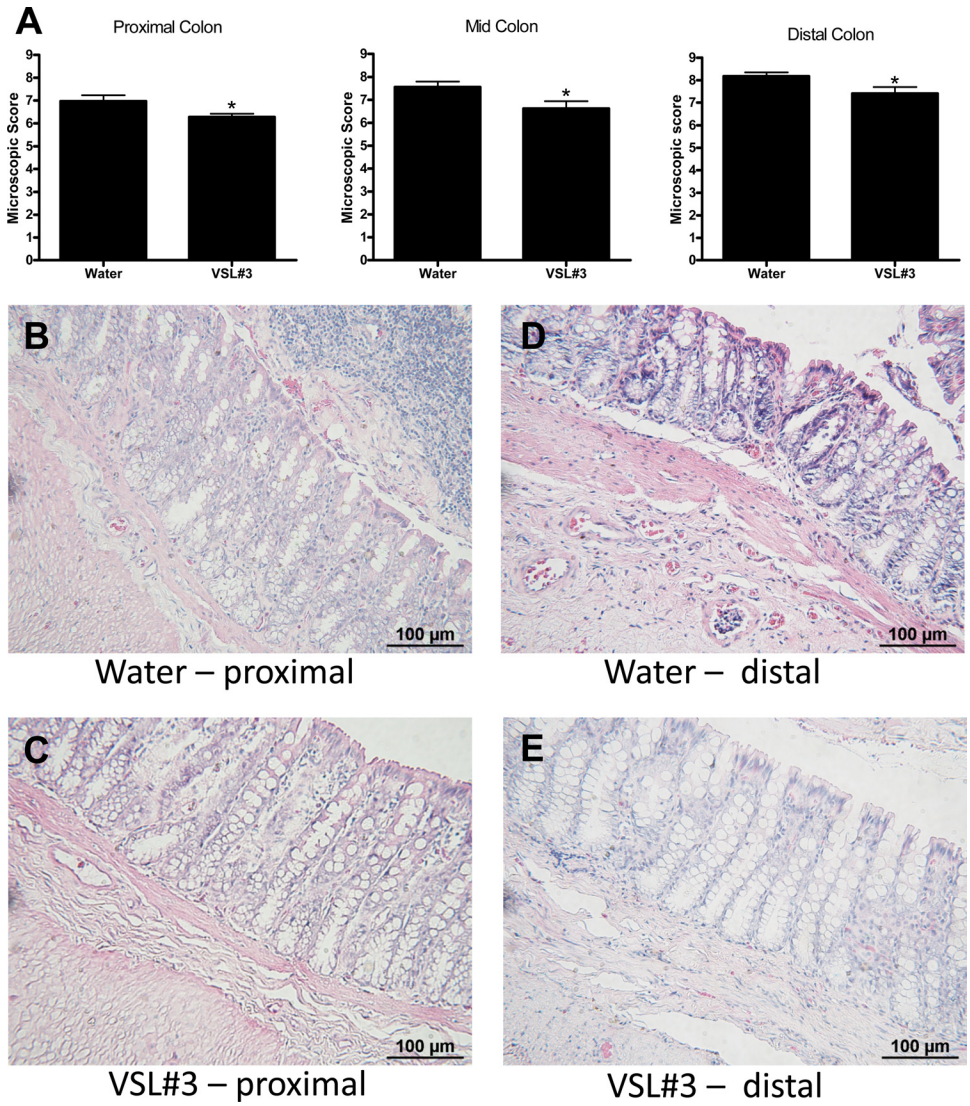

Fig. 2. Effect of VSL#3 on microscopic damage in colitis-associated dysplasia. *A*: total microscopic damage score was lower in the probiotic-treated group within each region of the colon (*$P < 0.05$, $n = 16–17$/group ± SE). Representative histologies from proximal and distal regions of colon from water-treated (*B* and *D*) and VSL#3-treated (*C* and *E*) animals, respectively; ×40.

VEGF levels in tissue could not be measured because bands were too faint, even after optimization, regardless of treatment group. These results were corroborated by measurement of VEGF in rat serum, which was also minimal, with no difference between the treatment groups (3.09 ± 1.04 in water-treated vs. 3.18 ± 1.33 pg/ml in VSL#3-treated animals; $n = 16–17$).

DISCUSSION

It is apparent that a link exists between chronic inflammation and cancer throughout the gastrointestinal system, but it is not yet clear how the presence of chronic inflammation results in neoplastic transformation and progression. As outlined earlier, several lines of evidence support the theory that the use of probiotics could potentially modify the transition of inflammation to dysplasia. Proposed mechanisms may include suppression of the growth of bacteria, which may convert procarcinogens to carcinogens, binding to mutagenic compounds, and modification of gut microflora (reviewed by Rafter 35). It has recently been demonstrated that administration of VSL#3, a probiotic preparation with eight different strains of bacteria, can modify both species richness and the microbial diversity found within the luminal microbial community (48). Moreover, damage scores in the TNBS model of chronic inflammation correlated with changes in species richness and diversity (48). Our results extend this finding, demonstrating that, in VSL#3-treated animals, tissue microbial species richness and diversity correlate with severity of dysplasia. It is possible that reduced richness and diversity in VSL#3-treated mice are the consequence of either selective expansion of cancer-protective or reduction of cancer-promoting, mucosal-associated bacteria. Identification of these mucosal-associated microorganisms will require 16S deep-sequencing analysis combined with various bacterial culture techniques. Alternatively, VSL#3 could directly modulate cancer development through intrinsic biological activities associated with these probiotic bacteria.

The VSL#3 probiotic preparation has already been demonstrated to attenuate intestinal inflammation in various animal models, including the IL-10 knockout mouse model (27, 36, 41), the DSS-induced colitis model (15), and the SAMP1/YitFc mouse model of ileitis (31). Two studies conducted on pouch biopsy samples obtained from patients with pouchitis before

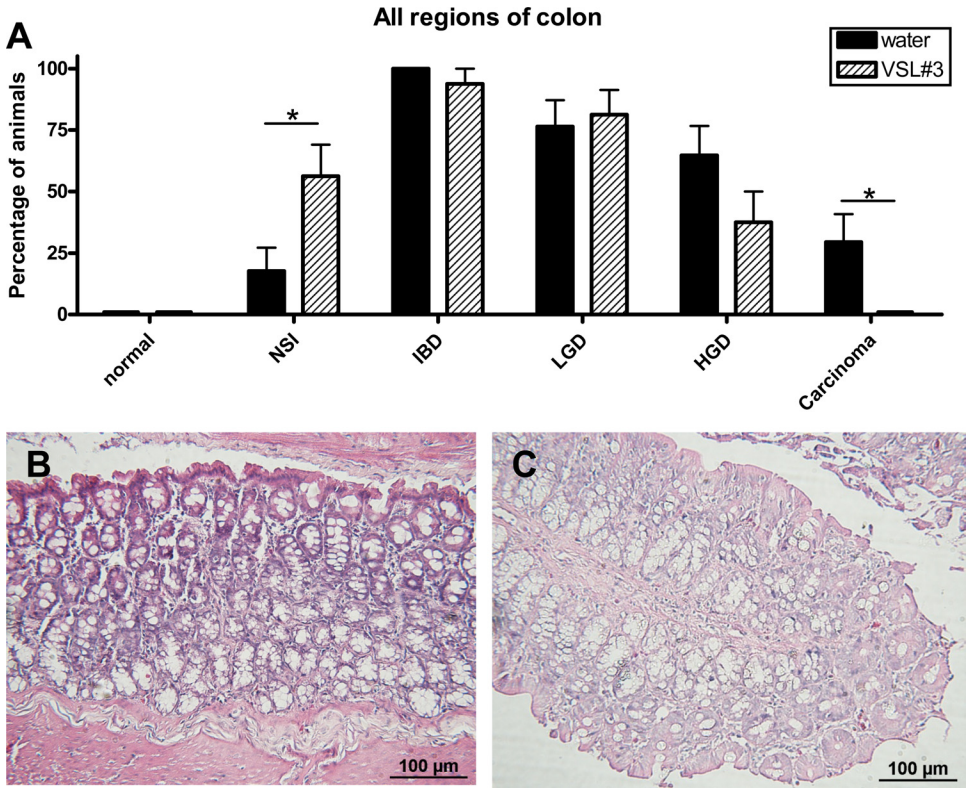

Fig. 3. Probiotic-treated animals did not progress to carcinoma. *A*: effect of VSL#3 on pathological analysis (*$P < 0.05$, n = 16–17/group ± SE). NSI, nonspecific inflammation; IBD, inflammatory bowel disease; LGD, low-grade dysplasia; HGD, high-grade dysplasia. *B*: LGD. *C*: HGD with carcinoma in situ. Atypical mitosis, no goblet cells, and irregular nuclei are shown; ×40.

and after VSL#3 treatment showed increased levels of IL-10 and CD4+CD25high and CD4+LAP-positive cells (34, 45). In subjects with UC, treatment with VSL#3 resulted in a decreased expression of Toll-like receptor-2 on dendritic cells, accompanied by an augmented production of IL-10 (30). In addition, recent multicenter clinical trials on patients with UC have confirmed previous data about the efficacy of VSL#3 in pouchitis, demonstrating that this probiotic can successfully induce remission in both adult and pediatric patients (20, 21, 28, 42, 44).

Our study demonstrates for the first time that pretreatment with this probiotic preparation not only reduces colonic inflammation but is also able to attenuate the transition from chronic inflammation to colon cancer in a model of colitis-associated dysplasia. None of the animals receiving VSL#3 developed carcinoma, which is in contrast to 30% of water-treated animals developing carcinoma. Treatment with the probiotic appeared to cause a shift to a less severe diagnosis, with no high-grade dysplasia found in either the proximal or mid colon of treated animals.

Some genera of bacteria, such as *Clostridium*, *H. pylori*, and *Bacteroides*, have been found in association with a higher incidence of neoplasia, whereas bifidobacteria and lactobacilli are usually considered to be "protective" against inflammation and damage. Although results from studies aimed at assessing the relationship between microbiota and colon cancer reduction have been difficult to interpret, the potential role of certain species in protecting the host from chronic inflammatory processes has been highlighted. In this context, regarding the observed protective effects of VSL#3 in our model, we can extrapolate several possible hypothetic mechanisms of action.

One possible mechanism is linked to the presence of a sphingomyelinase working at an alkaline pH (Alk-SMase) in the bacterial mix of VSL#3. Alk-SMase is an enzyme present in the gastrointestinal tract of mammals that catabolizes sph-

Table 2. *Effect of VSL#3 on percentage of animals with all pathology diagnosis per region*

Treatment	Normal	NSI	IBD	LGD	HGD	Carcinoma
Proximal region						
Water	0	5.88 ± 5.88	82.35 ± 9.53	70.59 ± 11.39	23.53 ± 10.60	29.41 ± 11.39
VSL#3	0	31.25 ± 11.97	62.50 ± 12.50	50.00 ± 12.91	0*	0*
Mid region						
Water	0	11.76 ± 8.05	82.35 ± 9.53	58.82 ± 12.30	29.41 ± 11.39	0
VSL#3	0	25.00 ± 11.18	68.75 ± 11.97	31.25 ± 11.97	0*	0
Distal region						
Water	0	5.88 ± 5.88	94.12 ± 5.88	52.94 ± 12.48	35.29 ± 11.95	0
VSL#3	0	18.75 ± 10.08	81.25 ± 10.08	62.50 ± 12.50	37.50 ± 12.50	0

Applicable values are means ± SE. *$P < 0.05$ vs. water treatment for same diagnosis within same region of the colon. NSI, nonspecific inflammation; IBD, inflammatory bowel disease; LGD, low-grade dysplasia; HGD, high-grade dysplasia.

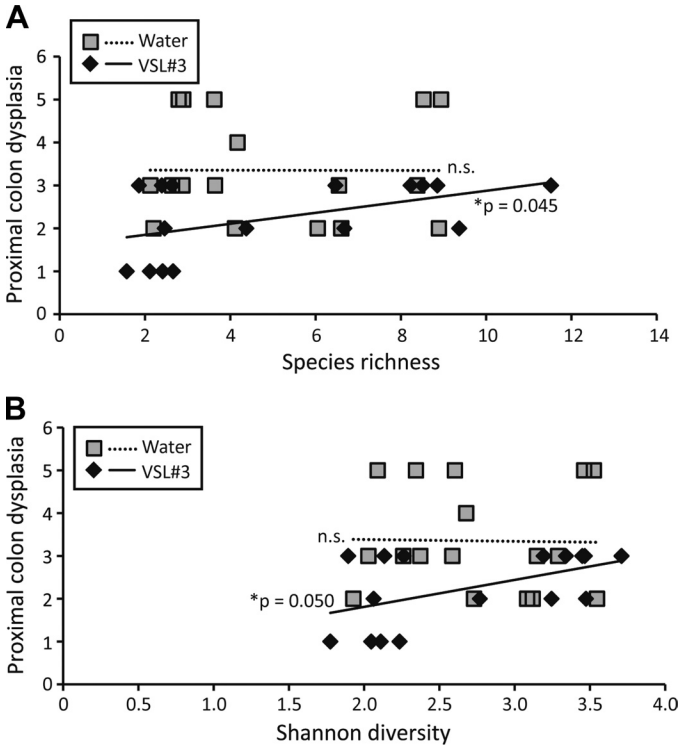

Fig. 4. Extent of proximal colon dysplasia in VSL#3-fed rats correlates with proximal colon tissue microbial species richness and diversity. *A*: colon tissue Margalef's richness vs. proximal colon dysplasia score. Pearson's correlation for water group, $P = 0.992$; for VSL#3 group, $*P = 0.045$. *B*: colon tissue Shannon diversity vs. proximal colon dysplasia score. Pearson's correlation for water group, $P = 0.944$; for VSL#3 group, $*P = 0.050$.

ingomyelin (11). Its activity can be decreased by 75% in colon carcinoma tissue compared with adjacent normal mucosa (18, 19). Until now, reduced levels of Alk-SMase have been reported in patients with IBD, colorectal adenomas, familiar adenomatous polyposis, and colon dysplasia (40). Recently, Alk-SMase activity in human stool has been proposed as a prognostic and diagnostic marker of colorectal neoplasia (10). Our results corroborate previous findings demonstrating that VSL#3 administration in a mouse model of colitis or in patients with UC upregulated the mucosal Alk-SMase activity and significantly ameliorated the histological injury score (41). Interestingly, substances such as 5-ASA, ursodeoxycholic acid, and psyllium, which are now generally considered to be candidates for colon cancer prophylaxis, are all able to upregulate the mucosal Alk-SMase activity.

Upregulation of alkaline phosphatase activity has previously been found in the inflamed intestine (37). Fecal levels of alkaline phosphatase, an enzyme found in the apical membrane of enterocytes, were lower in our probiotic-treated animals than in controls. Damage to the epithelial cell membrane releases alkaline phosphatase into the fecal material; thus our results suggest that those animals not receiving the probiotic may be shedding more alkaline phosphatase, which is being produced in higher amounts in an attempt to protect the colon (26).

Interestingly, low levels of vitamin D have been associated with an elevated risk of CRC. Vitamin D may reduce cell proliferation while stimulating apoptosis and cell differentiation, suggesting a significant, protective effect (22). The importance of vitamin D and VDR in regulating intestinal mucosal barrier integrity has previously been demonstrated (24). Expression of VDR has recently been shown to be decreased in patients with dysplasia and colitis-associated CRC, in line with the results presented here (51). The increased expression of VDR in our animals receiving VSL#3 suggests the ability of the bacteria to modulate this receptor, also supporting the notion that some autoimmune diseases may result from bacteria-induced VDR dysfunction (54).

Another possible mechanism is related to the hypothesis that VSL#3, by modifying the composition of the gut microflora, changes the balance between the enzymes of the microbiome, producing carcinogens and the enzymes converting the precarcinogens into inactive metabolites. Of interest could be the production of conjugated linoleic acids, a group of isomers of

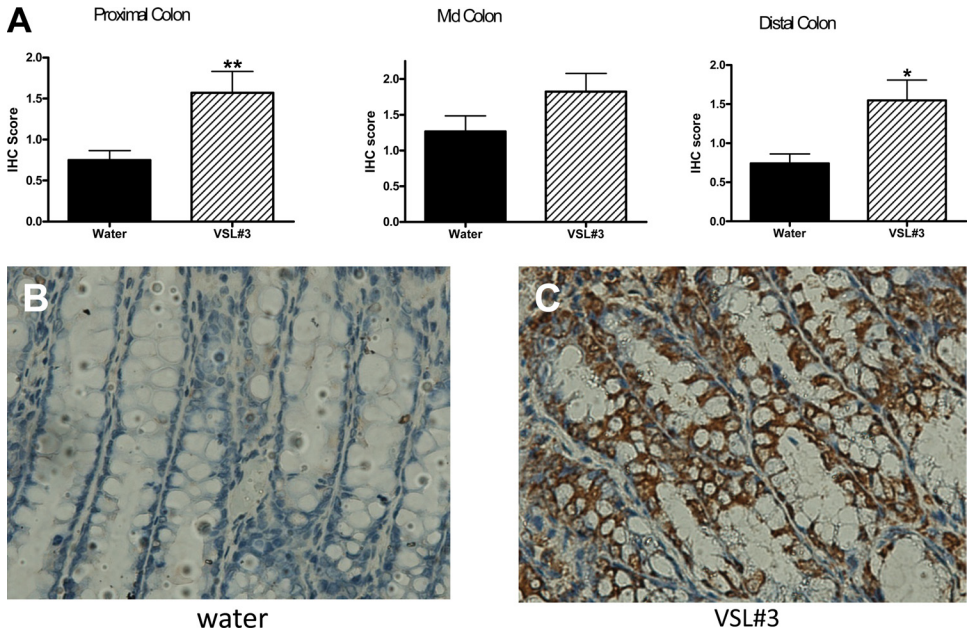

Fig. 5. Administration of the probiotic VSL#3 increases vitamin D receptor (VDR) expression. *A*: VDR expression was higher in VSL#3-treated animals in all regions of the colon ($*P < 0.05$, $**P < 0.01$, $n = 16–17$/group ± SE). Representative histologies from mid colon in a water-treated (*B*) and a VSL#3-treated (*C*) animal; ×40. IHC, immunohistochemistry.

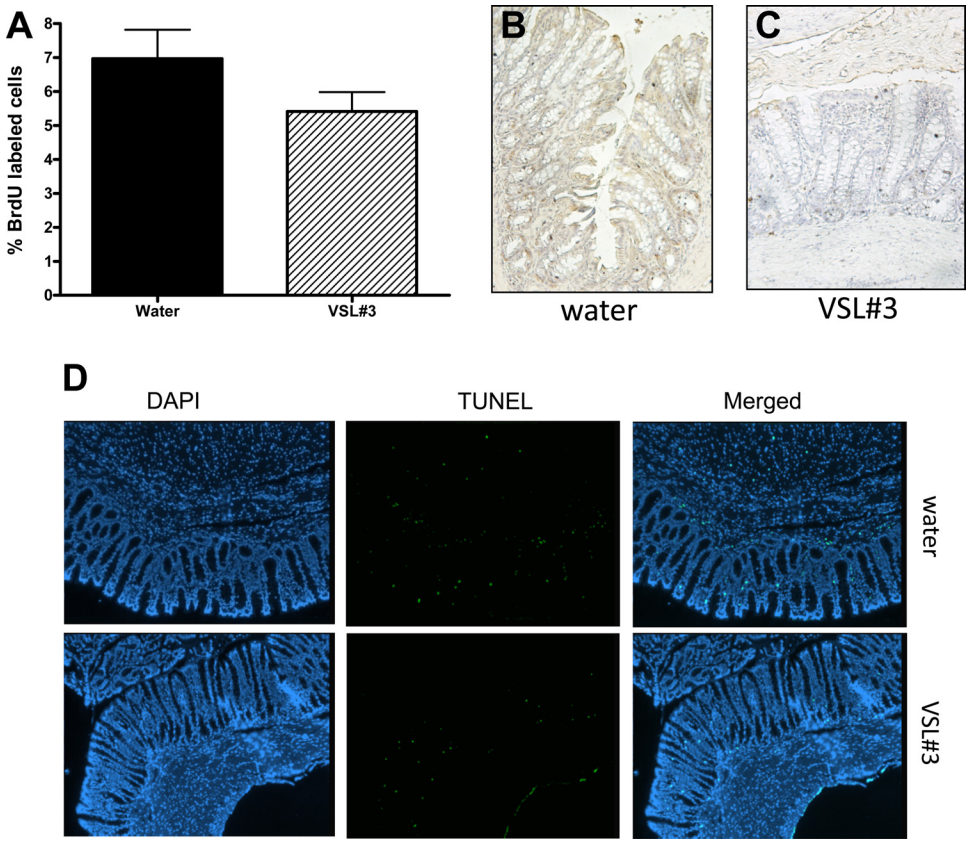

Fig. 6. Effect of probiotic treatment on crypt cell proliferation and apoptosis in colitis-associated cancer model. *A*: bromodeoxyuridine (BrdU) incorporation in distal colon of animals treated with water or VSL#3 (n = 6/group). Representative photos from distal colons of water-treated (*B*) and VSL#3-treated (*C*) animals. *D*: 4′,6-diamidino-2-phenylindole (DAPI) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining to assess the percentage of apoptotic cells showing no difference between treatment groups.

linoleic acid-possessing anti-inflammatory and anticarcinogenic properties, as well as a positive influence on the host's lipid profile and insulin resistance (52). All strains of VSL#3 have been shown to produce *cis-9*, *trans-11*, *trans-10*, and *cis-12* isomers of conjugated linoleic acids from linoleic acid, inducing the upregulation of PPARγ in HT-29 cells and reducing cancer cell viability in vitro (12). Such properties of VSL#3 have been suggested to have roles in attenuating inflammation and for preventing colon cancer.

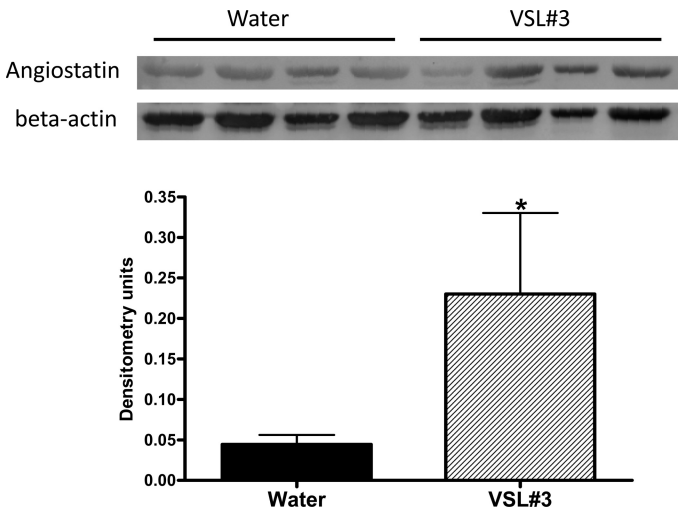

Fig. 7. Effect of probiotic treatment on expression of angiostatin (50 kDa) in an animal model of colitis-associated colon cancer. Tissues from proximal colons of treated animals were ground, and equal amounts of protein (100 μg) were separated by 15% SDS-PAGE before Western blot analysis. Gels show representative samples from 4 rats/group. Western blot bands were quantified by densitometry (*$P < 0.05$ vs. water, n = 10 rats/group ± SE).

Additional information stemming from our study is the role of this specific preparation when administered in an animal model of chronic inflammation on angiogenesis. Angiogenesis, the formation of new blood vessels, contributes to the neoplastic process and plays a role in the pathology of IBD (33). 5-ASA (mesalamine), a drug often used to treat IBD, in addition to effects of cyclooxygenase and lipoxygenase pathways, can alter the balance between angiogenic and antiangiogenic factors (32, 43). In this study we examined the impact of modulation of the microflora on levels of the angiogenic factor VEGF and the antiangiogenic factors endostatin and angiostatin. Angiostatin is an endogenous inhibitor of angiogenesis and is undergoing clinical trials in anticancer protocols. VEGF is a substance produced by the cells to stimulate the growth of new blood cells, and cancers that express VEGF are metastasizing ones. Interestingly, we found that angiostatin levels in VSL#3-treated animals were almost fivefold higher than those in the water-treated group, whereas VEGF levels were comparable between the different animal groups before and after treatment. The possibility that this mediator may help limit the further development of tumor growth in colitis-associated CRC requires further investigation.

In conclusion, the relationship between microbes and CRC, including colitis-associated CRC, has been increasingly scrutinized (16, 49) and the protective role for probiotics debated (7, 8). The results of the present study demonstrate that pretreatment with the probiotic VSL#3 can attenuate the development of colonic damage and delay the transition to

dysplasia and cancer. The minor effects induced by VSL#3 may also be more evident in chronic models of colonic inflammation, and such future studies should be performed. Regardless, our outcomes further support the theory that, if chronic inflammation is the main risk factor for developing CRC in patients with IBD, suppression of inflammation will decrease this risk (46). Moreover, our results open the debate about the role of probiotics in angiogenesis and cancer metastasis and hint at their potential therapeutic use in patients with longstanding colitis.

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DISCLOSURES

C. De Simone has stock ownership in VSL Pharmaceuticals. All other authors have nothing to disclose.

REFERENCES

1. **Alberts DS, Martinez ME, Hess LM, Einspahr JG, Green SB, Bhattacharyya AK, Guillen J, Krutzsch M, Batta AK, Salen G, Fales L, Koonce K, Parish D, Clouser M, Roe D, Lance P, Phoenix and Tucson Gastroenterologist Networks.** Phase III trial of ursodeoxycholic acid to prevent colorectal adenoma recurrence. *J Natl Cancer Inst* 97: 846–853, 2005.
2. **Appleyard CB, Morales M, Santiago C.** Chronic inflammation alters the contribution of neurokinin receptor subtypes to epithelial function in rat colon. *Dig Dis Sci* 53: 220–228, 2008.
3. **Appleyard CB, Wallace JL.** Reactivation of hapten-induced colitis and its prevention by anti-inflammatory drugs. *Am J Physiol Gastrointest Liver Physiol* 269: G119–G125, 1995.
4. **Bernstein CN, Blanchard JF, Kliewer E, Wajda A.** Cancer risk in patients with inflammatory bowel disease: a population-based study. *Cancer* 91: 854–862, 2001.
5. **Bibiloni R, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, De Simone C, Sartor RB.** VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am J Gastroenterol* 100: 1539–1546, 2005.
6. **Boivin GP, Washington K, Yang K, Ward JM, Pretlow TP, Russell R, Besselsen DG, Godfey VL, Doetschman T, Dove WF, Pitot HC, Halberg RB, Itzkowitz SH, Groden J, Coffey R.** Pathology of mouse models of intestinal cancer: consensus report and recommendations. *Gastroenterology* 124: 762–777, 2003.
7. **Capurso G, Marignani M, Delle Fave G.** Probiotics and the incidence of colorectal cancer: when evidence is not evident. *Dig Liver Dis* 38, *Suppl* 2: S277–S282, 2006.
8. **Davis CD, Milner JA.** Gastrointestinal microflora, food components and colon cancer prevention. *J Nutr Biochem* 20: 743–752, 2009.
9. **Deng X, Tolstanova G, Khomenko T, Chen L, Tarnawski A, Szabo S, Sandor Z.** Mesalamine restores angiogenic balance in experimental ulcerative colitis by reducing expression of endostatin and angiostatin: novel molecular mechanism for therapeutic action of mesalamine. *J Pharmacol Exp Ther* 331: 1071–1078, 2009.
10. **Di Marzio L, Di Leo A, Cinque B, Fanini D, Agnifili A, Berloco P, Linsalata M, Lorusso D, Barone M, De Simone C, Cifone MG.** Detection of alkaline sphingomyelinase activity in human stool: proposed role as a new diagnostic and prognostic marker of colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 14: 856–862, 2005.
11. **Duan RD.** Alkaline sphingomyelinase: an old enzyme with novel implications. *Biochim Biophys Acta* 1761: 281–291, 2006.
12. **Ewaschuk JB, Walker JW, Diaz H, Madsen KL.** Bioproduction of conjugated linoleic acid by probiotic bacteria occurs in vitro and in vivo in mice. *J Nutr* 136: 1483–1487, 2006.
13. **Farraye FA, Odze RD, Eaden J, Itzkowitz SH.** AGA technical review on the diagnosis and management of colorectal neoplasia in inflammatory bowel disease. *Gastroenterology* 138: 746–774, 2010.
14. **Fenoglio-Preiser CM, Noffsinger AE, Stemmermann GN, Lantz PE, Listrom MB, Rilke FO.** Inflammatory bowel disease. In: *Gastrointestinal Pathology, an Atlas and Text*, second ed. Philadelphia, PA: Lippincott Williams & Wilkins, 1999, p. 631–716.
15. **Fitzpatrick LR, Hertzog KL, Quatse AL, Koltun WA, Small JS, Vrana K.** Effects of the probiotic formulation VSL#3 on colitis in weanling rats. *J Pediatr Gastroenterol Nutr* 44: 561–570, 2007.
16. **Fukata M, Abreu MT.** Microflora in colorectal cancer: a friend to fear. *Nat Med* 16: 639–641, 2010.
17. **Gionchetti P, Rizzello F, Morselli C, Poggioli G, Tambasco R, Calabrese C, Brigidi P, Vitali B, Straforini G, Campieri M.** High-dose probiotics for the treatment of active pouchitis. *Dis Colon Rectum* 50: 2075–2082, 2007.
18. **Hertervig E, Nilsson A, Bjork J, Hultkrantz R, Duan RD.** Familial adenomatous polyposis is associated with a marked decrease in alkaline sphingomyelinase activity: a key factor to the unrestrained cell proliferation. *Br J Cancer* 81: 232–236, 1999.
19. **Hertervig E, Nilsson A, Nyberg L, Duan RD.** Alkaline sphingomyelinase activity is decreased in human colorectal carcinoma. *Cancer* 79: 448–453, 1997.
20. **Holubar SD, Cima RR, Sandborn WJ, Pardi DS.** Treatment and prevention of pouchitis after ileal pouch-anal anastomosis for chronic ulcerative colitis. *Cochrane Database Syst Rev* 20: CD001176, 2010.
21. **Huynh HQ, deBruyn J, Guan L, Diaz H, Li M, Girgis S, Turner J, Fedorak R, Madsen K.** Probiotic preparation VSL#3 induces remission in children with mild to moderate acute ulcerative colitis: a pilot study. *Inflamm Bowel Dis* 15: 760–768, 2009.
22. **Ingraham BA, Bragdon B, Nohe A.** Molecular basis of the potential of vitamin D to prevent cancer. *Curr Med Res Opin* 24: 139–149, 2008.
23. **Itzkowitz SH.** Molecular biology of dysplasia and cancer in inflammatory bowel disease. *Gastroenterol Clin North Am* 35: 553–571, 2006.
24. **Kong J, Zhang Z, Musch MW, Ning G, Sun J, Hart J, Bissonnette M, Li YC.** Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. *Am J Physiol Gastrointest Liver Physiol* 294: G208–G216, 2008.
25. **Loftus EV Jr, Sandborn WJ.** Epidemiology of inflammatory bowel disease. *Gastroenterol Clin North Am* 31: 1–20, 2002.
26. **López-Posadas R, González R, Ballester I, Martínez-Moya P, Romero-Calvo I, Suárez MD, Zarzuelo A, Martínez-Augustin O, Sánchez de Medina F.** Tissue-nonspecific alkaline phosphatase is activated in enterocytes by oxidative stress via changes in glycosylation. *Inflamm Bowel Dis* 17: 543–556, 2011.
27. **Madsen K, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C, Doyle J, Jewell L, De Simone C.** Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology* 121: 580–591, 2001.
28. **Miele E, Pascarella F, Giannetti E, Quaglietta L, Baldassano RN, Staiano A.** Effect of a probiotic preparation (VSL#3) on induction and maintenance of remission in children with ulcerative colitis. *Am J Gastroenterol* 104: 437–443, 2009.
29. **Munkholm P.** Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. *Aliment Pharmacol Ther* 18: 1–5, 2003.
30. **Ng SC, Plamondon S, Kamm MA, Hart AL, Al-Hassi HO, Guenther T, Stagg AJ, Knight SC.** Immunosuppressive effects via human intestinal dendritic cells of probiotic bacteria and steroids in the treatment of acute ulcerative colitis. *Inflamm Bowel Dis* 16: 1286–1298, 2010.
31. **Pagnini C, Saeed R, Bamias G, Arseneau KO, Pizarro TT, Cominelli F.** Probiotics promote gut health through stimulation of epithelial innate immunity. *Proc Natl Acad Sci USA* 107: 454–459, 2010.
32. **Pousa ID, Algaba A, Linares PM, Sanz-Cameno P, Maté J, Moreno-Otero R, Bermejo F, Gisbert JP.** Corticosteroids modulate angiogenic soluble factors in ulcerative colitis patients. *Dig Dis Sci* 56: 871–879, 2010.
33. **Pousa ID, Maté J, Gisbert JP.** Angiogenesis in inflammatory bowel disease. *Eur J Clin Invest* 38: 73–81, 2008.

34. **Pronio A, Montesani C, Butteroni C, Vecchione S, Mumolo G, Vestri A, Vitolo D, Boirivant M.** Probiotic administration in patients with ileal pouch-anal anastomosis for ulcerative colitis is associated with expansion of mucosal regulatory cells. *Inflamm Bowel Dis* 14: 662–668, 2008.
35. **Rafter J.** The effects of probiotics on colon cancer development. *Nutr Res Rev* 17: 277–284, 2004.
36. **Reiff C, Delday M, Rucklidge G, Reid M, Duncan G, Wohlgemuth S, Hörmannsperger G, Loh G, Blaut M, Collie-Duguid E, Haller D, Kelly D.** Balancing inflammatory, lipid, and xenobiotic signaling pathways by VSL#3, a biotherapeutic agent, in the treatment of inflammatory bowel disease. *Inflamm Bowel Dis* 15: 1721–1736, 2009.
37. **Sanchez de Medina F, Martinez-Augustin O, Gonzalez R, Ballester I, Nieto A, Gálvez J, Zarzuelo A.** Induction of alkaline phosphatase in the inflamed intestine: a novel pharmacological target for inflammatory bowel disease. *Biochem Pharmacol* 68: 2317–2326, 2004.
38. **Santiago C, Pagán B, Isidro AA, Appleyard CB.** Prolonged chronic inflammation progresses to dysplasia in a novel rat model of colitis-associated colon cancer. *Cancer Res* 67: 10766–10773, 2007.
39. **Schwiertz A, Jacobi M, Frick JS, Richter M, Rusch K, Köhler H.** Microbiota in pediatric inflammatory bowel disease. *J Pediatr* 157: 240–244, 2010.
40. **Sjöqvist U, Hertervig E, Nilsson A, Duan RD, Ost A, Tribukait B, Löfberg R.** Chronic colitis is associated with a reduction of mucosal alkaline sphingomyelinase activity. *Inflamm Bowel Dis* 8: 258–263, 2002.
41. **Soo I, Madsen KL, Tejpar Q, Sydora BC, Sherbaniuk R, Cinque B, Di Marzio L, Cifone MG, Desimone C, Fedorak RN.** VSL#3 probiotic upregulates intestinal mucosal alkaline sphingomyelinase and reduces inflammation. *Can J Gastroenterol* 22: 237–242, 2008.
42. **Sood A, Midha V, Makharia GK, Ahuja V, Singal D, Goswami P, Tandon RK.** The probiotic preparation, VSL#3 induces remission in patients with mild-to-moderately active ulcerative colitis. *Clin Gastroenterol Hepatol* 7: 1202–1209, 2009.
43. **Tuin A, Poelstra K, de Jager-Krikken A, Bok L, Raaben W, Velders MP, Dijkstra G.** Role of alkaline phosphatase in colitis in man and rats. *Gut* 58: 379–387, 2009.
44. **Tursi A, Brandimarte G, Papa A, Giglio A, Elisei W, Giorgetti GM, Forti G, Morini S, Hassan C, Pistoia MA, Modeo ME, Rodino' S, D'Amico T, Sebkova L, Sacca' N, Di Giulio E, Luzza F, Imeneo M, Larussa T, Di Rosa S, Annese V, Danese S, Gasbarrini A.** Treatment of relapsing mild-to-moderate ulcerative colitis with the probiotic VSL#3 as adjunctive to a standard pharmaceutical treatment: a double-blind, randomized, placebo-controlled study. *Am J Gastroenterol* 105: 2218–2227, 2010.
45. **Ulisse S, Gionchetti P, D'Alo'S, Russo FP, Pesce I, Ricci G, Rizzello F, Helwig U, Cifone MG, Campieri M, De Simone C.** Expression of cytokines, inducible nitric oxide synthase, and matrix metalloproteinases in pouchitis: effects of probiotic treatment. *Am J Gastroenterol* 96: 2691–2699, 2001.
46. **Ullman TA, Itzkowitz SH.** Intestinal inflammation and cancer. *Gastroenterology* 140: 1807–1816, 2011.
47. **Ullman T, Odze R, Farraye FA.** Diagnosis and management of dysplasia in patients with ulcerative colitis and Crohn's disease of the colon. *Inflamm Bowel Dis* 15: 630–638, 2009.
48. **Uronis JM, Arthur JC, Keku T, Fodor A, Carroll IM, Cruz ML, Appleyard CB, Jobin C.** Gut microbial diversity is reduced by the probiotic VSL#3 and correlates with decreased TNBS-induced colitis. *Inflamm Bowel Dis* 17: 289–297, 2011.
49. **Uronis JM, Jobin C.** Microbes and colorectal cancer: is there a relationship? *Curr Oncol* 16: 22–24, 2009.
50. **Viscido A, Bagnardi V, Sturniolo GC, Annese V, Frieri G, D'Arienzo A, Papi C, Riegler G, Corrao G, Caprilli R.** GISC: Italian Group for the Study of the Colon and Rectum. Survival and causes of death in Italian patients with ulcerative colitis, a GISC nationwide study. *Dig Liver Dis* 33: 686–692, 2001.
51. **Wada K, Tanaka H, Maeda K, Inoue T, Noda E, Amano R, Kubo N, Muguruma K, Yamada N, Yashiro M, Sawada T, Nakata B, Ohira M, Hirakawa K.** Vitamin D receptor expression is associated with colon cancer in ulcerative colitis. *Oncol Rep* 22: 1021–1025, 2009.
52. **Wahle KW, Heys SD, Rotondo D.** Conjugated linoleic acids: are they beneficial or detrimental to health? *Prog Lipid Res* 43: 553–587, 2004.
53. **Wang X, O'Gorman MR, Bu HF, Koti V, Zuo XL, Tan XD.** Probiotic preparation VSL#3 alters the distribution and phenotypes of dendritic cells within the intestinal mucosa in C57BL/10J mice. *J Nutr* 139: 1595–1602, 2009.
54. **Waterhouse JC, Perez TH, Albert PJ.** Reversing bacteria-induced vitamin D receptor dysfunction is key to autoimmune disease. *Ann NY Acad Sci* 1173: 757–765, 2009.
55. **Wolf JM, Rybicki LA, Lashner BA.** The impact of ursodeoxycholic acid on cancer, dysplasia and mortality in ulcerative colitis patients with primary sclerosing cholangitis. *Aliment Pharmacol Ther* 22: 783–788, 2005.