# Inhibition of Chronic Ulcerative Colitis-associated Adenocarcinoma Development in Mice by VSL#3

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**Background:** Colorectal cancer is the most severe complication in inflammatory bowel disease. This study aimed to investigate the effects of the probiotic VSL#3 when administered as either preventive or concurrent treatment in the progression from chronic colitis to colon cancer.

**Methods:** Mice were exposed to 5, 10, and 15 cycles of dextran sulfate sodium (DSS); each cycle consisted of 0.7% DSS for 1 week followed by distilled water for 10 days. VSL#3 was administered either from 2 weeks before the colitis induction or from the first day of the colitis until being killed. After each period, macroscopic and histological studies, as well as analysis of inflammatory and tumor biomarkers, were performed.

**Results:** Prophylactic or concurrent VSL#3 administration attenuated the disease activity index score and colon inflammation after 5, 10, and 15 cycles of DSS, as well as reduced the histological alterations and the incidence of colonic dysplastic lesions at the 3 periods studied. None of the animals receiving VSL#3 as a concurrent treatment developed carcinoma, which is in contrast to 5% and 20% of the mice following preventive VSL#3 administration, developing carcinoma at the 10th and the 15th cycles of DSS, respectively. In addition, the probiotic reduced the proliferating cell nuclear antigen labeling index, tumor necrosis factor alpha, interleukin-1β, interleukin-6 production, cyclooxygenase-2 expression, and increased interleukin-10 levels in colon tissue at the 3 periods assayed.

**Conclusions:** VSL#3 administration reduced chronic inflammation and prevented or delayed the development of dysplasia and carcinoma in a mouse model of chronic colitis-associated cancer.

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**Key Words:** VSL#3, chronic inflammation, colon carcinogenesis, cytokines, COX-2

Ulcerative colitis (UC) and Crohn's disease are a form of chronic inflammatory bowel disease (IBD) that usually takes a clinical course of repeated bouts of inflammation and remission. Individuals with IBD are at increased risk of developing colorectal cancer (CRC) over healthy individuals.[1] Although the molecular mechanisms underlying the association between chronic colitis and carcinogenesis are not completely understood, it has been shown that the rate of neoplastic changes increases with longer duration of colitis, as well as with the extent and/or severity of inflammation.[2] Compelling evidence in the literature has reported that chronic inflammation develops through the action of various proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-6. These mediators contribute to carcinogenesis by influencing the survival, growth, mutation, proliferation, differentiation, angiogenesis, and movement of tumor and stromal cells.[3] In addition, the inducible enzyme cyclooxygenase-2 (COX-2), involved in chronic inflammation, is also a key predisposing factor to CRC. This enzyme is induced during tumorigenesis by various stimuli including cytokines and growth factors. COX-2 is upregulated in several types of cancer, such as gastric and CRC, generating protumorigenic eicosanoids, in particular, prostaglandins that can promote cell growth, angiogenesis, and suppress immunity.[4,5] In addition, it has been reported that increased epithelial cell proliferation associated with repetitive cycles of inflammation, damage, and regeneration in case of UC might lead to carcinogenesis in the long run. In this context, colitis-associated cancer (CAC) can be modeled in mice by repeated exposure to dextran sulfate sodium (DSS), an agent with direct toxic effects on the colonic epithelium. This model has demonstrated that the clinical and histopathological features are similar to those seen in human UC and IBD-associated cancer.[6]

Some commensal and pathogenic organisms of the human enteric microflora play an important role in the pathogenesis of IBD and CRC.[7] Therefore, manipulating the gut bacterial composition by using probiotic bacteria has been explored as a promising

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avenue for therapeutic intervention against these pathologies. Probiotics are live microbial feed supplements that beneficially impact on host health through improvement of intestinal microbial balance, enhancement of epithelial barrier function, and modulation of immune cell responses.[8] Moreover, they have shown to have antioxidant and antitumor properties. The commercially available probiotic VSL#3 (VSL3 Pharmaceuticals, Inc., Gaithersburg, MD) is a mixture of 8 strains of lactic acid–producing bacteria (*Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii ssp. Bulgaricus*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium breve*). Numerous studies have reported the beneficial effects of VSL#3 in human IBD and in several experimental models of colitis.[9–13] These activities have been attributed to a wide range of effects including protection of gut epithelial barrier function,[10] changes in the composition of the intestinal microbiota, and reduction of oxidative stress.[11] In addition, VSL#3 has been reported to suppress nuclear factor-κB (NF-κB) activation as well as the levels of proinflammatory cytokines and inducible enzymes such as COX-2 and inducible nitric oxide synthase.[12,13] However, only a limited number of studies have dealt with the effect of VSL#3 on experimental models of CAC, and the results are contradictory. In this line, it has been shown that prophylactic VSL#3 administration prevented inflammation and carcinogenesis[14]; by contrast, VSL#3 administered after the onset of inflammation enhanced tumorigenesis in a model of CAC.[7]

The goal of this study was to further explore the effects of VSL#3 when administered as either preventive or concurrent treatment in the progression from chronic colitis to colon cancer, induced by repeated oral administration of DSS. We have analyzed, in different time points of the "inflammation-dysplasia-cancer" sequence, the effects of VSL#3 on proliferating cell nuclear antigen (PCNA) and COX-2 expression as well as proinflammatory cytokines levels, such as TNF-α, IL-1β and IL-6, and the anti-inflammatory cytokine IL-10. Our results report that VSL#3 probiotic mixture improves chronic colitis and prevents or delays the development of UC-associated carcinoma in mice, offering a potential therapeutic use in patients with chronic IBD.

## MATERIALS AND METHODS

### Experimental Animals

A total of 240 six-week-old female C57BL/6 mice supplied by Animal Services of the University of Seville (Spain) were used in this study. They were maintained in our Animal Laboratory Center under standard conditions (temperature of 24°C–25°C, humidity of 70%–75%, lighting regimen of 12L/12D) and were fed pellet standard diet and water ad libitum. Experiments followed a protocol observed by the Animal Ethics Committee of the University of Seville, and all experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 2010/63/EU).

### Induction of Colitis and Probiotic Treatment

Chronic UC was induced by the repeated oral administration of DSS (TdB Consultancy AB, Uppsala, Sweden), according to the method described by Yeo et al.[15] To evaluate the damage progression over time, the experimental protocol was conducted for 5, 10, or 15 cycles of DSS.

After an acclimation period, mice were stratified randomly into 5 experimental groups for each period assayed: 85 days (5 cycles), 170 days (10 cycles), and 255 days (15 cycles):

1. Water (10 mice per group): mice were administered ordinary tap water throughout the entire experiment.
2. VSL#3 (10 mice per group): mice were administered drinking water supplemented with VSL#3 throughout the entire experiment.
3. DSS (20 mice per group): mice received cycles of 0.7% DSS in distilled water, each for 7 days followed by 10 days of tap water.
4. DSS + pVSL#3 (20 mice per group): mice received pretreatment with VSL#3 in the drinking water from 2 weeks before the colitis induction until the time of being killed.
5. DSS + VSL#3 (20 mice per group): mice received treatment with VSL#3 in the drinking water from the day of the colitis induction until being killed.

VSL#3 (VSL3 Pharmaceuticals, Inc.) contains 333 billion colony-forming units of lyophilized bacteria per gram, including 8 different strains (*S. thermophilus* DSM24731, *L. acidophilus* DSM24735, *L. delbrueckii ssp. Bulgaricus* DSM24734, *L. paracasei* DSM24733, *L. plantarum* DSM24730, *B. longum* DSM24736, *B. infantis* DSM24737, *B. breve* DSM24732). Everyday, fresh VSL#3 was administered at a dose adjusted to ensure consumption of 5 billion colony-forming units bacteria per 100 g of body weight, based on a daily intake of 3600 billion bacteria for an adult human with a mean weight of 70 kg.[14] Extrapolated from daily water consumption, it was estimated that each mouse ingested the dose assayed. Animals were killed at the end of each experimental period by an overdose of intraperitoneal chloral hydrate.

### Evaluation of Severity of Colitis

The clinical activity of colitis was evaluated during experimentation to determine the disease activity index (DAI), as per Gommeaux et al[16] with minor modifications (see Table, Supplemental Digital Content 1, http://links.lww.com/IBD/A755); the presence of diarrhea, rectal bleeding, and weight loss were separately graded on a 0 to 3 scale, and the average of the 3 values constitutes the DAI.

### Macroscopic and Histopathological Evaluation

Colons were removed from the dead animals, slightly cleaned in physiological saline to remove fecal residues, weighed, and measured. An independent observer, who was unaware of the treatment, evaluated the severity of macroscopic damage.



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Photographs taken from colon samples were digitized using a Kodak D290 zoom camera (Eastman Kodak, Rochester, NY). Pieces of colon were collected and frozen in liquid nitrogen for measurement of biochemical parameters.

For histological determination, the colon was divided into 3 equal portions (proximal, middle, and distal). A section of each one of them, around 3 mm in diameter, was fixed in 4% buffered formaldehyde, dehydrated by increasing concentrations of ethanol, and embedded in paraffin. The paraffin sections were stained with H&E in accordance with the standard procedures for histological evaluation of colonic damage. The severity of colitis for each portion of the colon was histologically graded on a scale from 0 to 3 by an experienced pathologist and expressed using a pathological index corresponding to the following standard scoring system: 0, normal; 1, focal inflammatory cell infiltration including polymorphonuclear leukocytes; 2, gland loss with inflammatory cell infiltration or crypt abscess formation; 3, mucosal ulceration.[17] The results were expressed as the average scores of the 3 colonic sections.

Colitis-associated colonic neoplasms were analyzed microscopically and diagnosed as low-grade dysplasia, high-grade dysplasia, and adenocarcinoma, according to established criteria by Riddell et al.[18] Neoplastic lesion incidence was calculated as the number of neoplastic lesion-bearing mice divided by the total number of mice. Histopathological evaluation was determined by a pathologist who was unaware of the experimental protocol.

## Immunohistochemical Evaluation of PCNA

Staining of PCNA was performed using a streptavidin–biotin–peroxidase method. Seven-micrometer thick distal colon sections were dried in an oven at 37°C overnight, and then a pressure-cooking antigen retrieval system was used, in which the slides were boiled in citrate buffer (pH 6.0) at maximum pressure for 1 minute, followed by cooling at room temperature for 20 minutes. The endogenous peroxidase activity was inhibited, and then sections were incubated in normal horse serum (Vectastain Kit; Vector Laboratories, Burlingame, CA) for 20 minutes to reduce nonspecific staining and successively incubated with monoclonal mouse anti-PCNA (Dakocytomation, Denmark) at dilution 1:100 overnight at 4°C. Subsequently, slides were treated with anti-mouse IgG antibody for 30 minutes and incubated with the streptavidin–peroxidase complex for 30 minutes, at room temperature (Vectastain Kit; Vector Laboratories). The enzymatic activities were developed with 3,3′-diaminobenzidine (DAB), and the sections were counterstained with hematoxylin. Negative control sections were treated in the same way, omitting the primary antibody. Intensity and localization of PCNA immunoreactivity was examined on all sections using a microscope Olympus BX61 (Olympus Optical Co. Ltd. Tokyo, Japan). The proliferation index was determined by counting the number of PCNA immunostained-positive cells among at least 200 cells in the lesion and was indicated as percentages.[6]

## Colonic Cytokine Levels

TNF-α, IL-1β, IL-6, and IL-10 concentrations in colon tissues were measured by quantitative enzyme immunoassay kits according to the manufacturer's protocol (Diaclone, Besancon, France). A random subset of colon samples (8 per group) were weighed and homogenized at 4°C, after thawing, in 0.3 mL phosphate-buffered saline (pH 7.2), 1% bovine serum albumin containing 0.01 mg/mL leupeptin, 0.01 mg/mL pepstatin, 0.01 mg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Then, they were centrifuged at 12,000*g* for 10 minutes. The concentrations of cytokines were determined in duplicate. TNF-α, IL-1β, IL-6, and IL-10 values were expressed as picograms per milligram tissue.

## Quantitative Real-time Polymerase Chain Reaction Analysis

Levels of mRNA of the TNF-α, IL-1β, IL-6, and IL-10 genes were measured in the colon tissue by quantitative real-time polymerase chain reaction, following a previously described protocol.[19]

## Isolation of Cytoplasmic Proteins and Western Blot Analysis

A random subset of colon samples (8 per group) were weighed and homogenized in ice-cold buffer 50 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 5 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetra acetic acid, 0.5 mM EDTA, 0.01 mg/mL leupeptin, 0.01 mg/mL pepstatin, 0.01 mg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 250 mM NaCl. Homogenates were centrifuged (12,000*g*, 15 min, 4°C), and the supernatants were collected and stored at −80°C. Protein concentration of the homogenate was determined after the Bradford[20] colorimetric method. Aliquots of supernatants containing equal amounts of protein (50 μg) were separated on 10% acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibody: rabbit anti-COX-2 (Cayman Chemical, Michigan) at a dilution of 1:3000. Each membrane was washed 3 times for 15 minutes and incubated with the secondary horseradish peroxidase linked antirabbit (Pierce Chemical Company, Rockford, IL). To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Sigma-Aldrich, MO). Immunodetection was performed using an enhanced chemiluminescence light-detecting kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce, IL). Densitometric data were studied after normalization to the control (housekeeping gene). The signals were analyzed and quantified with a Scientific Imaging Systems (Biophotonics ImageJ Analysis Software, National Institute of Mental Health, Bethesda, MD).

## Statistical Analysis

All values in the figures, tables, and text are expressed as arithmetic means ± SEM. Data were evaluated with GraphPad Prism version 5.00 software (GraphPad Software, Inc., San Diego, CA). The statistical significance of any difference in each parameter



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among the groups was evaluated by one-way analysis of variance followed by Tukey's test. $P$ values of $<0.05$ were considered statistically significant. In the experiment involving histology, the figures shown are representative of at least 3 experiments performed on different days.

## RESULTS

### Effects of VSL#3 on Clinical Disease Activity and Macroscopic Damage

Figure 1A shows results from DAI analysis, which refer to external signs of colitis, for all of the experimental groups during the 15 cycles of DSS administration. No evidence of symptoms was found in animals that were administered only tap water or VSL#3 (without DSS). By contrast, mice exposed to DSS showed a dramatic rise in the DAI index starting at cycle 2. This index was gradually increasing as DSS cycles went forward (from the second to the 15th cycle). Prophylactic or concurrent VSL#3 administration significantly attenuated DAI score from the second cycle of DSS onward, with no significant differences between both dosing regimes (Fig. 1A). There were no differences in the volume of fluid consumed per day between the groups, and all VSL#3-pretreated and treated animals consumed 5 billion colony-forming units of bacteria per 100 g of body weight (data not shown). In line with the clinical findings, macroscopic examination of colon revealed visible thickening of the colon walls in all of the DSS-treated groups (Fig. 1C). A significant progressive increase in colonic weight/length ratio, a marker of tissue inflammation, was observed in mice administered 5, 10, and 15 cycles of DSS ($P < 0.001$ versus respective water group) (Fig. 1B). Mice following prophylactic or concurrent VSL#3 regimes exhibited a marked suppression in colon inflammation, as demonstrated by the reduction of the weight/length ratio of the colon at the 3 periods assayed. Interestingly, at the 10th cycle, VSL#3-treated animals displayed statistical differences in this parameter compared with the VSL#3-pretreated mice ($P < 0.05$).

### Effects of VSL#3 on Microscopic Damage and Dysplasia and Cancer Development

Colitis-associated histological features were examined on H&E–stained colonic sections (Fig. 2), and results from the histopathological score for all groups are shown in Table 1. Water-treated mice had morphologically normal colons without inflammation or ulceration (Fig. 2A). In contrast, after the fifth DSS cycle, the 3 portions of the colon (proximal, middle, and distal) exhibited mild inflammation, characterized by inflammatory cell infiltration (Fig. 2B). A higher inflammation score with loss of surface epithelium, crypt distortion, mucosal ulceration and erosion, and muscularis mucosa affectation, especially in middle and distal sections, was evident in mice administered 10 cycles of DSS (Fig. 2C) and 15 cycles of DSS in relation to 5 DSS cycles (Fig. 2D–E and Table 1). However, colonic microscopic damage was significantly lower in all regions of the colon in both VSL#3-pretreated and treated animals at the 3 periods studied, compared with their respective DSS groups ($P < 0.05$) (Fig. 2F–H). There were no differences in the histological parameters of colitis among the 2 VSL#3 groups (Table 1).

The data regarding incidence of neoplastic lesions are presented in Table 1 and are expressed as the number of malignant lesion-bearing mice divided by the total number of animals per group. The severity of dysplasia was positively correlated with the number of DSS cycles administered and the degree of inflammation. In these animals, the incidence of neoplastic lesions was higher in the middle and distal colon segments than in the proximal colon (data not shown). After 5 cycles of DSS, animals only developed low-grade dysplasia, detected in 80% of mice. A reduction of the incidence of low-grade dysplasia was observed in VSL#3-pretreated and treated mice compared with DSS group (75% and 65%, respectively). Animals exposed to 10 and 15 cycles of DSS presented high-grade dysplasia (35% and 65%, respectively, compared with 0% in 5 cycles of DSS-treated mice) (Fig. 2C–D and Table 1). Prophylactic or concurrent VSL#3 administration reduced the incidence of high-grade dysplasia up to 10% and 5%, respectively, at the 10th cycle, and up to 45% and 20%, respectively, at the 15th cycle. Development of high-grade dysplasia in both VSL#3 groups was limited to the distal region of the colon. Adenocarcinomas occurred in 5% of the mice administered 10 cycles of DSS and in 45% of the 15 cycles of DSS-treated mice (Fig. 2E) and were detected in the middle and distal colonic sections, with no presence in the proximal colon portions. When VSL#3 was administered as a preventive strategy, adenocarcinoma incidence was similar to that found in DSS group after 10 cycles (5%). However, a lower tumor incidence was detected in these animals at the 15th cycle compared with DSS group (20% versus 45%). Development of adenocarcinoma in VSL#3-pretreated mice occurred only in the distal colon segments. Notably, none of the animals receiving concurrent treatment with VSL#3 developed adenocarcinoma in any region of the colon; the most severe pathology found in these mice, after 10 and 15 cycles of DSS administration, was high-grade dysplasia (Table 1).

### Effects of VSL#3 on PCNA Expression

Immunohistochemical analysis was performed on distal colonic tissues from all of the experimental groups to detect the expression of PCNA. As summarized in Table 1, in normal colon, a low PCNA immunostaining was detected (Fig. 3A). However, the colonic mucosa of all of the DSS-treated groups (DSS-5, DSS-10, and DSS-15) showed a marked increase in PCNA-positive cell numbers as compared with water-treated mice ($P < 0.001$) (Fig. 3B, D and F). The highest PCNA-labeling index was detected in mice that received 15 cycles of DSS (Fig. 3F), demonstrating that hyperproliferation was associated with cancer development. Colons from both VSL#3-pretreated and treated mice showed a significant down-regulation of PCNA staining after 5, 10, and 15 cycles of DSS administration (Fig. 3C, E and G).

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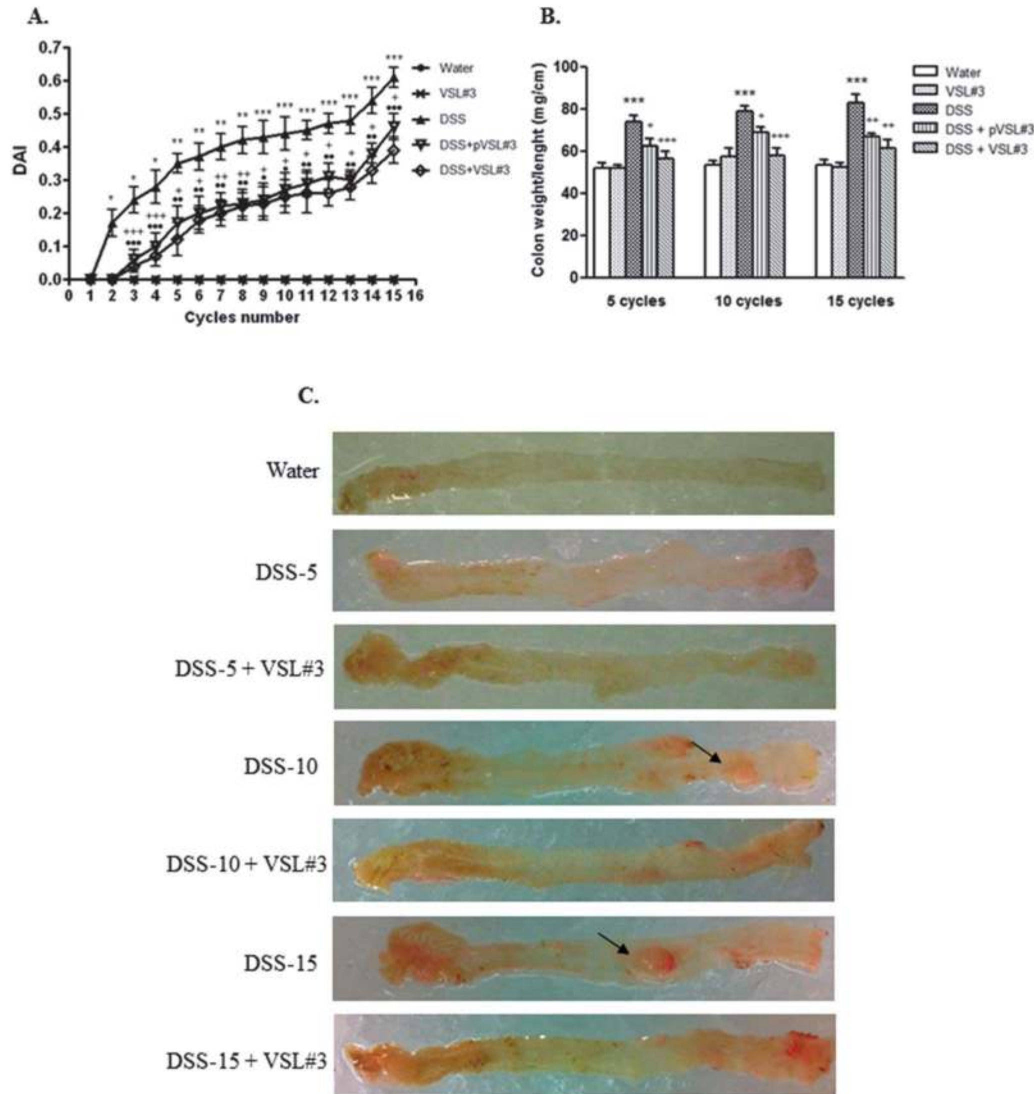


FIGURE 1. A, Effects of VSL#3 on clinical findings in DSS model of colitis-associated colon cancer in mice. DAI was calculated as the average of the scores of bleeding, weight loss, and stool consistency. B, Effects of VSL#3 on weight/length ratio of the colons after 5, 10, and 15 cycles of DSS treatment. C, Macroscopic view of large bowels from control animals, DSS-treated mice, and VSL#3-treated mice (mice starting VSL#3 treatment on the same day of colitis induction) after 5, 10, and 15 cycles of DSS. Gross polypoid lesions were evident on the middle and distal portions of the large intestine at the 10th and 15th cycles of DSS (arrows). Data are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus water group; +$P < 0.05$, ++$P < 0.01$, and +++$P < 0.001$, DSS + pVSL#3 versus DSS group; •$P < 0.05$, ••$P < 0.01$, and •••$P < 0.001$, DSS + VSL#3 versus DSS group.

## Effects of VSL#3 on Cytokines Levels

To support the beneficial effects of VSL#3 on IBD-associated colon cancer and investigate its possible mechanism of action, we examined the expression and production of the proinflammatory cytokines TNF-α, IL-1β and IL-6, which have been highly correlated with IBD, and the anti-inflammatory cytokine IL-10 (Fig. 4). PCR analysis at the 15th DSS cycle showed a significant increase in these cytokines expression in the colonic mucosa of DSS-treated mice compared with control animals ($P < 0.001$) (Fig. 4A, C, and E). In accordance with the suppression of the histological scores, the amounts of TNF-α, IL-1β, and IL-6 mRNA in the colon significantly decreased in both VSL#3-pretreated and treated animals after 15 cycles of DSS administration. In relation to IL-10 mRNA levels, although the colonic expression of this protein tended to increase after prophylactic or concurrent VSL#3 administration compared with mice exposed to 15 cycles of DSS, no significant differences were observed between any of the groups (Fig. 4G).

We next quantified the production levels of TNF-α, IL-1β, and IL-6 in colonic tissues following exposure to 5, 10, and 15 DSS cycles. Our findings showed a significant increase in all of these inflammation-related molecules at the 3 periods studied,



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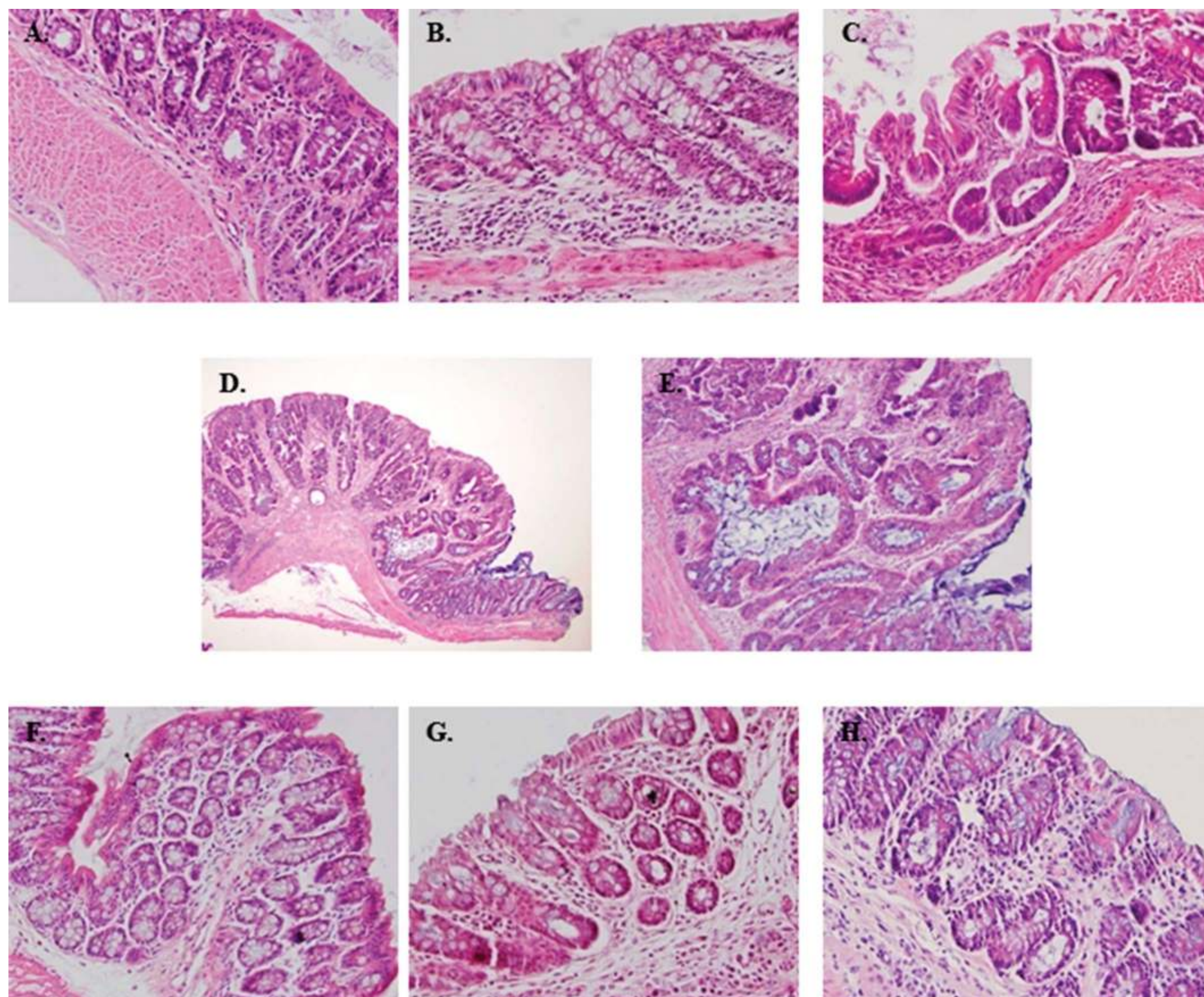


FIGURE 2. Representative histological sections of distal colon showing the effects of VSL#3 administration in the progression from chronic UC to carcinoma. A, Normal colon in control mice. B, Mild inflammation and low-grade dysplasia in DSS-treated mice at the fifth cycle. C, Severe inflammation and high-grade dysplasia at the 10th cycle. D and E, Adenocarcinoma in situ at 15th cycles of DSS. F–H, Low-grade dysplasia in VSL#3-treated mice exposed to 5, 10, and 15 DSS cycles, respectively. H&E stain. Original magnification ×200; D, ×100.

compared with their corresponding control mice that were administered ordinary tap water only (Fig. 4B, D and F). Prophylactic VSL#3 treatment significantly suppressed TNF-α and IL-6 levels only after 15 cycles of DSS ($P < 0.001$ and $P < 0.01$, respectively). When VSL#3 was administered as a concurrent treatment, a marked reduction in these cytokines values was detected at the 3 periods assayed (TNF-α, $P < 0.001$ and IL-6, $P < 0.05$ at the fifth cycle and $P < 0.01$ at the 10th and 15th cycle). Our cytokine analysis also showed that, at the 10th cycle, TNF-α levels were significantly reduced in VSL#3-treated mice in relation to VSL#3-pretreated animals ($P < 0.05$). As regards IL-1β levels, preventive or concurrent exposure to VSL#3 significantly attenuated the colonic production of this cytokine at 3 time points assayed (pVSL#3: $P < 0.05$ at the fifth and 10th cycle and $P < 0.001$ at the 15th cycle; VSL#3: $P < 0.01$ at the fifth and 10th cycle and $P < 0.001$ at the 15th cycle). Finally, prophylactic or concurrent VSL#3 administration resulted in a significant increase in IL-10 levels after exposure to 5, 10, and 15 DSS cycles, compared with their corresponding DSS groups (Fig. 4H). Interestingly, at the 15th cycle, IL-10 production was significantly higher in VSL#3-treated animals in comparison with VSL#3-pretreated animals ($P < 0.01$).

## Effects of VSL#3 on COX-2 Expression

Finally, we examined COX-2 expression in colonic mucosa because upregulation of this enzyme has been associated with human intestinal inflammation and CRC. Western blot analysis



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**TABLE 1.** Effects of VSL#3 on Neoplastic Lesions Incidence, Histological Score, and PCNA in DSS Model of Colitis-associated Colon Cancer in Mice

| | Group | Low-grade Dysplasia | High-grade Dysplasia | Adenocarcinoma | Colitis Score | PCNA-labeling Index (%) |
|---|---|---|---|---|---|---|
| 5 cycles | Water | 0 | 0 | 0 | 0 | 12.7 ± 1.1 |
| | VSL#3 | 0 | 0 | 0 | 0 | 12.5 ± 0.6 |
| | DSS | 16/20 (80%) | 0/20 (0%) | 0/20 (0%) | 1.82 ± 0.1* | 36.1 ± 1.8* |
| | DSS + pVSL#3 | 15/20 (75%) | 0/20 (0%) | 0/20 (0%) | 1.16 ± 0.2† | 28.7 ± 1.5† |
| | DSS + VSL#3 | 13/20 (65%) | 0/20 (0%) | 0/20 (0%) | 1.21 ± 0.1† | 26.0 ± 1.9† |
| 10 cycles | Water | 0 | 0 | 0 | 0 | 13.2 ± 1.2 |
| | VSL#3 | 0 | 0 | 0 | 0 | 13.7 ± 1.0 |
| | DSS | 17/20 (85%) | 7/20 (35%) | 1/20 (5%) | 2.12 ± 0.2* | 66.2 ± 2.8* |
| | DSS + pVSL#3 | 16/20 (80%) | 2/20 (10%) | 1/20 (5%) | 1.50 ± 0.1† | 52.7 ± 2.9‡ |
| | DSS + VSL#3 | 14/20 (70%) | 1/20 (5%) | 0/20 (0%) | 1.33 ± 0.1† | 50.0 ± 2.1§ |
| 15 cycles | Water | 0 | 0 | 0 | 0 | 13.7 ± 1.3 |
| | VSL#3 | 0 | 0 | 0 | 0 | 14.7 ± 1.2 |
| | DSS | 18/20 (90%) | 13/20 (65%) | 9/20 (45%) | 2.36 ± 0.2* | 82.8 ± 2.9* |
| | DSS + pVSL#3 | 17/20 (85%) | 9/20 (45%) | 4/20 (20%) | 1.76 ± 0.2† | 66.7 ± 1.6§ |
| | DSS + VSL#3 | 7/20 (35%) | 4/20 (20%) | 0/20 (0%) | 1.78 ± 0.1† | 62.0 ± 3.9§ |

Neoplastic lesions incidence: number of malignant lesions-bearing mice/total number of mice per group. Colitis score and PCNA-labeling index: The criteria used for the histopathological scoring and immunostaining evaluation of colonic mucosa are described in Materials and Methods. Data are expressed as the means ± SEM.
*$P < 0.001$ versus respective water group.
†$P < 0.05$ versus DSS group.
‡$P < 0.01$ versus DSS group.
§$P < 0.001$ versus DSS group.

revealed increased expression of this enzyme in inflamed colons of all DSS-exposed mice compared with their respective control mice with no inflammation (Fig. 5). However, COX-2 expression was significantly downregulated by the preventive or concurrent administration of VSL#3 at the 3 periods studied.

## DISCUSSION

CRC is one of the most serious complications of IBD, accounting for increased mortality in these disorders. The pathogenesis of CAC is widely believed to involve a stepwise progression from inflamed and hyperplastic epithelia through dysplasia to adenocarcinoma, but the mechanism is not yet clear.[21] During inflammation in patients with UC, different members of the Enterobacteriaceae family and different Clostridium species have been found to increase in accordance with a decrease in bifidobacteria and lactobacilli.[22] This alteration in the composition of the microflora, leading to an imbalance between potentially beneficial and adverse bacteria, is now regarded as a driving force behind the pathogenic features of IBD and seems to similarly impact the development of CRC. Several lines of evidence support the theory that the ingestion of probiotics could potentially modify the transition of inflammation to dysplasia and cancer.[23] Previous articles have evaluated the role of VSL#3 on different experimental models of CAC, but the effects of this probiotic mixture in the disease progression over time have not been investigated yet. It has been shown that the control of colon inflammation at early phase of disease may provide a therapeutic strategy to suppress its progression to colon cancer. In this context, the aim of this study was to assess the effects of preventive or concurrent administration of probiotic VSL#3 during the course of the disease, starting in an early phase of the inflammatory process, before cancer develops.

Our study has followed a model of CAC induced by repeated oral administration of DSS, in which the development of tumors is slow, caused by the chronic inflammation alone, with no additional activation of signaling pathways by a high dose of a chemical carcinogen. Mice undergoing long-term DSS exposure show a sequence of inflammation–dysplasia–cancer that has clinical and histopathological similarities to dysplasia and cancer development in humans with colitis.[6] Our findings confirmed a gradually increased inflammatory activity in mice exposed to DSS, indicated by a significant increase in the DAI from cycles 2 to 15. However, prophylactic and concurrent VSL#3 administration improved the DAI and reduced colon inflammation. These findings are in agreement with previous studies, which showed that the VSL#3 probiotic mixture suppressed colitis in various experimental animal models[12,13,24] and in patients with IBD.[9]

Microscopic findings of colon revealed histological abnormalities with low- and high-grade dysplasia and adenocarcinomas



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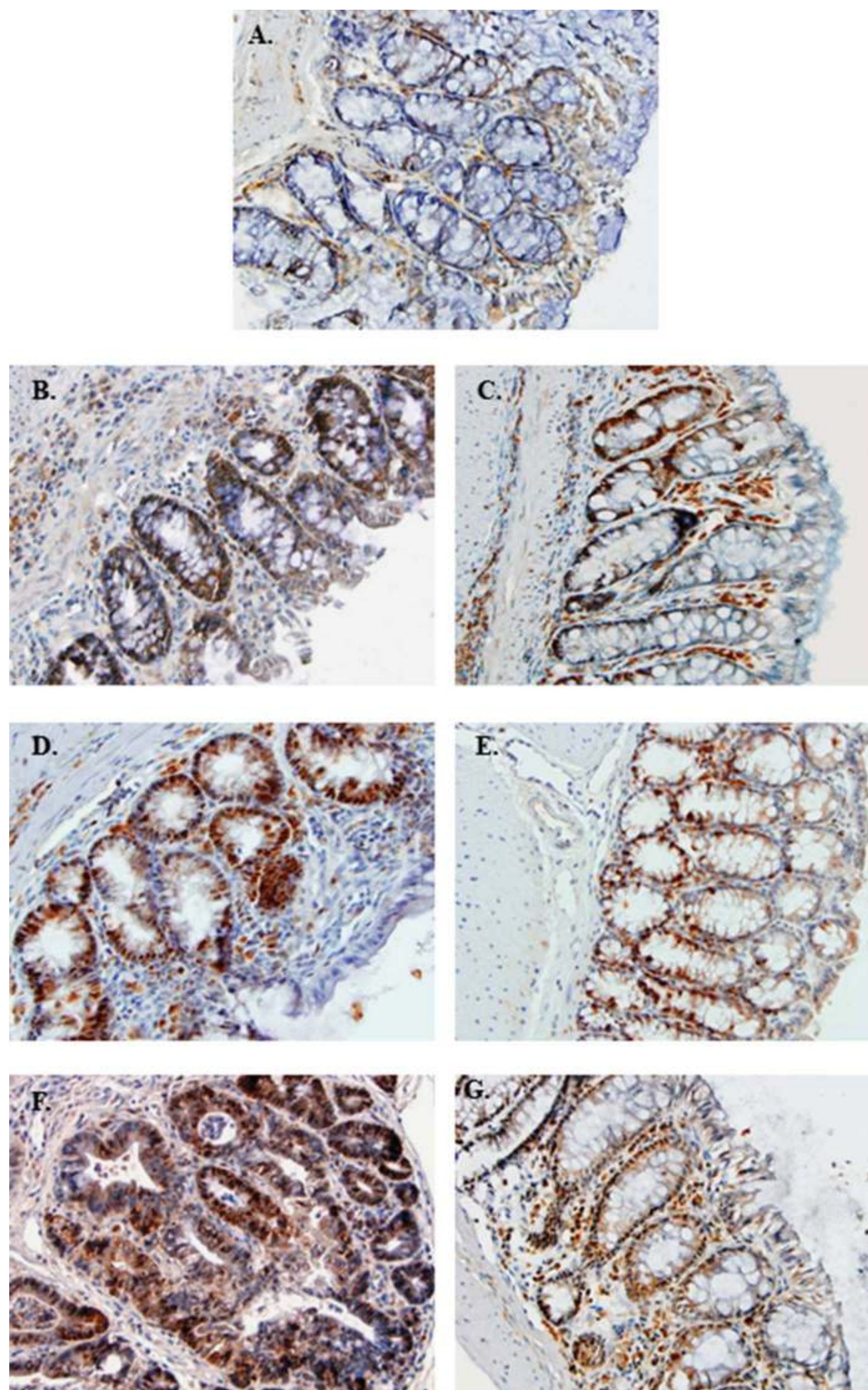


FIGURE 3. Effects of VSL#3 on PCNA expression in distal colon samples in DSS model of CAC in mice. Representative colon samples from the water group (A) and after 5 cycles (B), 10 cycles (D), and 15 cycles (F) of DSS treatment. Images from VSL#3-treated mice (mice starting VSL#3 treatment on the same day of colitis induction) are also shown. A marked reduction in PCNA-labeled proliferative cells was detected in these animals at the fifth, 10th, and 15th cycles of DSS (C, E, and G). Original magnification ×200.

in animals exposed to DSS cycles. The presence of dysplasia was correlated with the levels of inflammation and the number of cycles of DSS administered. The probiotic mixture VSL#3 significantly attenuated the histological alterations in the colon and reduced the incidence of dysplastic lesions in mice exposed to 5, 10, and 15 DSS cycles. Consistent with our findings, Appleyard et al[14] reported that pretreatment with this probiotic mixture delayed the transition from chronic inflammation to dysplasia and



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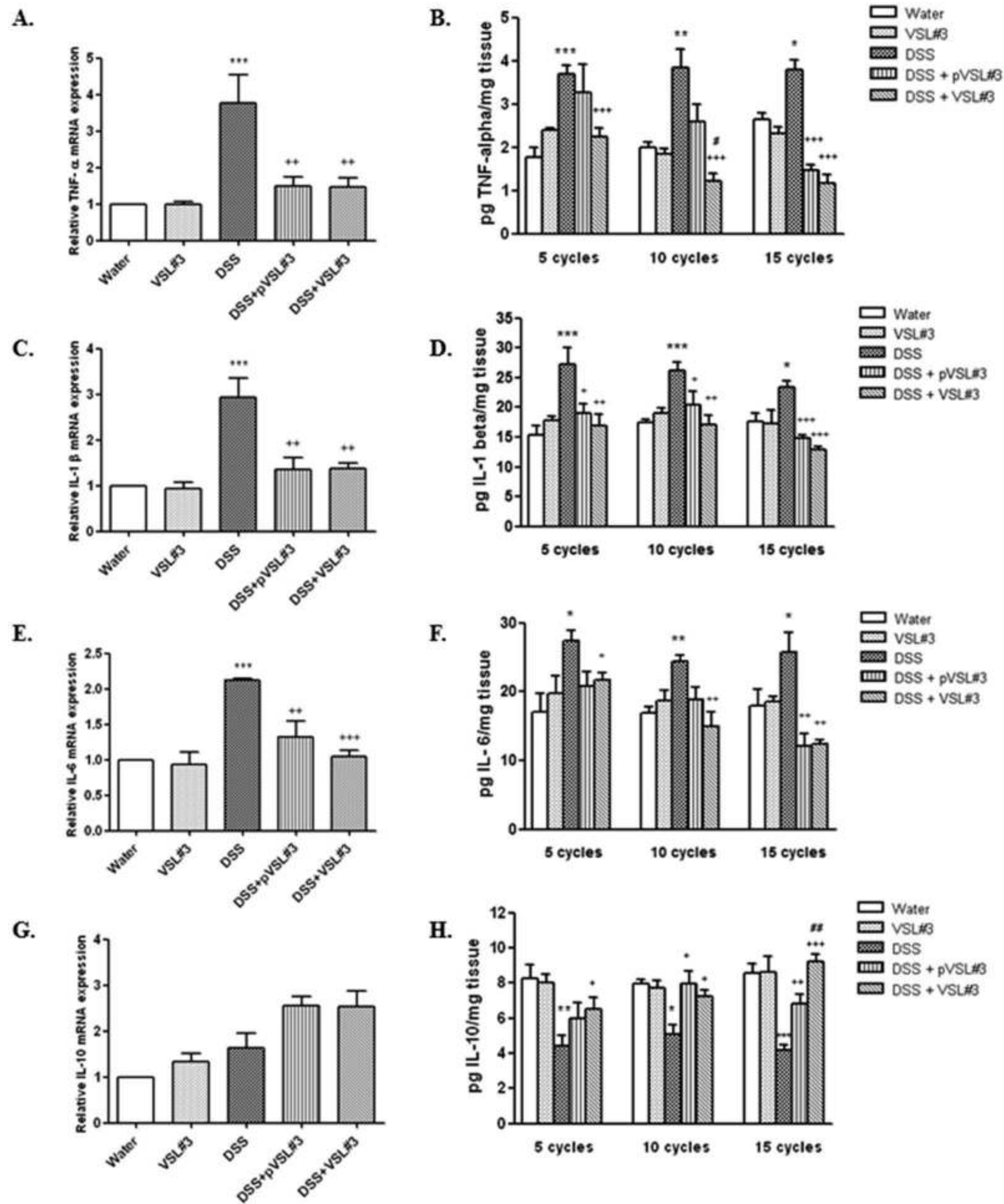


FIGURE 4. Effects of VSL#3 on gene expression and production of cytokines in colon tissue in DSS model of colitis-associated colon cancer in mice. TNF-α (A and B), IL-1β (C and D), IL-6 (E and F), and IL-10 (G and H) after 5, 10, and 15 cycles of DSS treatment. Data are expressed as the means ± SEM of 8 animals per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus respective water group; +$P < 0.05$, ++$P < 0.01$, and +++$P < 0.001$ versus respective DSS group; # $P < 0.05$ and ##$P < 0.01$ versus VSL#3-pretreated group.

colon cancer in the rat TNBS model of colitis-associated dysplasia. Similarly, prophylactic VSL#3 reduced adenoma and adenocarcinoma formation in the azoxymethane or DSS model.[25] However, our data seem to contradict the results of a recent study that observed an increased tumorigenesis and tumor invasion in azoxymethane/IL-10$^{(-/-)}$ mice after therapeutic administration of VSL#3.[7] These discrepancies between the effects of VSL#3 on CAC might be explained by the experimental protocols of damage induction performed in different laboratories.

It has been previously reported an increased proliferation in colon mucosa from both IBD patients[26] and animal models of UC.[27] This hyperproliferation under long-term chronic inflammation status correlates with high-grade dysplasia and cancer risk. Indeed, a previous study by our group reported a gradual increase in PCNA expression from dysplasia to adenocarcinoma in a DSS-induced CAC model.[6] In this study, VSL#3 significantly reduced the PCNA-labeling index in the colonic mucosa after 5, 10, and 15 cycles of DSS, thus suggesting that this probiotic suppresses



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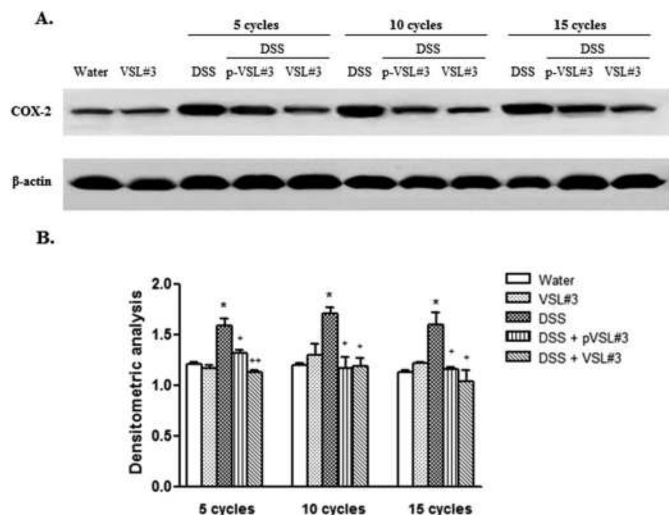


FIGURE 5. A, Effects of VSL#3 on colonic cyclooxygenase-2 (COX-2) expression in DSS model of colitis-associated colon cancer. B, Densitometric data were studied following normalization to the control (housekeeping gene, β-Actin). Data are expressed as the means ± SEM of 8 animals per group. $^*P < 0.05$ versus respective water group; $+P < 0.05$ and $++P < 0.01$ versus respective DSS group.

the abnormal proliferative activity of preneoplastic and neoplastic cells, thereby inhibiting progression from chronic colitis to carcinogenesis. This effect of VSL#3 on cell proliferation is consistent with other findings, which have shown that the probiotic mixture decreased the proliferation of normal colonic mucosa of rats[28] and epithelial cells in human tissue samples from patients with familial adenomatous polyposis.[29]

To better understand the mechanism underlying ameliorating activity of VSL#3 in the mouse DSS-induced colon carcinogenesis model, we assessed the production of proinflammatory cytokines such as TNF-α, IL-1β and IL-6, and the anti-inflammatory cytokine IL-10. It has been reported that blockade of TNF-α,[30] IL-1β,[31] or IL-6[32] resulted in the suppression of tumor growth and progression in different experimental models of CAC, indicating the crucial role of these cytokines in inflammation-mediated tumorigenesis. Previous articles have showed that VSL#3 treatment downregulated colonic expression of TNF-α, IL-1β, and IL-6 in various experimental models of acute colitis.[12,13] Our results extend these findings and demonstrate for the first time that administration of this probiotic mixture consistently decreases the release of these cytokines in the colon during the time course of the disease.

IL-10 is an essential immunoregulator in the intestinal tract that physiologically downregulates inflammation.[33] It has been reported that the treatment of IL-10–deficient mice with VSL#3 attenuated microscopic signs of colitis and colonic production of TNF-α and IFN-γ.[34] In addition, previous articles have reported that VSL#3 upregulated the levels of the anti-inflammatory cytokine IL-10 in both TNBS- and DSS-induced colitis models[12,35] and in patients with active UC.[36] Consistent with these reports, colonic levels of this cytokine in this study were found to be significantly increased in mice that received pretreatment or treatment with the probiotic mixture during the course of DSS administration. Altogether, these results strongly suggest that the probiotic mixture VSL#3 suppressed inflammatory reactions and the subsequent colon carcinogenesis, probably by downregulating TNF-α, IL-1β, and IL-6 secretion, and upregulating IL-10 production in the colon.

The inducible enzyme COX-2 has been shown to be overexpressed in patients with UC[37] and adenocarcinomas.[38] A large body of evidence reveals that COX-2 is mechanistically involved in colon carcinogenesis because this enzyme can activate procarcinogens, indirectly produce free radicals, promote angiogenesis and cell proliferation, and inhibit apoptosis.[5] A previous article demonstrated a marked reduction in colonic expression of COX-2 after VSL#3 administration to rats with DSS-induced acute colitis.[12] In our study, we have demonstrated for the first time, to the best of our knowledge, that VSL#3 pretreatment and treatment significantly downregulated the colonic expression of COX-2 during the gradual development of chronic colitis and its progression to colon cancer. These findings, at least partly, suggested that suppression of COX-2 expression may be involved in the preventive effect of VSL#3 on DSS-induced colitis and colon carcinogenesis.

In conclusion, this study demonstrates that VSL#3 reduces chronic inflammation and prevents or delays the development of dysplasia and carcinoma in a mouse model of chronic CAC, offering a potential therapeutic use in patients with chronic IBD. These actions may be associated with a decrease in inflammation-caused epithelial cell proliferation, as well as a downregulation of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 and an upregulation of the anti-inflammatory cytokine IL-10, accompanied by a suppression of COX-2 expression in colon tissue.

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