The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells

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The intestinal microbiota consists of more than 500 bacterial species that inhabit the human intestinal tract (44). In healthy individuals the composition of the microbiota is represented by both permanent and transient members that remain in constant equilibrium for long periods of time (36). However, the bacterial densities change in different parts of the intestine: it is estimated that enteric bacteria in the upper ileum reach a concentration of $10^4$ colony-forming units (cfu)/g and become more abundant ($10^{10}-10^{11}$ cfu/g) in the lower ileum and colon (25). In addition to the benefits that these bacteria provide to the host organism through the fermentation of nondigestible foods and synthesis of vitamins and other metabolites (23), the intestinal microbiota have been shown to play important immunomodulatory and homeostatic roles (42).

Another chief constituent of the gastrointestinal tract is the mucin protective layer, which is produced by goblet cells (11). These gel-forming glycopolypeptides act as lubricants and as a protective barrier between the body and the external environment (4), rigorously selecting the transport of nutrients across the epithelium and excluding the passage of harmful molecules and pathogens into the circulatory system. Mucin monomers are synthesized as rod-shaped apomucin cores that are posttranslationally modified by exceptionally abundant glycosylation, which can reach up to 80% (wt/wt) (28). The polypeptide backbone provides numerous sites for the addition of O-linked oligosaccharides through an abundance of serine and threonine/proline residue (3). The amino- and carboxy-terminal regions are poorly glycosylated but rich in cysteines, which are involved in establishing disulfide linkages within and among mucin monomers. The excessive glycosylation of mucins provides them with considerable water-holding characteristics and also renders them resilient to proteolysis. At least nine human mucin (MUC) genes have been identified, and MUC1, MUC2, MUC3, MUC4, and MUC5AC are expressed in the human colon (1). However, MUC2 is the major gel-forming mucin of the small and large intestines and is the main structural component of the mucus gel (11).

The intestinal microbiota and the mucosal lining are closely related components of the intestinal epithelial barrier. The interaction between these two elements is also important for the well-being of the intestinal epithelium (23). On one hand, clinical observations and animal experiments have suggested that intestinal bacteria can trigger ongoing mucosal inflammation in some susceptible individuals (26, 37). On the other hand, the administration of bacterial supplements, known as probiotics, can alter the composition of the intestinal bacteria and minimize the symptomology of these illnesses (38). Probiotic therapy, for example, can be utilized to prevent relapse of pouchitis (20, 30) and ulcerative colitis (8), decrease new or recurrent bacterial infections in high-risk patients (traveler’s diarrhea) (2), and prevent antibiotic-induced diarrhea (40) as well as Crohn’s disease (9, 16). Probiotic bacteria are naturally part of the intestinal microbiota. To better understand their beneficial consequences, it is also important to study the physiological effect of these bacteria in a normal environment. Probiotic components exert their beneficial effects through various mechanisms of action (12). Previous studies suggested that probiotics may induce mucin gene expression in colonic epithelium. Administration of probiotic bacteria, supplements composed of microbiota constituents, was previously shown to diminish symptoms in patients suffering from inflammatory bowel diseases. This raises the possibility that probiotics may play an active role in enhancing the intestinal barrier at the mucosal surface. In this study, we investigated whether the clinically tested VSL#3 probiotic formula and/or its secreted components can augment the protective mucus layer in vivo and in vitro. For in vivo studies, Wistar rats were orally administered the probiotic mixture VSL#3 on a daily basis for seven days. After treatment, basal luminal mucin content increased by 60%. In addition, we exposed isolated rat colonic loops to the VSL#3 probiotic formula, which significantly stimulated colonic mucin (MUC) secretion and MUC2 gene expression; however, MUC1 and MUC3 gene expression were only slightly elevated. The effect of the VSL#3 mucin secretagogue was also tested in vitro by use of LS 174T colonic epithelial cells. In contrast to the animal studies, cultured cells incubated with VSL#3 bacteria did not exhibit increased mucin secretion. However, the bacterial secreted products contained in the conditioned media stimulated a remarkable mucin secretion effect. Among the three bacterial groups (Lactobacilli, Bifidobacteria, and Streptococci) contained in VSL#3, the Lactobacillus species were the strongest potentiator of mucin secretion in vitro. A preliminary characterization of the putative mucin secretagogue suggested that it was a heat-resistant soluble compound, which is not sensitive to protease and DNase treatment. These findings contribute to a better understanding of the complex and beneficial interaction between colonic epithelial cells and intestinal bacteria.

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epithelial cell lines (27, 31); however, mucin secretion, as a consequence of stimulation with probiotics, remains poorly defined. The objective of this study was to determine whether the clinically tested VSL#3 probiotic formula could enhance the protective mucus barrier in animals and through in vitro studies. Furthermore, we determined which bacterial component of VSL#3 formula was responsible for the maximum stimulation of mucin secretion.

MATERIALS AND METHODS

VSL#3 bacterial and conditioned media preparations and cell cultures. VSL#3 (Seaford Pharmaceuticals) probiotic mixture contains four species of Lactobacilli: 3.1% (wt/wt) of L. plantarum, 7.3% of L. acidophilus, 16% of L. casei, and 8.4% of L. delbrueckii subsp. Bulgaricus; three species of Bifidobacteria: B. infantis, B. breve, and B. longum, representing 17.7% of the mixture; and 47.5% of Streptococcus salivarius subsp. Thermophilus. For all experiments, 0.01 g of the probiotic formula was reconstituted in 10 ml of serum/antibiotic-tococcus salivarius particles and diluted with MEM cell culture medium free of serum and m membrane (Millipore) to remove any insoluble separate the bacteria, and the resulting supernatant was filtered Sharpe (MRS, Difco) broth medium at 37°C without shaking. To single species were grown overnight in 10 ml of Man, Rogosa, and Sharpe (MRS, Difco) broth medium at 37°C without shaking. To prepare conditioned medium (CM), VSL#3 probiotic formula (0.01 g) or the single species were grown overnight in MRS medium at 37°C without shaking. The CM was centrifuged at 4,100 rpm for 10 min to separate the bacteria, and the resulting supernatant was filtered through a 0.22-μm membrane (Millipore) to remove any insoluble particles and diluted with MEM cell culture medium free of serum and antibiotics before use. Similarly, Streptococcus, Lactobacillus, and Bifidobacterium single-species CM were diluted in MEM culture medium to a final concentration of 30, 28, and 24%, respectively. The human colonic adenocarcinoma cell line LS 174T was obtained from the American Type Culture Collection (Rockville, MD) and was cultured in MEM cell culture medium containing 10% fetal calf serum, 10 U/ml penicillin, and 100 μg/ml streptomycin. LS 174T cells were seeded in 6- or 24-well tissue culture plates (Corning) and grown to 50% confluence over 6 days for subsequent mucin secretion experiments.

Animals and in vivo mucin secretion studies. Four- to 6-wk-old male Wistar rats (Charles River, Quebec, Canada) weighing ~350 g on average were used in this study. Animals were fed laboratory chow and water ad libitum. Rats were fed intragastrically for 7 days with either 0.15 mg/kg (3 × 109 bacteria) of live VSL#3 probiotic formula dissolved in 1 ml of Dulbecco’s phosphate-buffered saline (D-PBS) or D-PBS alone as a negative control. After a 7-day probiotic treatment, rats were injected intraperitoneally with 20 μCi of [3H]glucosamine (Amersham) in 0.5 ml of D-PBS (pH 7.2) and left for 3 h to metabolically label the newly synthesized mucin. The animals were killed, and the entire colon was resected and flushed with warm D-PBS to eliminate fecal material before surgical ligation. Ligated colonic loops 1 cm in length were made with black-braided nylon, leaving the mesenteric blood vessels intact. Two loops of rat were established by double ligation between loops to prevent cross-contamination of the intraluminal test substances. The colon 1 cm distal from the cecum and the entire rectum were not used. Immediately following ligation, the loops were inoculated with 1 ml of each condition: 0.15 mg/kg (3 × 109 bacteria) of VSL#3 reconstituted in D-PBS, D-PBS alone used as a negative control, and 1 mg/kg E. histolytica (clone HM1-IMSS) live trophozoites in D-PBS as a positive control for mucin secretion (7). Following 4-h incubation, rats were killed, and the [3H]-labeled glycoproteins from each loop were separately removed and adherent mucus was lightly scraped. Mucosal glycoproteins were collected, and activity was measured, and glycoproteins were subjected to gel filtration on a 12% separating gel and a 4% stacking gel under reducing conditions. The proteins contained in each fraction were quantified by Bradford protein assay (Bio-Rad). Proteins were submitted to SDS-PAGE with a 12% separating gel and a 4% stacking gel under reducing conditions.
probed with rabbit immune serum anti-MUC2 (7) diluted 1:20,000 followed by horse anti-rabbit immunoglobulins diluted 1:25,000. The results were developed by using ECL advance (Amersham).

**Statistical analysis.** Results are expressed as means and SD. Significant differences between means were determined by the two-tailed Student’s t-test and analysis of variance. *P* values < 0.05 were considered to be statistically significant.

**RESULTS**

Probiotics enhance mucin secretion and MUC2 gene expression in rat colon. Because the mucus barrier is the first line of host defense against noxious agents and infections (11), we determined the effect of the VSL#3 probiotic formula on baseline mucus secretion in rats subjected to a 7-day treatment with the bacterial preparation. As shown in Fig. 1A, rats fed VSL#3 increased basal total luminal glycoprotein content by ∼3.7-fold compared with PBS-fed controls. When the glycoproteins were analyzed by Sepharose 4B column chromatography, which separates high-molecular-weight mucins from low-molecular-weight nonmucin components (43), rats fed probiotics showed a 60% increase in mucin secretion [Fig. 1B, void volume (Vo) fractions 10–15] compared with PBS-fed controls. The increase in mucin secretion was concomitant with a fivefold (SD 1.85) increase in MUC2 gene expression compared with controls (Fig. 1C). Surprisingly, there was also a significant increase in the secretion of nonmucin glycoproteins (fractions 20–35) ranging from 50–660 kDa (Fig. 1B). To ensure that the observed mucin secretion effect was not due to intestinal injury, we examined the expression of ITF, which are small peptides involved in restitution of the gastrointestinal tract epithelium. ITF and mucins are both expressed by goblet cells, and it is thought that the expression of these proteins can be closely linked under certain circumstances (41). As shown in Fig. 1C, ITF gene expression was slightly increased (1.8-fold above control) but was not statistically significant (SD 0.95, *P* = 0.09).

The effect of VSL#3 on mucin secretion and MUC2 gene expression in rat colonic loops. Because feeding VSL#3 can enhance the basal levels of the total mucin pool (Fig. 1A), it was of interest to determine whether the probiotic mixture can directly stimulate mucin secretion. To do this, rat colonic loops were injected directly with cultured live VSL#3 bacteria and mucin secretion examined. As shown in Fig. 2, VSL#3 enhanced total colonic glycoprotein secretion by ∼2.2-fold and high Vo mucin isolated by Sepharose 4B chromatography by ∼2.6-fold (data not shown). Interestingly, the mucin secretagogue in the probiotic mixture was about 80% effective compared with the known mucin secretagogue *Entamoeba histolytica* (43). To investigate whether the probiotics were modulating mucin gene expression in addition to mucin secretion,
CM with maximal mucin secretogogue activity occurring with loops. Rats were injected IP with 20 μCi of [3H]glucosamine and left for 3 h to metabolically label the glycoproteins, including mucins. Ligated colonic loops were prepared and inoculated with 1 ml of D-PBS, or VSL#3 (3 × 10^8 cfu). Live Entamoeba histolytica trophozoites (1 × 10^7) were used as a positive control. Total glycoprotein was measured as CPM. Values are means and SD of 2 independent experiments (n = 5, *P < 0.05).

Fig. 2. Secretion of [3H]glucosamine-labeled glycoproteins in rat colonic loops. Rats were injected IP with 20 μCi of [3H]glucosamine and left for 3 h to metabolically label the glycoproteins, including mucins. Ligated colonic loops were prepared and inoculated with 1 ml of D-PBS, or VSL#3 (3 × 10^8 cfu). Live Entamoeba histolytica trophozoites (1 × 10^7) were used as a positive control. Total glycoprotein was measured as CPM. Values are means and SD of 2 independent experiments (n = 5, *P < 0.05).

total RNA was isolated from the colonic loop and MUC1, MUC2, and MUC3 gene expression was measured by real-time quantitative PCR. ITF was used as an injury marker. As shown in Fig. 3A, MUC2 gene expression in colonic loops treated with probiotic bacteria was increased 60-fold (SD 15.32) relative to the PBS-treated controls. MUC1 and VSL#3 CM gene expression levels were slightly elevated after probiotic treatment showing about fourfold (SD 0.95 and 0.25, respectively) increase relative to the PBS-negative control (Fig. 3B). ITF gene expression in the loops treated with VSL#3 bacteria was modest but not significant (P = 0.34) (Fig. 3A). However, in colonic loops treated with the parasite E. histolytica as a positive control, MUC2 and ITF gene expression was increased up to 60- (SD 24.43) and 750-fold (SD 279.3), respectively, indicating that increased intestinal mucin content was a consequence of enhanced secretion as a defense mechanism (Fig. 3A). These studies clearly demonstrate that VSL#3 bacteria can enhance luminal barrier function by stimulating mucin secretion and mucin gene expression in the absence of tissue injury.

VSL#3 CM induced mucin secretion in LS 174T colonic epithelial cells. We expanded the study and tested the effect of the probiotics on mucin secretion in vitro. LS 174T human adenocarcinoma cells were utilized because they constitutively express and secrete mucin in response to mucin secretagogues (6, 7, 15). Cellular mucins were metabolically labeled with [3H]glucosamine for 16 h before a challenge with VSL#3 bacteria, single species of VSL#3 bacteria, or CM from live VSL#3 bacteria. In pilot studies, we failed to see mucin secretion in response to live VSL#3 bacteria or several of its individual bacterial components regardless of the incubation time or concentration used (data not shown). In contrast, VSL#3 or the single-species CM markedly stimulated [3H]mucin glycoprotein secretions. We consistently found that VSL#3 CM was very acidic and cytotoxic to the colonic cells as determined by Trypan blue and monolayer destruction assays. Thus, to accurately access the mucin secretagogue effect of the VSL#3 CM, we diluted the CM in serum-free MEM culture medium. Data in Fig. 4A show a dilution curve for the VSL#3 CM with maximal mucin secretagogue activity occurring with 28% (vol/vol) of the bacterial CM at pH 4.9. At this dilution and pH, the VSL#3 CM stimulated mucin secretion in a time-dependent manner up to 7 h in a similar fashion to the positive control, calcium ionophore (Fig. 4B). Surprisingly, real-time PCR analysis revealed that both probiotics and VSL#3 CM treatments did not significantly increase MUC2 gene expression compared with the untreated control (data not shown).

To determine which bacterial species was responsible for evoking maximal mucin secretion, we tested the efficacy of the CM from individual cultures. As shown in Fig. 5, colonic cells stimulated with whole VSL#3 CM secreted up to 160% (SD 11.84) more 3H-labeled mucous glycoproteins than the untreated controls. Interestingly, the CM derived from Lactobacillus cultures was as efficient as the complete VSL#3 CM at potentiating mucin secretion. On the other hand, the CM of Bifidobacterium species and Streptococcus salivarius induced a relatively reduced effect. Mixing the CM from the different species did not synergistically enhanced mucin secretion (data not shown). Figure 6 shows the Sepharose 4B column chromatography elution profile for mucin and nonmucin secretions in response to the probiotic secreted products. VSL#3 CM and calcium ionophore (positive control) stimulated 170 and 250% increase, respectively, in secretory mucin content compared with the nontreated MEM controls. The marked increase in the secretion of nonmucin glycoproteins (fractions 17–27) in response to VSL#3 CM was similar to that shown with live bacteria in rat colons (Fig. 1B). Immunoblot analysis using an

Fig. 3. Mucin and ITF gene expression in rat colonic loops. Total RNA was isolated from VSL#3 or control treated colonic loop tissue. Expression of MUC2 and ITF (A) and MUC1 and MUC3 (B) genes was analyzed by real-time quantitative PCR. Each reaction contained cDNA derived from 2 μg total RNA was performed in triplicate and normalized with the housekeeping gene β-actin. The results were analyzed by the 2^-ΔΔCT method. Values are means and SD of 2 independent experiments (n = 5; *P < 0.05).
anti-MUC2 antibody raised against purified colonic mucin confirmed the presence of mucin only in the high-molecular-weight Vo fractions (Fig. 6B).

VSL#3 mucin secretagogue is heat labile and resistant to protease and DNase treatment. Figure 7 shows various biochemical and physical properties of the putative mucin secretagogue derived from VSL#3 CM. Regardless of the biochemical treatment, we could not inhibit the mucin secretagogue effect. Remarkably, the mucin secretagogue was found not to be heat labile and was resistant to DNase and protease treatment. This suggests that the putative mucin secretagogue(s) may be a lipoprotein or polysaccharide.

**DISCUSSION**

The mucus gel layer is a structural component of the gut that lubricates and protects the gastrointestinal tract against harmful agents. The integrity of the mucin protective layer is also crucial for healing processes during inflammatory bowel disease (IBD) (13). Previous studies have shown that the VSL#3 probiotic formula positively regulates epithelial barrier functions (31). The ability of these organisms to survive passage through the stomach and further establishment in the gut (25, 39) makes them suitable for administration through the oral route. There is growing evidence that such probiotic supplements may be helpful in the treatment of various states of human intestinal disease, including pouchitis (20), ulcerative colitis (8, 25), and Crohn’s disease (16). However, it remains...
elusive how probiotics promote healing or protection of the epithelial lining of the gut.

In the present study, we provide compelling evidence that probiotics can enhance colonic mucin gene expression and secretion in vivo and in vitro. Oral administration of the VSL#3 probiotic formula to rats increased MUC2 gene expression as well as mucin protein accumulation in the colonic lumen. Furthermore, we were able to reproduce this effect by direct application of VSL#3 to rat colonic loops. Our results suggest that treatment of the colonic loops with VSL#3 correlates with increased transcription of the major secreted gel-forming mucin, MUC2. The expression levels of two other mucin types, MUC1 and MUC3, which are membrane associated, were only slightly altered compared with MUC2. To confirm that increased accumulation of mucin in the colonic loops was not a mere consequence of epithelial injury due to exposure to large titers of bacteria or as a result of the surgical procedure, we examined gene expression levels of the injury marker ITF. As expected, exposure of intestinal tissue to a pathogen, E. histolytica, led to elevated expression of MUC2 as well as ITF. On the other hand, VSL#3 bacterial treatment only induced MUC2 gene expression. This strongly suggests that nonpathogenic probiotic bacteria upregulate mucin expression without stressing the intestinal epithelium. It has been reported that the induced expression of ITF is associated with goblet-cell proliferation and differentiation in vitro (33). Since the ITF gene expression remained relatively unaltered after VSL#3 treatment, it is very likely that the mucin secretion effect we observed in the treated rats is due only to an increase in the activity of already differentiated goblet cells, which suggests that their number remained constant during the treatment. However, we cannot completely exclude the possibility that there may be an increase in the density of goblet cells after VSL#3 treatment since other reports have demonstrated that goblet cells from germ-free rodents are less numerous and smaller in size relative to those derived from conventionally raised animals (19) and that metabolites produced owing to fermentation in the intestinal microflora may play a role in the growth and maturation of goblet cells (5). It was previously shown that mucin expression in colonic epithelial cells followed the adherence of probiotics (24). Our data and those of others support the idea that probiotics may modulate mucin expression and secretion as a strategy for intestinal colonization, which can coincide with a beneficial effect for the host. VSL#3 bacteria may selectively upregulate MUC2 expression, to allow for the proper establishment of the microbiota (10, 14).

We attempted to reproduce the mucin secretion effect of VSL#3 or one of its subunit bacterial cultures in vitro using cultured LS 174T and another colonic cell line, T84 (data not shown). Stimulation of cells with VSL#3 or the individual bacterial cultures did not have any significant effect on mucin secretion. One likely explanation is that our cell conditions did not promote the proper growth of probiotic bacteria, which are mostly anaerobic. In addition, because of the technical limitations of such an in vitro experiment, we were unable to extend the incubation periods of VSL#3 bacteria with cell cultures more than 7 h. Since previous reports had shown that bacterial metabolites may have a stimulatory effect on mucin secretion (5), we considered the possibility that the mucin secretagogue effect may be present in the CM. The mucin secretion effect in LS 174T cells treated with VSL#3 CM was similar to that obtained from the in vivo experiments, however, real-time PCR analysis revealed that VSL#3 CM treatments did not significantly increase MUC2 gene expression in LS 174T cells. The reason for this discrepancy is not clear. It is important to note that the effect of VSL#3 in the in vivo studies is not directly comparable to the in vitro experiments owing to the absence of luminal or epithelial accessory elements in the latter instance, which may contribute to the efficacy of the probiotic bacteria. Our data suggest that, during the first few hours of exposure, VSL#3 CM acts like a secretagogue inducing only the discharge of mucins from the goblet cell vesicles without necessarily influencing mucin gene expression. Similar effects have been observed when gobletlike colonic epithelial cells were stimulated with certain mucin secretagogues such as calcium ionophores A23187. This chemical has been shown to induce mucin secretion without elevating mucin gene expression (29, 46). We further compared the mucin secretion effect of the CM from the different bacterial species present in the probiotic formula. Lactobacillus CM was as effective as the VSL#3 complete formula CM, while the CM of Bifidobacterium and S. salivarius induced a minimal mucin secretion effect. Although it was suggested that a synergistic effect may exist among the different species contained in this formula (25), our results indicate that the three tested probiotic bacteria do not have an additive mucin secretion effect (data not shown).

The ability of the VSL#3 CM to induce mucin secretion suggests that it contains at least one active secretagogue element. Our efforts to partially characterize this mucin secretagogue were inconclusive. Treatment of VSL#3 CM with proteases or heat did not lead to a reduction in CM-induced mucin secretion, whereas application of DNase I led to a modest, yet nonsignificant ($P = 0.26$), decrease in mucin secretion. DNA extracted from probiotic bacteria was previously shown to actively participate in the modulation of proinflammatory responses in the gut and, although some researchers suggested DNA as a possible replacement for treatment with live bacteria (18, 34), others have strongly disagreed (35). Because the reduction in mucin secretion was not significant following the treatment of CM with DNase, the secretagogue in the CM is...
not likely constituted by bacterial DNA only. At the present time, we cannot exclude the possibility that the secretagogue elements are proteins since short peptides and highly glycosylated proteins are typically resilient to protease treatment or lipids, which are resistant to heat. Further research will be required to better determine the active secretagogue.

We also demonstrated that the S4B mucin elution profiles obtained from cellular supernatants released into the media after VSL#3 CM stimulation and from the intestinal lumen of rats after VSL#3 or CM treatments in LS 174T cells are highly similar. In both instances, we noticed a significant increase in the nonmucin fraction. Although we did not characterize the glycoproteins contained in the low-molecular-weight fractions, we confirmed the absence of any truncated or digested mucin fragments using Western blotting techniques. These results suggest that the mucin secretion effect exhibited by VSL#3 or VSL#3 CM treatment is triggered by the same mechanism.

In patients suffering from IBD, the increased mucin secretion associated with the colonization of probiotic bacteria may help restore and reinforce the epithelial barrier function. Identification of the secretagogue will allow for the development of more efficient therapies for IBD treatment. Administration of selected strains or genetically engineered strains (45) of probiotics that secrete high levels of the mucin secretagogue or administration of purified secretagogue to patients suffering from IBD may improve the therapeutic quality of probiotic supplements and reduce the treatment periods. At present, most evidence suggests that the anti-inflammatory and healing properties of probiotics are an outcome of the plethora of previously evidenced benefits and properties of probiotics that are associated with the colonization of probiotic bacteria in the gastrointestinal tract. A determination of the secretagogue and the contributions of each of the VSL#3 subunit strains to these effects will be required to better understand their therapeutic values.

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