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

C. Caballero-Franco,[1] K. Keller,[1] C. De Simone,[2] and K. Chadee[3]

[1]*Institute of Parasitology, McGill University, Saint Anne de Bellevue, Quebec, Canada;* [2]*University of L'Aquila, L'Aquila, Italy; and* [3]*Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada*

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Caballero-Franco C, Keller K, De Simone C, Chadee K. The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 292: G315–G322, 2007. First published September 14, 2006; doi:10.1152/ajpgi.00265.2006.—Several studies have stressed the importance of the microbiota in the maintenance of the gastrointestinal 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.

probiotics; intestinal mucosa; mucin secretion; mucin secretagogue

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 10^2-10^3 colony-forming units (cfu)/g and become more abundant (10^{10}-10^{12} 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 glycoproteins 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 post-translationally 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

Address for reprint requests and other correspondence: K. Chadee, Dept. of Microbiology and Infectious Diseases, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1 (e-mail: kchadee@ucalgary.ca).

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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. *Thermofilus*. For all experiments, 0.01 g of the probiotic formula was reconstituted in 10 ml of serum/antibiotic-free MEM (GIBCO) cell culture medium. After serial dilutions, bacterial concentration was measured by spectrometry and adjusted to an absorbance of $0.25^{600\ OD}$ (10^7-10^8 cfu/ml). In addition, VSL#3 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 HEPES at 37°C, 5% CO_2; 6×10^5 cells/well 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 (3×10^9 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 [^{3}H]glucosamine (Amersham) in 0.5 ml of D-PBS (pH 7.2) and left for 3 h to metabolically label the gastrointestinal mucin pool. The animals were killed, and the colons were excised and slit opened with fine scissors into a Petri dish and the mucosal surface was brushed slightly to remove the adherent mucus. Mucosal proteins were placed in 3 ml of D-PBS and vortexed on high speed for 10 min, and then the supernatant was clarified by centrifugation (1,000 *g* for 10 min). The cell-free supernatant was reserved; glycoproteins were precipitated with equal volumes of 10% trichloroacetic acid (TCA) and 1% phosphotungstic acid (PTA) overnight at 4°C, solubilized in distilled water and neutralized to pH 7.0–7.4 with 0.1 mol/l NaOH. Five milliliters of scintillation cocktail (UniverSol) was added, and ^{3}H activity (a measure on mucus secretion) was determined in a scintillation counter. To confirm the identity of the high-molecular-weight mucin following incubation with probiotics or the secreted products, the secreted [^{3}H]glucosamine-labeled glycoproteins produced in response to the probiotics and untreated controls were subjected to Sepharose-4B (Sigma) column chromatography. To do this, the 10% TCA-1% PTA-precipitated glycoproteins were dissolved in column buffer and applied to a S4B column previously equilibrated with 0.01 mol/l Tris·HCl. Fractions (30–40 fractions of 0.4 ml) were collected, and ^{3}H activity of each fraction was determined by liquid scintillation counting. The results are expressed as percentage of counts per minute (CPM) recovered, which was determined after calculating the percentage of the CPM from each fraction over the total CPM initially loaded into the column. The column was calibrated using the following molecular weights standards: blue dextran (BD; 2,000 kDa), thyroglobulin (669 kDa) and BSA (67 kDa) (Amersham).

To test the direct effect of the probiotics on mucin secretion in rat colons, we utilized the colonic loop method (43). Male Wistar rats 4–5 wk old fasted for 24 h were injected intraperitoneally with 20 μCi of [^{3}H]glucosamine in 0.5 ml of D-PBS (pH 7.2) and left for 3 h to metabolically label the newly synthesized mucin. Rats were anesthetized with pentobarbital sodium (35 mg/kg), the abdomen cavity was opened, and the colons were 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 per 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 (3×10^9 bacteria) of VSL#3 reconstituted in D-PBS, D-PBS alone used as a negative control, and 1×10^6 *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 ^{3}H-labeled glycoproteins from each loop were separately removed and adherent mucus was lightly scraped. Mucosal glycoproteins were collected, ^{3}H activity was measured, and glycoproteins were subjected to gel filtration S4B column chromatography fractionation to quantify the newly released mucins. Mucin profiles are expressed in percentage of CPM recovery, which was determined after calculation of the percentage of the CPM from the mucin fractions over the total CPM loaded into the column. All protocols in this study were carried out with the approval of the McGill University Animal Care Committee.

In vitro mucin secretion studies. LS 174T cells were fed with fresh medium containing 2 μCi/ml [^{3}H]glucosamine overnight to label the newly synthesized mucin pool. Before the experiment, cells were washed three times with warm fresh medium free of antibiotics and serum. VSL#3 probiotic mixture (10^7 cfu/ml) or the diluted secreted products were added to the medium and [^{3}H]mucin secretion was determined by liquid scintillation counting at various times. The same protocol was also performed individually for each bacterial species contained in the VSL#3 mixture. For these experiments calcium ionophore A23187 (Sigma) 20 μmol/l was used as a positive control for mucin secretion.

Quantitative real-time PCR analysis. RNA isolation was performed using TRIzol (Invitrogen) following DNase I (Invitrogen) treatment (1 U/ml) according to the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA using Oligo-dT primer and M-MLV RT enzyme (Invitrogen). Subsequently, real-time PCR for MUC1, MUC2, MUC3, and intestinal trefoil factor (ITF) gene (Table 1) was performed using SYBR Green super mix (Qiagen) in a Rotor-Gene detection system (Corbette Research). Each PCR was performed in triplicate and normalized with the housekeeping gene β-actin. The results were analyzed using $2^{-\Delta\Delta CT}$ method (22).

SDS-PAGE and Western blot analysis. LS 174T cells were stimulated with VSL#3 CM for 4 h. After incubation, cell supernatants were collected and the glycoproteins were precipitated with 10% TCA-1% PTA for subsequent fractionation by S4B column chromatography. 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. Proteins were transferred to a 0.22-μm pore size PVDF membrane (Bio-Rad) for subsequent Western blot analysis. Membrane was

Table 1. *Sequences of PCR rat primers and expected product sizes*

Gene	Primer	Sequence (5′–3′)	Product Size
MUC1	Forward	cgccgaaagagctatg	170 bp
	Reverse	taagagagaccgctactgcc	
MUC2	Forward	gccagatcccgaaacca	127 bp
	Reverse	tataggagtctcggcagtca	
MUC3	Forward	aalgtcagttgcagcgaagt	170 bp
	Reverse	ggagaacacagcgaggatca	
ITF (TFF3)	Forward	agccttctggacaaccct	137 bp
	Reverse	atgtgacagtggggtagtta	
β-Actin	Forward	atatcgctgcgctcgtcgtc	174 bp
	Reverse	tcttgctctgggcctcgtc	

MUC, mucin; ITF, intestinal trefoil factor (TFF).

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. 1*A*, 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. 1*B*, 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. 1*C*). Surprisingly, there was also a significant increase in the secretion of nonmucin glycoproteins (fractions 20–35) ranging from 50–660 kDa (Fig. 1*B*). 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. 1*C*, 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. 1*A*), 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,

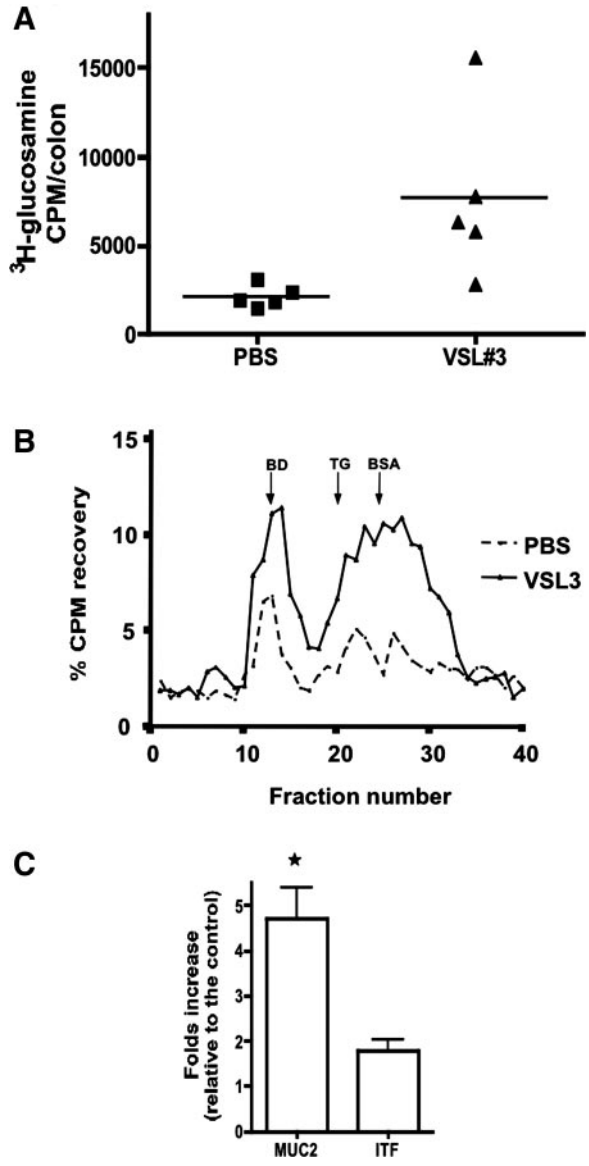

Fig. 1. Secretion of ^{3}H-labeled mucins and MUC2 gene expression after probiotic treatment. Rats were fed with VSL#3 (3×10^9 cfu/kg) or sterile Dulbecco's PBS (D-PBS) for 7 days. Four hours before the experiment, the animals were injected intraperitoneally (IP) with 20 μCi of [^{3}H]glucosamine. Colons were excised and the mucosal proteins were collected and precipitated with 10% TCA, 1% PTA. *A*: total ^{3}H-labeled glycoproteins were measured as total counts per minute (CPM)/colon. *B*: sepharose 4B (S4B) column chromatography of the ^{3}H-labeled secreted glycoproteins. The ^{3}H-labeled glycoproteins secreted into the colonic lumen were acid precipitated and fractionated by S4B column chromatography. Higher mucin void volume (Vo) content (fractions 10–15) eluted with the blue dextran molecular marker (BD; 2,000 kDa). Other molecular markers included thyroglobulin (TG; 669 kDa) and bovine serum albumin (BSA; 67 kDa). *C*: MUC2 and intestinal trefoil factor (ITF) gene expression after probiotic treatment. Total RNA was isolated from colonic tissue after the rats were fed with probiotics for 7 days. Gene expression was determined by real-time PCR. The change in expression of the target gene (MUC2 and ITF) was measured relative to the expression of the control gene (β-actin). The results were analyzed by the $2^{-\Delta\Delta CT}$ method. Values are means and SD of 2 independent experiments $n = 5$ (*$P = 0.03$).

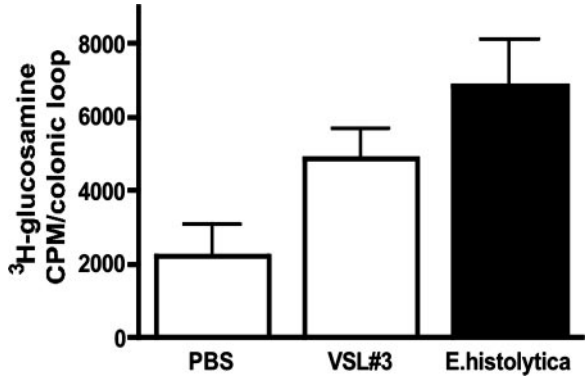

Fig. 2. Secretion of [^{3}H]glucosamine-labeled glycoproteins in rat colonic loops. Rats were injected IP with 20 μCi of [^{3}H]glucosamine and left for 3 h to metabolically label the glycoproteins, including mucins. Ligated colonic loops were prepared and inoculated with 1 ml of D-PBS, or VSL#3 (3×10^9 cfu). Live *Entamoeba histolytica* trophozoites (1×10^6) 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. 3*A*, 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 MUC3 gene expression levels were slightly elevated after probiotic treatment showing about fourfold (SD 0.95 and 0.25, respectively) increase relative to the D-PBS-negative control (Fig. 3*B*). ITF gene expression in the loops treated with VSL#3 bacteria was modest but not significant ($P = 0.34$) (Fig. 3*A*). 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. 3*A*). 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 [^{3}H]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 [^{3}H]mucus 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. 4*A* show a dilution curve for the VSL#3 CM with maximal mucin secretogogue 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. 4*B*). 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 ^{3}H-labeled mucus 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. 1*B*). Immunoblot analysis using an

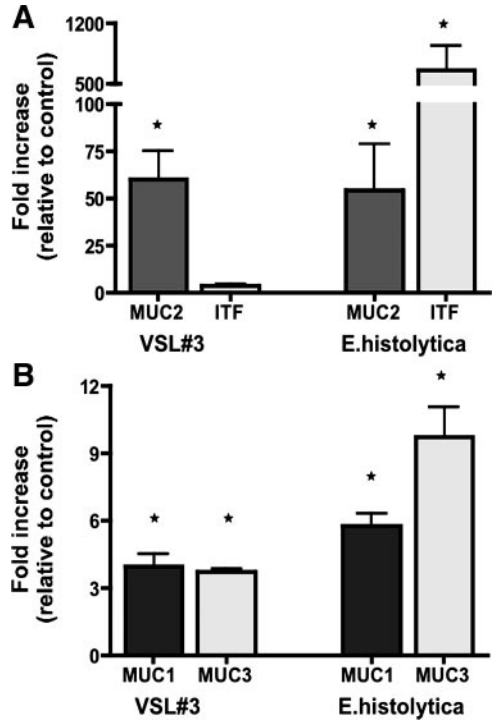

Fig. 3. Mucin and ITF gene expression in rat colonic loops. Total RNA was isolated from VSL#3 or control treated colonic loop tissue. Expression of MUC2 and ITF (*A*) and MUC1 and MUC3 (*B*) genes was analyzed by real-time quantitative PCR. Each reaction contained cDNA derived from 2 μg total RNA was performed in triplicate and normalized with the housekeeping gene β-actin. The results were analyzed by the $2^{-\Delta\Delta CT}$ method. Values are means and SD of 2 independent experiments ($n = 5$; $^*P < 0.05$).

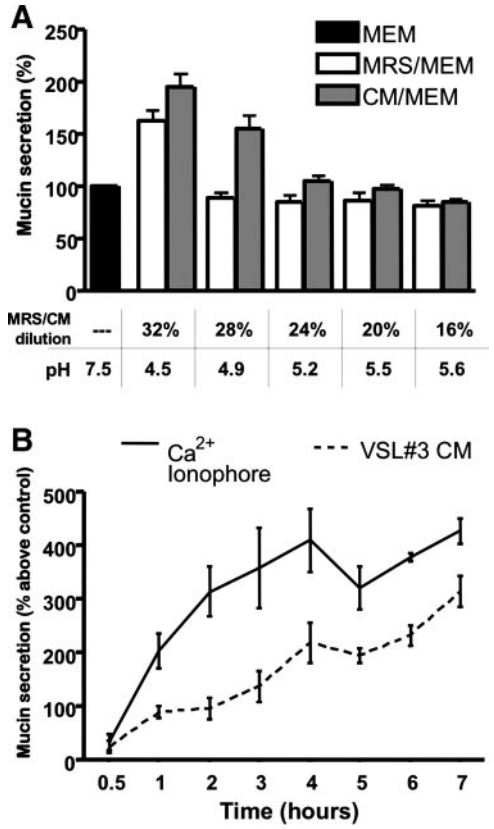

Fig. 4. Mucin secretion effect of VSL#3 conditioned media (CM) on colonic epithelial cells. LS 174T mucin was metabolically labeled with 2 μCi/ml of [^{3}H]glucosamine before incubation with CM. *A*: dose-dependent effect of probiotic CM on mucin secretion. ^{3}H-labeled LS 174T cells were incubated for 4 h with VSL#3 CM or Man, Rogosa, and Sharpe (MRS) culture medium at different dilutions in MEM cell culture medium. *B*: time-dependent effect of VSL#3 CM on mucin secretion. LS 174T cells were incubated with VSL#3 CM at a concentration of 28% CM in MEM (vol/vol). ^{3}H activity from labeled glycoproteins released into the media was measured at the indicated time points. Calcium ionophore A23187 (20 μmol/l) was used as a positive control. Values are means and SD of 3 independent experiments.

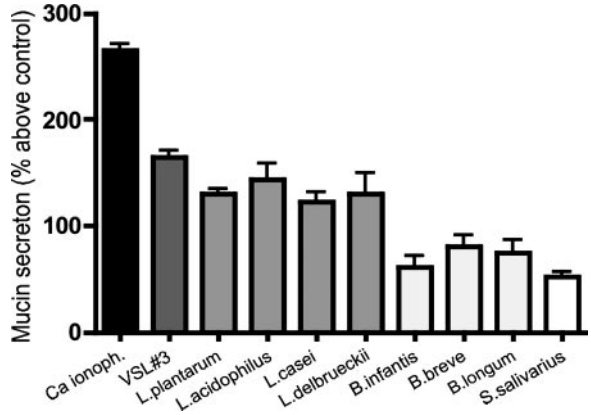

Fig. 5. Comparative effect of the CM from each bacterial component of the VSL#3 formula. LS 174T cells were incubated with the CM from VSL#3 or from each bacterial species contained in this formula for 4 h. [^{3}H]glucosamine-labeled glycoproteins released into the media were measured as CPM. Bars represent the percentage mucin secretion relative to the negative control. Calcium ionophore A23187 (20 μmol/l) was used as a positive control. Values are means and SD of 3 independent experiments.

anti-MUC2 antibody raised against purified colonic mucin confirmed the presence of mucin only in the high-molecular-weight Vo fractions (Fig. 6*B*).

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

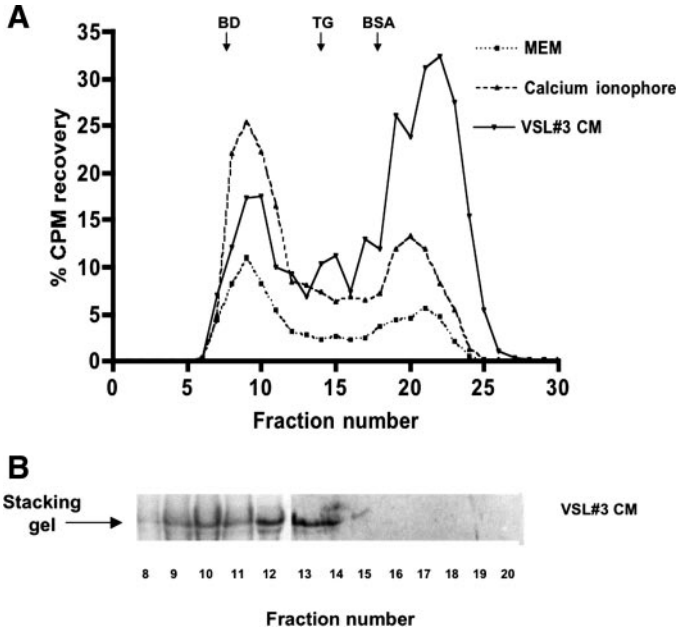

Fig. 6. Sepharose 4B chromatography of the ^{3}H-labeled mucin. ^{3}H-labeled LS 174T cells were stimulated with VSL#3 CM or calcium ionophore A23187 (20 mmol/l); untreated cells were kept as a negative control. The glycoproteins released into the supernatants were recovered after 4 h incubation for further precipitation and fractionation by S4B gel filtration column chromatography. Vo mucin volumes (fractions 7–12) from each group of treated cells were compared. *A*: percentage of [^{3}H] activity in each fraction relative to total CPM of the sample before fractionation. *B*: anti-MUC2 Western blot of S4B fractions. Cells were stimulated with VSL#3 CM for 4 h, and the supernatant was recovered for subsequent fractionation as described above. Fractions were concentrated and subjected to immunoblotting using anti-MUC2 rabbit serum. Gel-forming mucins remain in the stacking gel (fractions 7–11).

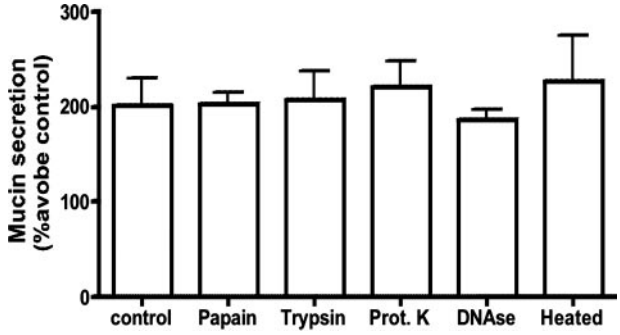

Fig. 7. Probiotic mucin secretagogue is resistant to protease and DNase and heat treatment. ^{3}H-labeled LS 174T colonic epithelial cells were stimulated with pretreated VSL#3 CM. VSL#3 CM was either boiled for 15 min or preincubated for 30 min at 37°C with 45 mg of trypsin, papain, proteinase K (Prot. K; 55°C), or DNase I (5 U/ml); proteinase activity was stopped by the addition of EDTA-free inhibitor cocktail tablets. The mucin secretagogue effect of CM was measured by monitoring the release of ^{3}H-labeled glycoproteins in the supernatant. Untreated CM was used as a control. Values represent means and SD of 3 independent experiments ($P \geq 0.26$, not significant).

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 culture 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 characterized interactions among bacteria, epithelial cells, other cell types (e.g., immune cells) (21) and/or luminal molecules (17, 32, 34). Although our in vitro experiments suggest that the CM contains a secretagogue sufficient for potentiation of mucin secretion, this does not preclude the fact that the mucin secretion effect could be further bolstered by the complex interaction between probiotic bacteria and the intestinal environment. 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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