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Effects of Probiotic Bacteria (VSL#3) on the Polyamine Biosynthesis and Cell Proliferation of Normal Colonic Mucosa of Rats

MICHELE LINSALATA[1*], FRANCESCO RUSSO[1*], PASQUALE BERLOCO[1], ANNA MARIA VALENTINI[2], MARIA LUCIA CARUSO[2], CLAUDIO DE SIMONE[3], MICHELE BARONE[4], LORENZO POLIMENO[4] and ALFREDO DI LEO[4]

[1]Laboratory of Biochemistry and [2]Department of Pathology, National Institute for Digestive Diseases IRCCS "Saverio de Bellis", Castellana G., Bari; [3]Department of Experimental Medicine, University of L'Aquila; [4]Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, Italy

Abstract. *Background: Probiotics seem to possess tumour inhibitory properties, but few studies have investigated their actions on the cell proliferation of normal colonic mucosa. The effects of a probiotic mixture (VSL#3) on polyamine biosynthesis, Ki-67 levels and apoptosis in the normal colon of rats were studied. Materials and Methods: For a 4-week period, 20 rats were fed a VSL#3 solution and 20 rats a saline solution. Samples from the colonic mucosa were collected at the end of treatment. Polyamines were detected by HPLC, ornithine decarboxylase activity by a radiometric technique, and apoptosis and Ki-67 by histochemical and immunohistochemical methods. Results: VSL#3 caused a significant decrease in colonic polyamine levels, ornithine decarboxylase activity and Ki-67 compared to controls. A significant increase in the apoptotic index was also observed. Conclusion: Probiotics could also reduce proliferation rates in a condition not affected by hyperproliferative or neoplastic growth, when the normal control mechanisms are still completely effective.*

Probiotics have been defined as living organisms that, upon oral ingestion, exert benefits on human and animal health (1). Most are non-pathogenic bacteria, normally present in the human intestine such as *lactobacilli*, *bifidobacteria* and *enterococci*. The gastrointestinal tract is considered the major target of probiotoic therapy, assuming that the organisms resist degradation by gastric acid and bile and are not inactivated by the intestinal immune system (2). Nevertheless, the modes of action of probiotics are still under extensive investigation. Some possible mechanisms have been proposed, such as receptor competition with pathogens at the intestinal epithelium (3) and stimulation of the local immune system (4). In this connection, specific cellular components in *Lactobacilli* strains seem to induce strong adjuvant effects, including modulation of cell-mediated immune responses, activation of the reticuloendothelial system and regulation of several cytokines (5, 6). Few data, derived mainly from studies performed in pathological conditions, have also suggested an active role of probiotic bacteria in controlling cell growth mechanisms (7, 8).

Among the substances actively involved in cell proliferation and differentiation, the polyamines (putrescine, spermidine and spermine) are a group of polycations found in high concentrations both in normal and neoplastic rapidly proliferating cells (9). Their functions are linked to the regulation of DNA, RNA, protein synthesis, membrane stability and cyclic AMP-independent protein kinase (10). In mammalians, polyamine synthesis starts from arginine, which is catabolyzed by arginase into ornithine and urea; ornithine is subsequently decarboxylated, this step being governed by ornithine decarboxylase (ODC), an enzyme representing the first and rate-limiting step in the polyamine biosynthetic pathway (11, 12).

Some studies have emphasized a relationship between polyamine biosynthesis and probiotic action in carcinogenesis and tumor growth. In mice, it has been observed that the administration of *Bifidobacterium longum* cultures significantly inhibited azoxymethane-induced cell proliferation, ODC

*These authors equally contributed to the work.

Correspondence to: Michele Linsalata, Laboratory of Biochemistry, IRCCS "Saverio de Bellis", 70013 Castellana Grotte (Bari), Italy. Tel: +390804960317, Fax: +390804960340, e-mail: irccsbiochimica@libero.it

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activity, as well as expression of the ras-p21 oncoprotein (13). Recently, evidence has been provided that *Lactobacillus brevis* strains induce apoptosis of Jurkat cells and it has been hypothesized that the apoptotic death-inducing ability of these bacterial samples could be associated with polyamine synthesis (14). Also, in pre-neoplastic conditions, characterized by a hyperproliferative state, such as during the infection of gastric mucosa by *Helicobacter pylori* (15, 16), high oral doses of *L. brevis* have been proved to significantly decrease the ODC activity and polyamine levels (17).

The claim for probiotics, as the quintessential functional foods capable of enhancing mucosal defences along with other health beneficial actions (18), has raised enormous interest in their potential in both diseases and human well-being. However, in view of the supposed link between probiotics and the polyamine metabolism in pathological conditions, and given that unperturbed polyamine biosynthesis is needed for unperturbed normal cell-cycle progression (19), it was of interest to study the effects of high doses of probiotics on polyamine biosynthesis directly in a healthy normal colonic mucosa. No relevant data are available in the literature. Therefore, the present study was designed to evaluate, in normal colonic mucosa of rats, the effects of high doses of a combination of different probiotic bacteria, as found in the VSL#3 compound, at the single and total polyamine levels and ODC activity. To further elucidate the role of these probiotics in modulating mucosal cell growth, their actions on the Ki-67 expression and apoptotic index were also investigated.

Materials and Methods

Animals and diets. Forty male (F-344) rats of 8 weeks of age, weighing 150-200 g, were purchased from Charles River, Calco, Italy. The rats were housed in pairs in stainless steel wire-mesh-bottomed cages and maintained under a 12-h light-dark cycle. The animals were kept in a temperature controlled room and received *ad libitum* access to standard diet (Mucedola srl, Settimo Milanese, Milan, Italy) and sterile filtered water, for at least 1 week before experimentation. The standard diet was composed of 535 g/kg carbohydrates, 185 g/kg protein, 30 g/kg lipids, 60 g/kg cellulose, 120 g/kg moisture, 70 g/kg vitamins and inorganic compounds and provided 315 Kcal/100 g of diet.

All the rats received adequate attention in compliance with the "*Guiding principles in the care and use of animals*" approved by the Council of the American Physiological Society.

VSL#3 therapy. After one week on the standard diet, the rats were randomly assigned to 2 groups: treated (n=20) and control (n=20). The rats in the first group were intragastrically gavaged daily for 4 weeks with a suspension of $2.8x10^8$ cfu/mL of a probiotic cocktail dissolved in saline. The probiotic compound, VSL#3, (VSL Pharmaceuticals, Inc., Ft Lauderdale, FL, USA) contains $9x10^{10}$ cfu/g of viable, lyophilized bifidobacteria (*B. longum*, *B. infantis* and *B. breve*), $8x10^{10}$ lactobacilli (*L. acidophilus*, *L. casei*, *L. delbrueckii* subsp. *L. bulgaricus* and *L. plantarium*), and $20x10^{10}$ of *Streptococcus salivarius* (subsp. *thermophilus*). The solution was prepared in the morning immediately before administration. The control group received saline gavage only for the same period (4 weeks).

Tissue preparation. At the end of the treatment, all animals underwent surgery under metaphane anesthesia, between 8.30 and 9.30 a.m. Sections of the right and left colon were rapidly dissected and the colonic contents were removed by thoroughly flushing the lumen with ice-cold saline (0.9% NaCl). The colonic sections were then opened longitudinally.

In order to evaluate the polyamine levels and ODC activity, a mucosal sample from the longitudinal segment of the proximal colon and one from the distal colon were weighed and stored at –80˚C until the assay.

Other longitudinal segments from the proximal and distal colon were fixed in 10% formalin, dehydrated and embedded in paraffin. Then, 4-μm sections of tissues were utilised for the cell proliferation assay and histochemical determination of apoptosis. The slides had been previously coated with silane.

Polyamine analysis. Each tissue sample (approximately 10-15 mg) of proximal and distal colon mucosa was homogenized in 700 μl of 0.9% NaCl mixed with 5 μl (174 nmol/ml) of the internal standard 1,10-diaminodecane (1,10-DAD). The total protein content was measured by Lowry's method (20).

In order to precipitate the proteins, 50 μl of perchloric acid (PCA) 3 mol/L were added to the homogenate. After 30 min of incubation in ice, the homogenate was centrifuged for 15 min at 7000 x g.

The supernatant was filtered (Millex-HV_{13} pore size 0.45 mm, Millipore, Bedford, MA, USA) and lyophilized.

The residue was dissolved in 250 μl of HCl (0.1 mol/L). Dansylation and the extraction of dansyl-polyamine derivatives were performed as previously described (21). After extraction, aliquots of 200 μl were injected into a high performance liquid chromatography system (LKB, Bromma, Sweden) equipped with a reverse-phase column (Nova-Pak C_{18} cartridge; 100x5 mm; 4-μm particle size, Waters, Milford, MA, USA) and a precolumn with the same characteristics (Nova-Pak C_{18}; 20x3.9 mm, 4-μm; Waters). Polyamines were eluted by linear gradient ranging from acetonitrile-water (50:50, v:v) to acetonitrile (100%) for 30 min. The flow was 0.5-1.0 ml/min from 0 to 12 min and then set at a constant rate (1.0 ml/min) until the thirtieth min. The fluorescent intensity was monitored by spectrofluorimeter (LS50, Perkin Elmer Ltd., Beaconsfield, UK), with excitation at 320 nm and emission at 512 nm. Dansyl derivatives of putrescine, spermidine, spermine and 1,10-DAD were identified by their retention times and quantified by comparison with standards. Recovery of 1,10-DAD in each sample (normally >90%) was used for calculations. Polyamine levels were expressed as concentration values in nmol/mg of protein.

ODC activity. ODC activity was measured in duplicate with a radiometric technique that estimated the amount of $^{14}CO_2$ liberated from DL-[1-^{14}C]-ornithine (specific activity, 42.47 mCi/mmol, New England Nuclear, USA) (22).

Tissue samples were homogenized in 0.6 ml ice-cold Tris-HCl buffer (15 mol/L, pH 7.5) containing 2.5 mol/L dithiothreitol, 40 μmol/L pyridoxal-5'-phosphate and 100 μmol/L EDTA and then centrifuged at 30,000 x g for 30 min at 4˚C.

An aliquot of supernatant (200 μl) was added to a glass test tube containing 0.05 μCi DL-[1-^{14}C]-ornithine and 39 nmol DL-ornithine.

Table I. *The effects of a VSL#3-enriched diet on single and total polyamine levels in the proximal and distal colonic mucosa of rats.*

	Proximal colon		Distal colon	
	Controls	VSL#3	Controls	VSL#3
	nmol/mg prot.			
Putrescine	0.05±0.014	0.03±0.003	0.04±0.010	0.02±0.001
Spermidine	1.81±0.28^{ab}	1.34±0.15^{ab}	1.38±0.10^{b}	1.07±0.08^{a}
Spermine	2.06±0.28^{ab}	1.48±0.15^{ab}	1.96±0.14^{b}	1.41±0.085^{a}
Total Polyamines	3.94±0.56^{ab}	2.87±0.30^{ab}	3.39±0.24^{b}	2.49±0.15^{a}

For a 4-week period, VSL#3 rats were fed a probiotic mixture; control rats received a saline solution. Polyamines were detected by high performance liquid chromatography. All data are expressed as Mean±SE, n=20. The means in a row with superscripts without a common letter differ significantly ($p<0.02$, Mann-Whitney rank sum test).

After incubation for 60 min at 37˚C, the reaction was stopped by adding trichloro-acetic acid (TCA) to a final concentration of 50%. $^{14}CO_2$ liberated from DL-[1-^{14}C]-ornithine was trapped on filter paper pre-treated with 40 μl NaOH (2 mol/L), which was suspended in a center well above the reaction mixture. Radioactivity on the filter papers was determined by a liquid scintillation counter (model 1219 Rackbeta, LKB-Pharmacia, Uppsala, Sweden). ODC activity was expressed as pmol CO_2/h/mg of protein.

Cell proliferation assay (Ki-67). Four-μm-thick sections were cut, deparaffinized and rehydratated. After the step of endogenous peroxidase block with methanol-peroxide for 30 min, the sections were retrieved by means of non-enzymatic epitope retrieval with citrate buffer solution (pH 6) in a microwave oven. The slides were cooled for 15 min and then incubated with MAb MIB1 (Dako, Glostrup, Denmark) for 30 min in an automatic immunostainer (Dakoautostainer, Dako Corporation, Carpenteria, CA, USA).

After incubation, the sections were processed using the Envision peroxidase system (Dako). Positive controls consisted of ductal breast cancer. Negative controls were obtained by replacing the primary antibody with another of the same class, but with different specificity.

The Ki-67 index was obtained by counting the number of positive nuclei per crypt (in a minimum of 10 well-orientated crypts for each specimen), dividing the number by the total number of nuclei crypts, and multiplying this fraction by 100 (23).

Histochemical determination of apoptosis. DNA fragments of apoptotic cells were visualized by an enzymatic reaction that involves a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) procedure, based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to the 3'-OH ends of DNA breaks, as previously described (24).

After deparaffination and rehydration, the tissue sections were digested with proteinase K (10 μg/ml) at 37˚C for 15 min. Following the application of an equilibration buffer, the sections were incubated in working strength TdT enzyme that contained deoxyuridine triphosphate (d-UTP)-biotin under a coverslip for 30 min at 37˚C.

After removing the coverslip, the slides were incubated with streptavidin-conjugated alkaline phosphatase for 30 min at 37˚C and then with nitroblue tetrazolium for 30 min at 37˚C. Sections of normal lymph nodes were used as the positive control for the TUNEL method. In negative controls, the TdT enzyme was omitted from the nucleotide mixture. The apoptotic signal was recorded as positive when either a diffuse-type or a granular-type nuclear dark blue staining was apparent.

The TUNEL Index was calculated (in a minimum of 10 well-orientated crypts for each specimen) by dividing the number of apoptotic nuclei per crypt by the total number of crypt nuclei and multiplying this fraction by 100.

Statistical analysis. Deviation of values from the Gaussian distribution was tested with the Kolmogor-Smirnov test. The significance of differences between corresponding segments of colon of rats treated with placebo or VSL#3 were analyzed by the Mann-Whitney rank sum test. The Wilcoxon rank sum test was applied when analyzing proximal and distal colonic segments of rats within the same group of treatments.

To assess correlations between single parameters, Spearman correlation coefficients were computed. Values of $p<0.05$ were considered significant. The STATA (STATA ver 4.0 Statistical Software, Stata Corporation) software package was used for all analysis. The results are reported as means±SEM.

Results

Body weight change and general conditions of rats. The body weights of the animals were recorded on day 1 and day 29. On each occasion, no differences were found between the control and the VSL#3 groups. Besides, during the treatment, no animal suffered for diarrhea.

Effect of VSL#3-enriched diet on the polyamine biosynthesis in colonic mucosa. The effects of the VSL#3-enriched diet on single and total polyamine levels in the proximal and distal colonic mucosa of rats are shown in Table I.

VSL#3 administration caused a reduction in the polyamine levels in both the proximal and distal colon of the treated rats. The reduction was statistically significant for spermidine, spermine and total polyamine levels in the

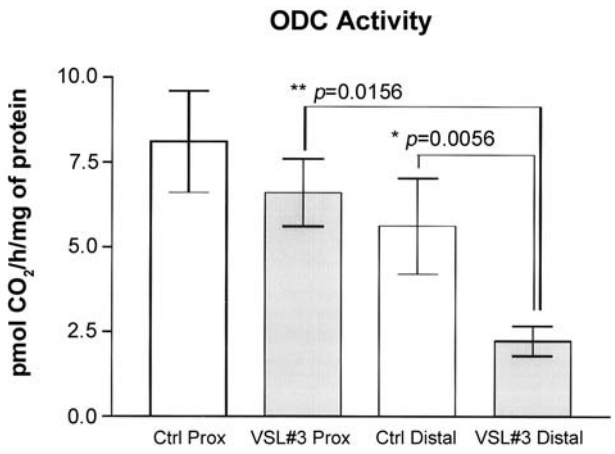

Figure 1. *The effects of a VSL#3-enriched diet on ODC activity in the proximal and distal colonic mucosa of rats. The ODC activity was evaluated by a radiometric technique and is expressed as pmol CO_2/h/mg of protein (Mean±SE). *p=0.0056 (Mann-Whitney rank sum test) distal colon of rats fed control solution vs. distal colon of rats fed VSL#3. **p=0.0156 (Wilcoxon rank sum test) proximal colon vs. distal colon of rats fed VSL#3.*

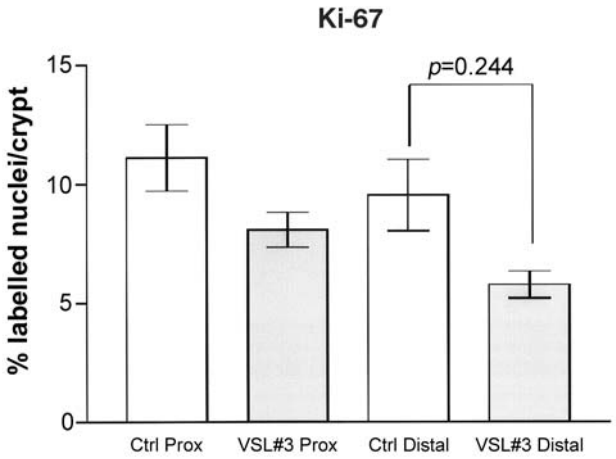

Figure 2. *The effects of a VSL#3-enriched diet on Ki-67 values in the proximal and distal colonic mucosa of rats. Ki-67 was evaluated by immunohistochemical method and its values are expressed as % labelled nuclei/crypt (Mean±SE). p=0.0244 (Mann-Whitney rank sum test). Distal colon of rats fed control solution vs. distal colon of rats fed VSL#3.*

distal colon ($p<0.02$, Mann-Whitney rank sum test). Besides, in both control and treated rats no significant differences in polyamine levels between the proximal and distal colon were found.

As regards ODC activity, rats fed the VSL#3-enriched diet showed a lower ODC activity in both the proximal (6.61±1.0) and distal colon (2.24±0.43) compared to the proximal (8.1±1.47) and distal colon (5.63±1.41) of control rats (Figure 1). The effect was enhanced in the distal colon where the difference was statistically significant ($p=0.0056$; Mann-Whitney rank sum test).

ODC activity was lower in the distal colon than in the proximal one in both the control and VSL#3 groups, but statistical significance was reached in the VSL#3-treated group ($p=0.0156$; Wilcoxon rank sum test).

Effect of VSL#3-enriched diet on the Ki-67 expression and apoptotic index in colonic mucosa. The effects of the VSL#3-enriched diet on the Ki-67 expression and apoptotic index in the proximal and distal colonic mucosa of rats are shown in Figures 2 and 3, respectively.

Probiotics affected the Ki-67 levels, which were lower in both the proximal (8.11±0.7) and distal colon (5.78±0.57) of rats fed the VSL#3-enriched diet than in the proximal (11.1±1.4) and distal colon (9.56±1.5) of controls. The difference reached statistical significance in the distal colon ($p=0.0244$; Mann-Whitney rank sum test). In either control or treated rats, no significant differences in Ki-67 expression between the proximal and distal colon were found. (Figure 2).

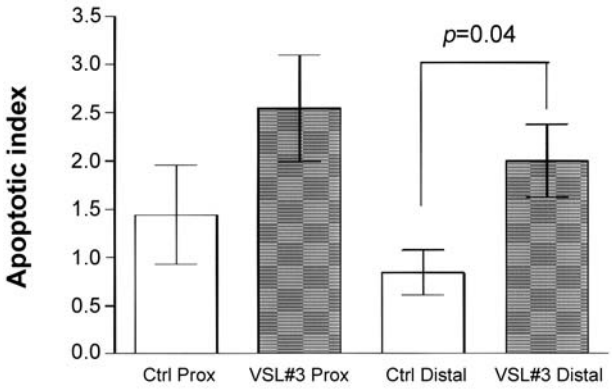

Figure 3. *The effects of a VSL#3-enriched diet on the apoptotic index in the proximal and distal colonic mucosa of rats. The apoptotic index is expressed as number of apoptotic bodies/cm^2 examined section (Mean±SE). p=0.04 (Mann-Whitney rank sum test). Distal colon of rats fed control solution vs. distal colon of rats fed VSL#3.*

On the contrary, higher values of apoptotic index were observed in both the proximal (2.54±0.56) and distal colon (2.01±0.38) of rats fed VSL#3 compared to the proximal (1.44±0.51) and distal colon (0.84±0.24) of control rats. The difference was significant when distal colonic segments of control and VSL#3-fed rats were compared ($p=0.04$; Mann-Whitney rank sum test) (Figure 3).

Finally, no correlation among single and total polyamines, ODC activity, Ki-67 expression and apoptotic index was found (data not shown).

Discussion

In recent years, much attention has been paid to the beneficial effects of probiotics in the gastrointestinal tract, but the mechanisms by which these bacteria exert their effects *in vivo* have not been clearly defined. In this study the effects of the probiotic compound VSL#3, containing different lyophilized *bifidobacteria*, *streptococci* and *lactobacilli* strains, were investigated in the normal colonic mucosa of rats.

The rationale for utilizing a cocktail of probiotic strains such as VSL#3 is in the body of evidence indicating that a probiotic mixture is much more effective than single *lactobacilli* in improving a series of intestinal diseases (*e.g.* colitis, acute pouchitis), in preventing postoperative recurrence of Crohn's disease, as well as in normalizing colonic epithelial functions (6, 25).

Several lines of evidence support the tumour inhibitory properties of lactic acid bacteria (7, 13, 26, 27), but few studies have investigated their actions on the cell proliferation of normal colonic mucosa. In this context, no study has considered the effects exerted by probiotics on polyamine metabolism, that plays a crucial role in cell proliferation and differentiation. This aspect may be of particular interest, since it is known that the supply of luminal polyamines is almost insignificant in normal colonic conditions compared to the neoplastic or adaptative states (28, 29).

The main finding from the present work is that administration of VSL#3 caused a decrease in the polyamine levels and ODC activity of normal colonic mucosa of rats, with a more evident effect in the distal segments of the colon.

Putrescine, spermidine and spermine are widely distributed organic cations actively engaged in both several mammalian normal and neoplastic rapidly proliferating cell systems. Polyamines biosynthesis is known to have specific peaks of induction during cell cycle progression, these molecules being required at different phases of the cycle. Particularly, polyamine synthesis represents an early event occurring during the G1-phase of cell cycle and prior to cell division, and there is a recognised relationship between polyamine levels and tissue cell proliferation rates (10). In this connection, also the results from the evaluation of Ki-67, a static index of proliferation, are in agreement with the polyamine behaviour in our rats. Ki-67, a protein that identifies a nuclear antigen associated with the cell cycle (30), decreased in the colonic mucosa following probiotic administration with a more marked decline in the distal bowel segments.

Additionally, higher values of the apoptotic index were observed in the colon of VSL#3-treated rats compared to control ones. Also in this case, the difference reached statistical significance in the distal colonic segments. The increase in the apoptotic index after probiotic administration is in agreement with the decrease of polyamine biosynthesis. In fact, it is known that a reduction in the polyamine content can have repercussions in the cell proliferative homeostasis, driving cells to the apoptotic pathway (31).

The precise mechanisms by which probiotic bacteria influence polyamine biosynthesis and, more generally, cell proliferation are not sufficiently clear, but it is likely that these effects may proceed through diverse metabolic and physiological modifications.

The present findings could be due, at least in part, to a peculiar enzymatic activity presented by some probiotic strains such as arginine deiminase, an enzyme normally confined to the prokaryotic kingdom (32). This enzyme catalyzes the irreversible conversion of arginine to citrulline and ammonia and a variety of bacteria is able to utilize arginine as a sole source of energy for growth (33). In mammals, polyamine synthesis starts from arginine, which is catabolyzed by arginase into ornithine and urea; ornithine is subsequently decarboxylated by ODC. As already postulated in studies performed both *in vitro* (14) and *in vivo* (34), the arginine deiminase activity following probiotic administration could cause arginine deficiency, thus preventing polyamine biosynthesis.

Secondly, the intrinsic characteristics of the cell components of probiotics could reduce polyamine biosynthesis and cell proliferation. In fact, it has been observed that cytoplasmic extracts derived from different probiotic bacteria (*L. rhamnosus GG*, *B. lactis*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *St. thermophilus*) can suppress mitogen-induced proliferation of peripheral blood mononuclear cells, whether the enzymatic activity was inactivated or not by heating (35).

Other evidence from this study is that the decrease in proliferative rates of the colonic mucosa of rats fed VSL#3 was more marked in the distal than proximal colonic segments. This could be due to the longer transit time in the distal colon (36) that represents an important factor in bacterial metabolism in the large intestine (37), since it could allow bacteria to exert their actions for a more protracted time than on the proximal colon. Also, the presence of different patterns in the cell proliferation in various sites around the colon (38) may justify, at least in part, the enhanced effect of probiotics on cell proliferation in the mucosa of the distal colon, given that this region has been demonstrated to possess a significantly lower cell turnover than the caecum and right colon (30).

In conclusion, this study provides data about the ability of a cocktail of probiotics, administered for 4 weeks, to reduce polyamine biosynthesis and cell proliferation in the normal colonic mucosa of rats. This could be considered as an undesirable effect, especially in a mucosa with an intense cellular turnover such as that in the colon. Thus, it seems

mandatory to investigate in depth the mechanisms of action as well as the times of administration of the single probiotic strains on cell proliferation of normal mucosa of the gut. In fact, it has been observed that the administration of other probiotic strains (namely, *L. casei* and *Clostridium butyricum*) for 1 week can induce an increase in the crypt cell production rates in the gut of laboratory animals (39).

The peculiar metabolic features of the administered probiotic strains, different survival times in the lumen (*i.e. St. thermophilus* does not survive this transit) (40), the period of administration, as well as the different parameters examined could all be involved in affecting the proliferation rates in colonic mucosa. On this basis, and in view of the potential offered by probiotics of affecting the proliferative activity of colonic mucosa, the possible implications in humans remain to be fully elucidated.

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