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ORIGINAL ARTICLE

Estimation of the Potential Antitumor Activity of Microencapsulated *Lactobacillus acidophilus* Yogurt Formulation in the Attenuation of Tumorigenesis in Apc(Min/+) Mice

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Abstract There is a strong correlation between orally administered probiotics and suppression of the low-grade inflammation that can lead to restoration of normal local immune functions. We studied the potential immunomodulatory and antitumorigenic properties of microencapsulated probiotic bacterial cells in a yogurt formulation in Min mice carrying a germline *APC* mutation. Daily oral administration of microencapsulated *Lactobacillus acidophilus* bacterial cells in the yogurt formulation mice resulted in significant suppression of colon tumor incidence, tumor multiplicity, and reduced tumor size. Results show that oral administration of microencapsulated *L. acidophilus* contributed to the stabilization of animal body weight and decreased the release of bile acids. Histopathological analyses revealed fewer adenomas in treated versus untreated animals. Furthermore, treated animals exhibited fewer gastrointestinal intra-epithelial neoplasias with a lower grade of dysplasia in detected tumors. Results suggest that oral administration of microencapsulated probiotic *L. acidophilus* exerts anti-tumorous activity, which consequently leads to reduced tumor outcome.

Keywords Microencapsulation · Probiotic bacteria · Colon cancer · Fecal bile acids · Oral delivery

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Introduction

Colorectal cancer is the second most common cause of cancer death in men and women. Although 70% of tumors are resectable when detected early, 25% of patients will have recurrent disease [1]. The current available treatments include chemotherapy and surgery, but they contribute vastly to a loss in the quality of life. Therefore, there is an immense need for an alternative preventative treatment.

Lactic acid bacteria (LAB) are commonly used probiotics ubiquitously found, for instance, in yogurts and other functional foods. The immunomodulating and immunostimulating properties of yogurt and fermented milks have been well documented [2–4]. Recent in vitro and in vivo studies have shown that the growth of transplantable and chemically induced tumors was inhibited by yogurt and other lactic acid bacteria [5–7]. Several researchers have attempted to elucidate the inhibitory effect of yogurt on colon tumors [7–10]. Moreover, the probiotic effect on various forms of intestinal inflammations has been evaluated in mice [11–14]. Probiotics like lactobacilli and bifidobacteria have been claimed to deconjugate and absorb bile acids. In doing so, they contribute to the overall reduction in the colonic mucosal secretion of mucin and fluids, which may lead to changes in colonic bacterial flora [15]. Bile acids have been reported to cause oxidative stress, DNA damage, and mitochondrial membrane instability in intestinal lining, leading to the formation of cancerous cells [16, 17].

IL-6 is an important regulatory cytokine with multiple actions on immune functions such as being involved in the differentiation of cytotoxic T lymphocytes and regulations of T-cell proliferation [18].

Microencapsulation in specialized ultra-thin semi-permeable polymer membranes has been successfully shown to protect live bacterial cells in oral and other delivery applications. Various methods of microencapsulation of probiotics as therapeutics in the prevention and treatment of various disorders, including GI diseases, have been thoroughly described in a review by Prakash and Bhathena [19]. Whilst the numerous studies and methods described were very promising, the overall therapeutic effect was diminished by the fact that only 1% of free bacteria ingested survive GI transit [20]. Lim and Sun [21] first proposed the alginate-poly-L-lysine-alginate (APA) microcapsule membrane in 1980 and since then, microencapsulation has proven to be an effective strategy for cell implantation and cell-based gene therapy for the treatment of diabetes, metabolic or neurologic disorders, and cancer [22–25].

This study investigates a novel approach for the oral delivery of microencapsulated probiotic bacteria *Lactobacillus acidophilus* in yogurt as a potential carrier, and investigates its potential performance in the prevention of colon cancer and in the amelioration of gastrointestinal health.

Materials and Methods

Chemicals

Sodium alginate (low viscosity), poly-L-lysine (MW = 27,400; lot 71K5120), and calcium chloride (desiccant, 96+%, A.C.S. reagent, FW 110.99, d 2.15, batch # 05614AC) were purchased from Sigma-Aldrich (Oakville, ON, Canada). MRS AGAR Difco ™ Lactobacilli and MRS BROTH Difco ™ Lactobacilli were purchased from Becton, Dickinson and Company (Sparks, MD, USA). Liberty plain yogurt 2% M. F. containing active *Acidophilus* and *Bifidus* cultures was procured from a local grocery store.

Bacteria and Culture Conditions

Lactobacillus acidophilus (ATCC 314) cells were cultivated and serially propagated three times in the MRS medium before experimental use. Incubations were performed at 37°C in a Professional Sanyo MCO-18 M Multi-Gas Incubator in anaerobic conditions (1–2% CO_2, Atmosphere Generation System AnaeroGen™; Oxoid, Hampshire, England). Bacteria to be encapsulated were isolated after 20 h of the third passage. Microcapsules containing live bacteria were homogenized manually and re-suspended in 0.9 mL of saline. Samples were serially 10-fold diluted in diluent (saline). Duplicate plates were inoculated with 0.4-mL samples from the appropriate dilutions and incubated under 5% CO_2 at 37°C for 48 h before counting.

The cell count was kept constant at an average of 10^{10} cfu/mL throughout the experiment.

Microencapsulation Method

The bacterial strains were microencapsulated into alginate-poly-L-lysine-alginate (APA) membranes. All membrane components were filter-sterilized through a 0.22-μm Sterivex-GS filter prior to use. Grown cultures were centrifuged at 3,000 *g* for 15 min at 25°C and the supernatant broth was decanted. The pellet of wet cells was weighted and suspended in 0.85% saline, pooled, and slowly added to a gently stirred sterile 3.3% sodium alginate solution (diluted 50% with 0.85% saline). The entire procedure was performed under sterile conditions in a Microzone Biological Containment Hood (Microzone Corporation, Ottawa, ON, Canada) and all solutions were autoclaved with the exception of poly-L-lysine which was 0.22 μm sterile-filtered prior to usage. APA microcapsules were prepared aseptically using an Inotech Encapsulator® IER-20 (Inotech Biosystems International, Switzerland). Freshly prepared microcapsules were washed twice with 0.85% saline and stored at 4°C. Parameters for microencapsulation were as follows: gelation time in CaCl2—30 min, coating time—10 min, nozzle diameter—300 μm, vibrational frequency—918 Hz, voltage >1.00 kV, and current 2 A.

Treatment Formulation Preparation

The APA microcapsules loaded with *L. acidophilus* bacterial cells were blended with Liberty plain yogurt 2% M.F. and 0.85% saline in the proportions of 3:1. Empty APA microcapsules were suspended in 0.85% saline using the same formulation. Alginic acid and its salts are considered to be Generally Recognized as Safe (GRAS) according to the Food Additive Status List [26] and have been used in the food industry as thickening agent, preservative, antioxidant, flavoring agents, as well as an encapsulant material, because it has the benefit of being nontoxic [27]. A treatment group consisting of animals being fed yogurt only was not included in this study as previous reports have tested its potential in colorectal cancers [10]; also, our focus was to test the efficacy of APA microencapsulated probiotic bacteria.

The Mouse Colorectal Cancer Model

Multiple intestinal neoplasia (Min) mice are heterozygous for *Apc* (Min/+), a germline-truncating mutation at codon 850 of the *Apc* gene and spontaneously develop numerous

pretumoric intestinal neoplasms [28]. The *Apc (*Min/+) mouse is a popular animal model for studies on human colorectal cancer [29]. It is used to study the effects of genetics, diet, or chemical compounds on the incidence and development of precancerous intestinal lesions, the adenomas [30]. The germline mutations in the APC gene lead to familial adenomatous polyposis (FAP), but inactivation of APC is also found in 80% of sporadic colorectal cancers [31].

Male heterozygous C57BL/6 J-*Apc*$^{Min/+}$ mice [32], weighing 20–25 g, were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The animals were kept in the Duff Medical Building Animal Care Facility under the conditions of a 12-h light–dark cycle with controlled humidity and temperature and maintained in a barrier facility. They were allowed sterile water and the laboratory rodent diet 5001 from Purina Land O'Lakes ad libitum. The overall health of the animals was monitored daily.

The protocol was approved by the Animal Care Committee of McGill University and the animals were cared for in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

Animal Protocol

Mice 7 or 8 weeks old were used. The life span of these mice is 119 ± 31 days [33]. The mice were separated into three experimental groups:

1. Control—the animals were gavaged empty APA microcapsules suspended in 0.85% saline.
2. Treatment 1—animals were gavaged APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt.
3. Treatment 2—gavaged APA microencapsulated *L. acidophilus* bacterial cells suspended in 0.85% saline.

Upon arrival, animals were randomly placed in the cages and allowed a 1-week acclimatization period. Based on initial serum IL-6 values the animals were ranked and randomly block assigned to the aforementioned groups. There were 11 animals per group. Animals were weighed individually every week; the saphenous vein was bled every 4 weeks and fecal samples were collected at specific intervals throughout the experiment. There were three end points at weeks 8, 10, and 12 of treatment, at which 9, 9, and 15 animals were sacrificed respectively.

Analytical Techniques

Interleukin-6 Determination

Interleukin-6 (IL-6) is a cytokine secreted by diverse cell types under homeostatic and inflammatory conditions [34]. IL-6 mRNA expression in general is low in normal, adenomatous, and cancerous human colon mucosa; except in rather undifferentiated lesions, in which IL-6 is overexpressed. IL-6 has been shown to be associated with cancer development. However, its role in gastric cancer has never been investigated.

For this test, blood samples were collected every 4 weeks into heparinized tubes, which after blood collection were centrifuged at 5,000 *g* for 20 min to yield plasma, which was used in further testing. The release of IL-6 from plasma samples into the culture medium was quantified by enzyme-linked mouse immunosorbent assay (ELISA; Biosource, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 50 μL of plasma plus 50 μL of standard diluent buffer were added to each well and incubated for 3 h and 30 min at room temperature. Upon completion of the assay procedure, the plate was read at a wavelength of 450 nm using a Perkin Elmer Victor microtiter plate reader.

The Hemoccult SENSA Test

This test was used according to Beckman Coulter's instructions. Briefly, using the applicator provided, small fecal samples were collected, a thin smear was applied covering Box A. The second applicator was used to obtain a second sample from a different part of the feces, covering Box B. Three days later, the samples were developed by applying one drop of Hemoccult SENSA Developer between the positive and negative Performance Monitor areas. The results were read within 10 s.

Fecal Bile Acids Determination

Feces were collected at specific intervals throughout the experiment and the analysis was performed per group per cage. Total fecal bile acids were determined as previously described [35, 36] with the following modifications. Twenty-five microliters of sample were used to determine the total bile acid concentration enzymatically, as previously described [37], using a commercially available kit (Sigma Diagnostic Bile Acids 450A; Sigma Diagnostics, St. Louis, MI, USA). Calibration was carried out according to the bile acid internal standard included in the same kit.

Enumeration, Classification, and Histopathology of Adenomas

Mice were euthanized by CO_2 asphyxiation, and the small, large intestine and cecum were excised. Upon removal, the intestines were infused with 10% phosphate buffered formalin (PBF), after which the Swiss Roll was performed. The mice were placed in cassettes and immersed in 10%

PBF as a fixative. Paraffin-embedded sections measuring 5 μm were stained with H&E for histological evaluation.

Polyp scoring was performed by a veterinary pathologist who was blinded to the treatment. The lesions observed were divided into two categories, mostly based on the size of the lesion: gastrointestinal intraepithelial neoplasia (GIN) (<1 mm) and adenomas (>1 mm). The standards for the histological assessment were established from the MMHCC-sponsored symposium and are detailed on the MMHC website: (http://emice.nci.nih.gov/emice/mouse_models/organ_models/gastro_models/murine_intestinal_neoplasia/mo dels_colorectal_cancer).

Statistical Analyses

The Statistical Analysis System (SAS Enterprise Guide 4.1 [4.1.0.471] by the SAS Institute, Cary, NC, USA) was used to analyze the data. Data were expressed as means ± SEM. Differences in body weight, IL-6 concentration, adenomas, and gastrointestinal intraepithelial neoplasia number between the groups were analyzed statistically using ANOVA Mixed Models. Data were considered significant at $P \leq 0.05$.

Results

Microencapsulation and Impact of Treatment on the Body Weight of Min Mice

The microencapsulation technique used yielded spherical alginate microcapsules with a narrow size distribution of 433 ± 67 μm. Figure 1 displays photomicrographs of fresh microcapsules loaded with *L. acidophilus* bacterial cells under light microscopy (×77 magnification in a and ×112 magnification in b). Using the optimal settings, microencapsulation yielded a consistent bacterial cell load of approximately 10^{10} cfu/mL in all batches throughout the experiment.

After an acclimatization period of 1 week, the mice were randomly assigned using a block design into three groups, each consisting of 11 animals. Body weights were recorded on weekly basis (Fig. 2a). Results show a steady drop in the body weight of control group animals (24.6 ± 0.48 to 22.0 ± 1.47 g over 12 weeks). However, a stable body weight was observed in animals receiving treatment: 24.2 ± 0.47 to 24.0 ± 1.32 g (treatment 1) and 24.3 ± 0.36 to 24.0 ± 0.71 g (treatment 2). After 17 weeks, a rapid decline in body weight was observed in control group animals compared with treated animals.

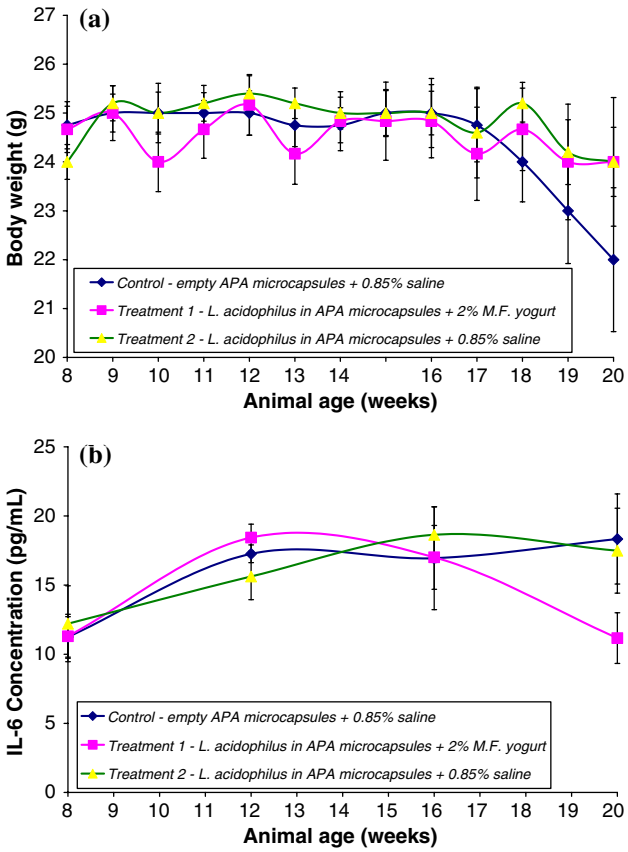

Fig. 2 The effect of treatment in the C57BL/6 J-*Apc*$^{Min/+}$ mice examined at different time intervals on (**a**) animal body weights and (**b**) the concentration levels of anti-inflammatory interleukin-6. Data represent the mean ± SEM of concentration levels per group

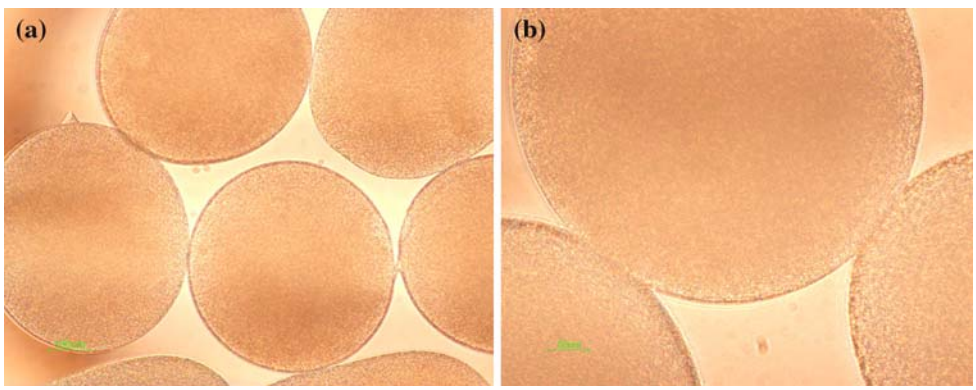

Fig. 1 Photomicrograph of APA microcapsules loaded with *Lactobacillus acidophilus* bacterial cells at (**a**) ×77 magnification and (**b**) ×112 magnification (size 433 ± 67 μm)

Interleukin- 6 Levels in Experimental Min Mice

Results show that the average levels of anti-inflammatory interleukin-6 (IL-6) were 11.17 ± 1.59 for the treatment 1 group, 17.45 ± 2.74 for the treatment 2 group, and 18.33 ± 1.46 pg/mL for the control group at the time of sacrifice (Fig. 2b). The expression levels increased steadily in the animals of the control group and decreased after the 16th week in animals of treatment group 1, and remained stable in the animals of treatment group 2.

Detection of Fecal Blood in Min Mice (Hemoccult SENSA Test)

Abnormal bleeding is associated with gastrointestinal disorders; the fecal samples from individual cages were collected at the beginning and end of the treatment period and analyzed for fecal blood using the Hemoccult SENSA test. The Hemoccult SENSA is a qualitative test with a higher sensitivity than standard guaiac tests. The occult blood test was performed in triplicate. All tests were positive and the coloration intensities were qualitatively scored by three blinded observers, + being the least intense and +++ being the most intense. The results are displayed in Tables 1 and 2. The results show that all animals had blood in their fecal samples on arrival. After the experimental period, the control group displayed a higher blood content in their fecal samples compared with the two treatment groups. Furthermore, treatment 2 was more effective in controlling fecal blood than treatment 1.

Fecal Bile Acid Levels in the Experimental Animal Model

To determine the effect of microencapsulated probiotic bacteria on luminal bile acids, the levels of bile acids in fecal samples from individual cages of each group were measured. Using the bile acid standard with a concentration of 100 µmol/L, the correlation of the determinant factors (R^2) of 0.9955 was obtained and used in this experiment. There was a constant drop in fecal bile acid observed in all groups (Fig 3), with the greatest reduction observed in treatment group 2 where animals were gavaged with *L. acidophilus* bacterial cells in APA microcapsules suspended in saline (448 ± 2.82–105 ± 21.36 [nmol/g fecal sample/100 g body weight]). A decrease in luminal bile acids (442 ± 4.87–210 ± 3.66 [nmol/g fecal sample/100 g body weight]) was also observed in treatment group 1 receiving microencapsulated *L. acidophilus* bacterial cells in 2% M.F. yogurt. Total fecal bile acid values averaged over time are presented in Table 1b. Results show an average decrease of 310.25 ± 75.22 (nmol/g fecal sample/100 g body weight) in treatment group 1 and 229.15 ± 101.95 (nmol/g fecal sample/100 g body weight) in treatment group 2 compared with control animals (358.44 ± 53.93 [nmol/g fecal sample/100 g body weight]) receiving no treatment.

Table 2 Comparison of average total fecal bile acid per group and the associated *p* values

	Average total fecal bile acid (nmol/g fecal sample/100 g body weight)	Repeated measures ANOVA using mixed models analysis *p* values
Control (C)	358.44 ± 53.93	0.0296 (T1 + C)
Treatment 1 (T1)	310.25 ± 75.22	0.0187 (T1 + T2)
Treatment 2 (T2)	229.15 ± 101.95	0.0037 (T2 + C)

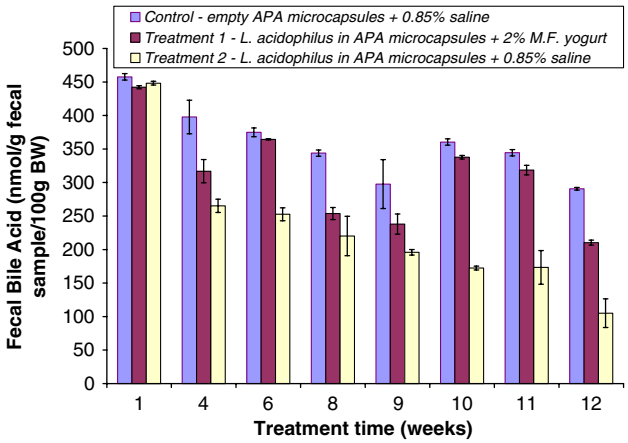

Fig. 3 Effect of treatment on total fecal bile acid levels. Data represent the mean ± SEM of concentration levels per group

Table 1 Detection of fecal blood using the Hemoccult SENSA test

	Animal age 8 weeks			Animal age 20 weeks		
	Cages 1–3	Cages 4–6	Cages 7–9	Cages 1–3	Cages 4–6	Cages 7–9
Control (C)	+	+	++	+++	+++	+++
Treatment 1 (T1)	+	++	+	++	+++	++
Treatment 2 (T2)	++	+	+	++	++	++

Reduction in Adenomas in the Treated Animals: Classification and Histopathology

The number of adenomas, low-grade dysplasia, high-grade dysplasia, and gastrointestinal intraepithelial neoplasias (GIN) were scored for each treated and nontreated animal group in both the small and large intestine. The numbers were averaged per animal in a given group. In the large intestine, 0.8 adenomas were found in the control group compared with 0.4 and 0.7 in treatment groups 1 and 2 respectively (Fig. 4a).

In the small intestine, 28 adenomas were found in the control group versus 13 and 18 in treatment groups 1 and 2 respectively (Fig. 4c). In the large intestines, there were 0.3 GIN found in the control group versus 0.2 and 0.1 in treatment group 1 and treatment group 2 respectively (Fig. 4b). In the small intestine, there were 8 GIN found in the control group versus 4 and 6 in treatment groups 1 and 2 respectively (Fig. 4d). Control group animals had a statistically higher number of adenomas and GIN in the small intestine than treatment group 1 mice. Although there were no statistically significant changes in numbers of adenomas or GIN in the large and small intestine between treatment groups, there was a trend toward fewer polyps compared with controls.

To evaluate the overall impact of the treatment procedure, after 12 weeks of treatment, histology of the colon lesions was analyzed and compared with that of control animals (Fig. 5). Results show that the tumors were mostly well-differentiated pedunculated adenomas with a high grade of dysplasia in a representative animal of the control group (Fig. 5a) compared with the treatment groups (Fig. 5b–d; arrows indicate the areas affected most). A clear trend in controlling colon lesions was observed in the treatment groups. Histological analysis of treatment group 2 (Fig. 5c, d) indicates that the animals had tumors that were at a more advanced stage characterized by a low grade of dysplasia (Fig. 5c) and broad-based adenomas (Fig. 5d). In contrast, the animal in treatment group 1 had mostly microadenomas (Fig. 5b).

Discussion

The development of colorectal cancer, one of the most frequent cancers, is influenced by the inflammatory state, with changes in mucosal function and structure as well as changes in the colonic bacterial flora [15]. Studies have shown that yogurt and other probiotic foods reduce the incidence of colorectal cancer and other gastrointestinal diseases [5]. The main objective of this study was to show the efficacy of microencapsulated probiotic bacteria.

There is a strong correlation between diet and disease occurrence. Furthermore, there is a relationship between food intake and age. As mentioned before, the life span of Apc-Min^{+} mice is 119 ± 31 days. After 119 days (17 weeks), no weight gain was observed due to a pronounced decline in food intake. The decline in food intake at advanced ages is usually due to social factors such as depression and social isolation, physical factors such as changes in taste and smell, and medical conditions including gastrointestinal disease, malabsorption syndromes, and acute and chronic infections [38].

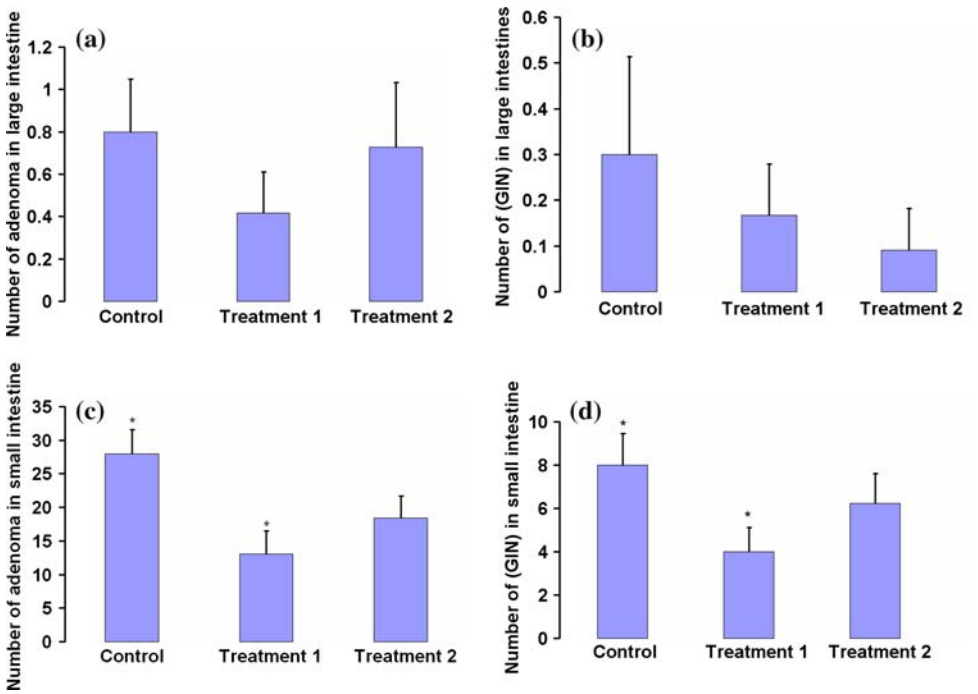

Fig. 4 The number of (**a**, **c**) adenomas and (**b**, **d**) gastrointestinal intraepithelial neoplasias (GIN) for the three groups—control (empty APA microcapsules + 0.85% saline), treatment 1 (*L. acidophilus* bacterial cells in APA microcapsules + 2% M.F. yogurt), and treatment 2 (*L. acidophilus* bacterial cells in APA microcapsules + 0.85% saline—found in (**a**, **b**) large and (**c**, **d**) small intestines. Data represent the mean ± SEM per group. *Asterisks* statistical differences ($P < 0.05$; Mann–Whitney *U* test)

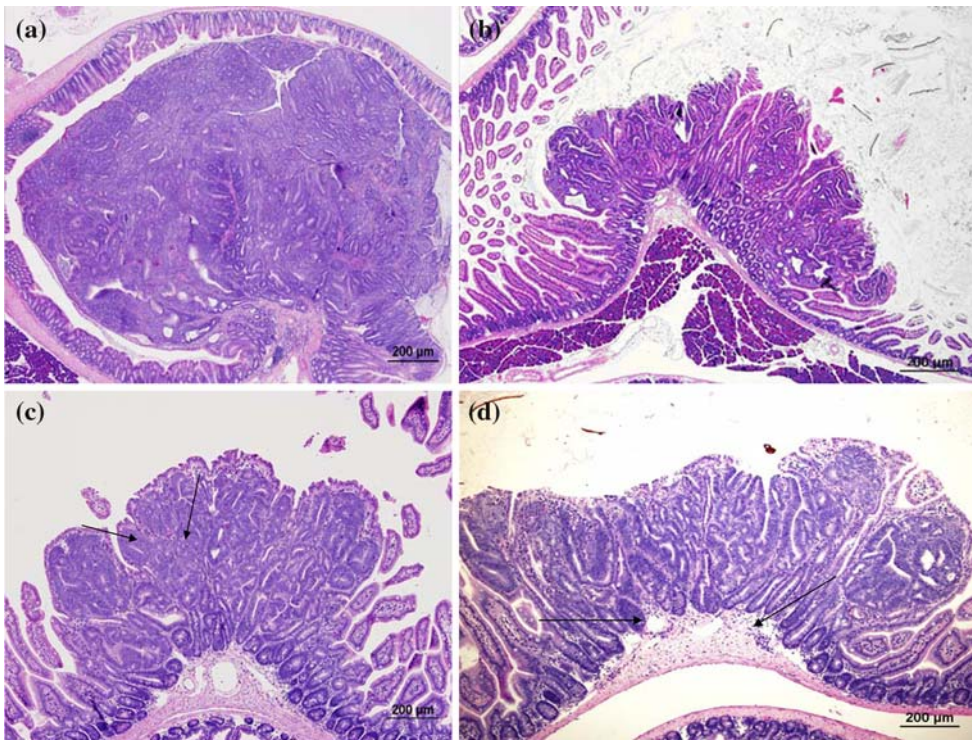

Fig. 5 Histological sections showing intestinal changes in C57BL/6 J-*Apc*$^{Min/+}$ mice. (**a**) A representative tumor of the colon found in a control (untreated) mouse shows pedunculated (polypoid) adenomas with a high grade of dysplasia. Original magnification ×40. (**b**) Gastrointestinal intraepithelial neoplasia (microadenomas) of the small intestine found in a treatment group 1 mouse gavaged with *L. acidophilus* bacterial cells in APA microcapsules + 2% M.F. yogurt. Note the increased nuclear/cytoplasmic ratio, the nuclear crowding, and the hyperchromasia of these glands (*arrow*). Original magnification ×100. (**c**) Papillary adenoma in the small intestine, sessile with a low grade of dysplasia (*arrows*; sessile adenomatous polyp) found in a treatment group 2 mouse gavaged with *L. acidophilus* bacterial cells in APA microcapsules + 0.85% saline. Original magnification ×100. (**d**) Broad-based adenomas of the small intestine found in a treatment group 2 mouse gavaged with *L. acidophilus* bacterial cells in APA microcapsules + 0.85% saline. Original magnification ×100. All tissues were stained with hematoxylin and eosin

In our study, older mice had a greater degree of intestinal obstruction in the form of polyps and were therefore less likely to maintain food consumption levels due to an inability to properly excrete.

Secretion of IL-6 is strongly associated with the pathogenesis of inflammatory bowel diseases (IBD) [39, 40] and overproduction of IL-6 by intestinal epithelial cells is thought to play a part in the pathogenesis of IBD [41]. IL-6 can initiate the innate immune response by inducing the acute phase of inflammation [42, 43] and appears to be involved in malignant transformation, tumor progression, and tumor-associated cachexia, as reported in studies on Kaposi's sarcoma [44], multiple myeloma [45], renal cell carcinoma [46], prostate cancer [47], ovarian cancer [48], and breast cancer [49]. The results of the present study indicate an overall trend of decreasing IL-6 in the two treatment groups compared with the control group (p values: T1/T2 = 0.55, C/T2 = 0.99 and C/T1 = 0.57). This indicates that an anti-inflammatory state correlates with the beneficial effect of the probiotic bacteria on the immunomodulatory mechanisms involved. The differences in inflammation levels among the treatment groups could be the result of the prevention of pathogenic bacterial growth, the binding to or penetration of mucosal surfaces by pathogens, the stimulation of the mucosal barrier function, or the alteration of immunoregulation (decreasing proinflammatory and promoting protective molecules).

Fecal occult blood testing (FOBT) is a screening method for colorectal cancer that has the most evidence of efficacy and is also the cheapest approach [50]. It helps to reduce mortality by detecting early signs that might lead to the formation or presence of polyps in the colon or rectum. Rectal bleeding was observed in animals on arrival and the test was repeated at the end of the experiment to verify whether the treatment has an effect on decreasing the bleeding from the GI tract. This qualitative test detected the presence of blood in the feces in all animal cages at the end of the treatment, but did not reveal any significant differences among groups. Although this test is more sensitive than other occult blood tests, it is considered to be a qualitative screening rather than a decisive test; therefore, it cannot provide conclusive evidence of the presence or absence of gastrointestinal bleeding or pathology.

Bile acids contribute to colonic carcinogenesis by disturbing the fine balance between proliferation, differentiation, and apoptosis in colonic epithelial cells [51–53]. Secondary bile acids have been implicated as an important etiological factor in colorectal cancer. In addition, the bile acids in the feces act as a promoter of colon cancer; in particular, deoxycholic acid (DCA), a secondary bile acid. The ratio of DCA/cholic acid (CA) in feces is also said to have diagnostic significance in colon cancer [54]. It has been established previously that lactobacilli are unable to bind the major conjugated bile acid, glycocholic acid (GCA) [55]. Further, it is also known that the colonic microflora probably exhibits bile salt hydrolase (BSH) activity, which causes the breakdown of conjugated bile acids to the secondary bile acids, most notably DCA [15]. Testing in the lab revealed that *L. acidophilus*, which was used in this study, had no significant BSH activity (data not shown). Owing to the fact that DCA is the primary bile acid measured in feces using the total bile analysis kit, the overall decreasing trend may indicate that a minor amount of primary bile acids were deconjugated to secondary bile acids. Also, we postulate that this trend could result in the replacement of BSH-positive colon flora with one that exhibits less BSH activity, over the course of the experiment. Although more studies are needed, our results indicate that microencapsulated bacterial cells may have an influence in tumorigenesis.

Histological examinations are the most reliable methods for the evaluation of the tumor status and therapy efficacy. The number of adenomas found in each of the treatment groups in the colon and in the small intestine indicates that treatment 1 had a greater impact than treatment 2. This may be explained in two ways: first, the synergistic effect between bacterial cultures and yogurt, along with its nutrients (i.e., calcium, vitamins A and D), may have a superior effect on bacterial cells suspended in saline solution; second, the protective effect of the 2% fat contained in the yogurt could have effectively shielded microcapsules and encased bacteria, protecting them from environmental stresses, thereby enhancing overall efficacy. In addition, the number of gastrointestinal intraepithelial neoplasias (GIN) found in the colon was lower than in the small intestine. Although there were no statistically significant differences between groups in the large intestines, there was a statistical difference between the control group and treatment group 1 in the small intestine with regard to the number of adenomas and GIN (Fig. 4c, d).

Among the organs examined in this study, there was only one malignant tumor (adenocarcinoma) found in the small intestine of a control animal. The nuclei were enlarged and pleomorphic with variable loss of their polarity. The glandular structure was distorted and resembled that seen in overt colonic carcinoma. The most tissue damage was observed in the colon of control group animals under the same conditions applied to the other tissues. This is probably due to the fact that the colonic wall, including the mucosa and the submucosa, is much thinner than that of the other organs.

Most of the adenomas found were to be sessile/broad-based and were composed of papillary projections of lamina propria covered by an epithelium. There were no lesions or adenomas found in the ceca. The greatest loss in mucin secretion was displayed in severely dysplastic glands of control group animals sacrificed in the 12th week of the experiment. The glands were closely packed and a structural atypia, e.g., "back to back" arrangement was more prominent. Nuclei were plump but still uniform and smaller than those in carcinomatous glands. Cytological abnormalities detected included cellular and nuclear pleiomorphism and loss of polarity. Architectural abnormalities included the presence of intraglandular papillary projections and of cribriform and solid epithelial areas. There were, however, no major differences in animal tissues collected from animals sacrificed at different time periods. The tumors found in both treatment groups showed some features of papillary carcinoma-grooved nuclei and papillary architecture, but these were not consistent (Fig. 5). Future work could incorporate additional cellular markers to reveal immunocytochemical indices of tumor growth dynamics, such as proliferation-associated antigens (e.g., Ki-67, PCNA) and to examine reciprocal correlations between the intensity of apoptosis and the expression of pro- and anti-apoptotic cell markers such as caspase-3 (cas-3), MT, and the Ki-67 antigen.

The present study therefore demonstrates that microencapsulated probiotic bacteria in yogurt exert beneficial action by maintaining the constant body weight, minimizing intestinal inflammation, and delaying overall polyp progression in experimental Min mice. This study will have implications for colon cancer, IBD, and other GI diseases.

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