Abstract. Background: Increased intake of probiotic dietary fibre reduces colonic cancer risk. Modified citrus pectin (MCP) requires optimal bioactivity to inhibit galectin-3 (GAL-3) and vascular endothelial growth factor (VEGF). This study evaluated the preventative effect of modified pectin alginate (MCPA) probiotic microbeads on azoxymethane (AOM)-induced colonic carcinogenesis in Balb/c mice. Materials and Methods: Optimization of AOM dose duration: 10-15 mg/kg was administered for 2-4 weeks. The optimal AOM dose was initiated prior to intake of MCPA, alginate probiotic (AP) microbeads and MCP in Balb/c mice for 16 weeks; samples were analyzed for colonic histopathology and immunohistochemistry. Results: AOM at 15 mg/kg for 4 weeks induced optimal GAL-3 and VEGF immunostaining. Furthermore, MCPA treatment reduced GAL-3 expression in the colon of AOM-treated mice compared to MCP. Conclusion: MCPA probiotic microbeads increase bioactivity and chemopreventative effect against pre-cancerous colonic lesions and adenocarcinoma through inhibition of GAL-3 and VEGF in the Balb/c mouse model of colonic carcinogenesis.

Colonic cancer is the third most common cancer worldwide, with high morbidity and mortality (1). Worldwide deaths from colonic cancer have increased from 608,000 in 2008 to 694,000 in 2012 (2). This trend is projected to rise, with an estimated 13.1 million deaths in 2030 (3). Treatments include either single or combination therapies such as chemotherapy, radiation, surgery and biologically targeted management (4). However, these therapies are less effective during metastasis, therefore prevention and earlier detection are key factors to reducing the risk of colonic cancer (2). Recent attention has been focused on increasing dietary intake of probiotic fibre in order to reduce colonic cancer risk (5).

In vitro trials have suggested that lactobacilli may reduce the progression of colonic cancer through increased apoptosis, immunomodulation, antioxidant activity and antiproliferative effect (6). Probiotics are defined as “live microorganisms which when administered orally in adequate amount confer health benefit on the host” (7). Probiotics can be encapsulated with a coating matrix to protect them from adverse environmental effects such as stress, oxygen tension (8), high temperature during processing and storage (8, 9), as well as pH and intestinal enzymatic activity (10).

Modified citrus pectin (MCP) is a polysaccharide dietary fibre broken-down into smaller fragments. In humans, the unique bioactivity of MCP against carcinogenesis is linked to sugar β-galactose inhibiting the cell signalling protein marker, galectin-3 (GAL-3) (11, 12). The degree of GAL-3 expression has been related to the stage of colonic cancer development in several animal models (13, 14). GAL-3 is intimately involved in endothelial cell morphogenesis and angiogenesis (15). Vascular endothelial growth factor (VEGF) receptors contribute to survival and proliferation of endothelial cells (16). The up-regulation of VEGF signalling can lead to intestinal inflammation and release of leukocytes in the vascular endothelium, thus upsetting the cytokine milieu in favour of cancer growth (16).

Azoxymethane (AOM) is a potent specific carcinogen used to induce colonic cancer in mice and rats (17, 18). Diverse dosage regimens of AOM administered through...
several routes have revealed aberrant crypt foci (ACF) in mice several weeks (12-36 weeks) after AOM initiation (19-22), with post-exposure subcutaneous doses of 15 mg/kg and intraperitoneal (i.p.) dose of 20 mg/kg being toxic (23, 24).

The bioactivity, bioavailability and uptake of MCP may be improved through a novel approach if conjoined with a supplement such as a probiotic. In an attempt to improve colonic cancer outcome via GAL-3 and VEGF inhibition, the aim of this study was to investigate the synergistic inhibitory effect of Lactobacillus acidophilus ATCC 4356 combined with alginate with/without MCP using a commercial representative preparation, PectaSol-C (ecoNugenics Inc., Santa Rosa, California, USA) to form biopolymer microbeads in a Balb/c mouse model of AOM-induced colonic carcinogenesis Preceding this investigation, we determined the optimal AOM dosage and duration of treatment that increased GAL-3 and VEGF immunoreactivity in the colon of the Balb/c mice.

Materials and Methods

Chemicals. AOM (13.4 Molarity, ≥98%), alginate sodium, chitosan, NaCl and glycerol (all analytical grades) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), De Mann-Rogosa-Sharpe (MRS) from Merck Biolab (Darmstadt, Germany), Hi-maize resistant starch from National Starch Food Innovation (Wadeville, South Africa) and canola oil from Willowton Group (KwaZulu-Natal, South Africa).

Animal model. After Institutional ethics approval (063/13/Animal and 084/14/Animal), this experiment was carried out at the Biomedical Research Unit (BRU), University of KwaZulu-Natal. The BRU-approved standard protocols for animal treatment were followed. Male Balb/c mice (i.e. aged 7 weeks) weighing 20-25 g were bred in-house under a controlled condition of humidity (50 ± 10%) and temperature (23±2˚C) on a 12 h light/12 h dark cycle with free access to water and food. During the experiment, mice were carefully observed for any toxic effect and abnormal behaviour. Additionally, their body weight was recorded.

Preparation of MCP alginate L. acidophilus ATCC 4356 microbeads. Microencapsulation of the probiotic was performed aseptically at room temperature. Frozen stock culture of L. acidophilus ATCC 4356 from Microbiologics (St. Cloud, MN, USA) was rehydrated and grown in MRS broth at 37˚C for 48 h. Fresh cell suspensions of about 9-10 log10cfu/g were prepared. Modified citrus pectin alginate (MCPA) and alginate calcium (AP) microbeads were produced using a modified emulsification method (25). A mixture of alginate sodium (2%), hi-maize resistant starch (2%) and MCP (8.5%) was agitated in distilled water for 10 min. Cell suspension of L. acidophilus ATCC 4356:canola oil–lecithin (1:10) was added to the mixture. This was adjusted to 300 ml with canola oil–lecithin (0.1%) and was then emulsified at a constant agitation of 1,400 rpm for 40 min. Calcium chloride (0.1 M) solution was added to the MCPA and AP microbeads were harvested and coated with chitosan. The coated microbeads were retrieved, washed and stored in sodium glycerol (0.9% NaCl, 5% glycerol) at 4˚C.

Table I. Category of treatments administered.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Modified citrus pectin alginate (MCPA) microbeads</td>
</tr>
<tr>
<td>2</td>
<td>Alginate calcium (AP) microbeads</td>
</tr>
<tr>
<td>3</td>
<td>Modified citrus pectin (MCP) solution</td>
</tr>
<tr>
<td>4</td>
<td>Water (Control)</td>
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Carcinogenic treatment of Balb/c mice. Experiment I: Selection of optimum dose duration of AOM. The study population (n=36) consisted of six groups of Balb/c mice (n=6 each). Groups 1 and 2 received 10 and 15 mg/kg AOM i.p., respectively, once a week for 2 consecutive weeks. Groups 4 and 5 received 10 and 15 mg/kg AOM i.p., respectively, once a week for 4 weeks. Group 3 and 6 (control mice) were injected with a 0.9% saline solution for 2 and 4 weeks, respectively. All groups were observed for 9, 12 and 16 weeks, at the end of which a colonic sample was obtained.

Experiment II: Investigation of chemopreventative efficacy of MCPA and AP microbead treatment. The optimum dose and time response selected from Experiment I was adopted in Experiment II. The total study population (n=40) consisted of four groups (n=10 each). Each group was administered 0.2 ml MCPA, AP probiotic microbeads, MCP or water, respectively for 16 weeks (Table I). Following euthanasia, macroscopic tumour detection and histopathological analysis of the entire gastrointestinal tract was performed. Immunohistochemistry for GAL-3 and VEGF was also performed.

Immunohistochemistry. Tissue preparation: The distal colonic tissues were fixed in 10% buffered formalin. Tissue processing was performed on a LEICA ASP 200S (SMM Instruments (Pty) Ltd., Midrand, South Africa) and wax embedding was carried out on a LEICA EG 1150H embedding station. Sections (3-5 μm) were cut on a rotary microtome (LEICA RM 2135) and collected onto poly-L-lysine-coated slides. This was followed by de-paraffinisation and rehydration in a descending series of ethanol.

Immunostaining: Immunostaining was performed using the EnVision™ FLEX reagents (Dako, Glostrup, Denmark) and EnVision™ FLEX staining technique. Sections were incubated in a preheat EnVision™ FLEX target antigen retrieval solution for 20 min at 95-99˚C. Endogenous peroxidase was blocked using 3% hydrogen peroxide blocking for 5 min. Post washing, sections were incubated at 4˚C in primary monoclonal antibodies (Mab) against GAL-3 (1:50 dilution, clone A3A12; Thermoscientific, Waltham, MA, USA) and VEGF (1:100 dilution, clone VG1; DAKO) for 24 h and 30 min, respectively. A pre-formed avidin biotinylated horseradish peroxidase macromolecular complex was then linked with a secondary antibody for 15 min at room temperature. Detection of reaction was carried out with diaminobenzidine as the chromogen. Slides were viewed with an Axioscope A1 photomicroscope (Zeiss, Gillitts, Durban, South Africa).

Semi-quantitative image analysis. Expression levels of GAL-3 Mab A3A12 and VEGF markers were based on the proportion of immunopositive cells in a field and the average staining intensity of immunopositive cells. The semiquantitative score adopted for the
percentage of immunopositive cells was: 0: 0-10%, 1: 10-39%, 2: 40-69% and 3: >70%; B, and for staining intensity was: 0: no staining, 1: weak staining, 2: moderate staining, and 3: strong staining.

**Data analysis.** The statistical package IBM®SPSS® (Statistics version 22 for Windows, Released 2013; IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Parametric and non-parametric data were used directly in analyses using the Kruskal–Wallis test and Mann–Whitney U-tests. Both Turkey’s test and analyses of variance (ANOVA) were used to compare expression of antibodies and probiotic treatments amongst groups. A value of *p*<0.05 indicated statistical significance.

**Results**

**Experiment I. Tumor type and incidence:** A high percentage of pre-cancerous lesions in the AOM-treated mice was recorded in the distal compared to the proximal region of the colon. Normal colon and ACF to microadenoma were noted (Figure 1). Crypts were found to be abnormally large with prominent nuclei. Moderately to severely dysplastic microadenoma was observed in which goblet cells were markedly reduced or absent. Colonic mucosal ulceration with focal dysplasia was marked with deep erosion inflammation mostly of crypt abscess formation. ACF developed in 4/12 (10 mg/kg) and 3/12 (15 mg/kg) AOM-treated mice, while microadenomas were observed in 5/12 mice treated with 15 mg/kg AOM; no microadenomas developed in mice treated with 10 mg/kg AOM. Almost 67% of mice in the group administered 15 mg/kg AOM for 4 weeks developed pre-cancerous tumors (*p*<0.05). The control group had morphologically normal colon with no signs of pathology.

**Immunostaining:** Gal-3 and VEGF immunostaining was observed in the colonic mucosae, crypts and endothelial cells of vessels within the lamina propria. The subsequent semi-quantitative evaluation of immunoeexpression of GAL-3 and VEGF in in the colon of AOM-treated Balb/c mice is shown.

*Figure 1. Colonic neoplasm from Balb/c mice treated with 15 mg/kg azoxymethane for 4 weeks. A: Normal colonic mucosa showing negative expression of galectin-3 (GAL-3). B: A single aberrant crypt at week 9 showing weak expression of GAL-3. C: Aberrant crypt foci (indicated by arrows) at week 12 showing moderate expression of GAL-3. D: Microadenomas invading the submucosa at week 16 showing moderate expression of GAL-3.*
in Figures 2 and 3. At week 9 (treatment + observation) of carcinogenesis, the expression of GAL-3 in the groups treated with AOM for 2 weeks was weakly positive. Immunoreactivity, however, varied from moderate to strongly immunopositive in the groups treated for 4 weeks. At week 12 (treatment + observation), although GAL-3 immunopositivity was observed in groups treated with AOM at both 10 and 15 mg/kg (2 and 4 weeks), it was not intense. At week 16 (treatment + observation), GAL-3 was more obvious in the superficial crypts of the colonic mucosa (Figure 4). Immunostaining of GAL-3 was intense in 25% of the mice which were dosed with 15 mg/kg AOM- and 17% of the 10 mg/kg AOM (4 weeks)-treated groups \((p<0.05)\). The expression of GAL-3 in the control group was weakly-positive in 33% and negative in 67% of the mice. The goblet cells in the colonic mucosa were not stained in AOM-treated nor control groups.

The immunostaining of VEGF in both AOM-treated groups increased progressively between weeks 9-16, with the results ranging from weak to strongly positive (Figure 5). VEGF expression in both AOM-treated groups was intensely positive in 50% of the colonic tissue. A weakly immunopositive reaction was observed in 50% of mice which were dosed with 15 mg/kg AOM- and 33% of 10 mg/kg AOM-treated groups \((p<0.05)\). None of the mice within the control group showed an intense immunopositivity for VEGF. There was no animal death during the experiment.

Experiment II. General observation: The body weight of mice in the MCPA/AP probiotic, MCP-treated and control groups increased with no significant difference \((p>0.05)\). Some mice from the treatment and control groups developed dim vision and eye inflammation. Vision was improved and inflammation was reduced in the probiotic and MCP-treated groups, while the control group showed no signs of improvement. No rectal bleeding was observed and animal behaviour did not change in the course of probiotic treatment.

Tumor incidence: The percentage of colonic pre-cancerous lesions in the control (untreated) and MCP-treated groups was high compared to the MCPA and AP probiotic-treated groups. The lowest percentage of lesions was observed in the MCPA-treated group (20%) compared to AP- and MPC-
treated groups (40-50%). However, there was significant reduction of tumour in the treated groups compared to the control group ($p<0.05$).

**Immunostaining:** The immunoexpression of GAL-3 and VEGF after MCPA, AP probiotic and MCP treatments is shown in Table II and Figures 6 and 7. In the MCPA probiotic-treated group, the immunoexpression of GAL-3 in the crypts and endothelium was almost negative, with 15-20% of the crypt cells barely stained. The epithelium of the villi was not stained, while that close to the sub-mucosa was faintly positive. In the case of AP probiotic- and MCP-treated groups, about 30% of the crypts and the epithelial luminal surface were weakly immunopositive. The intensity of GAL-3 was less in the crypts of the MCP-treated group. Conversely, almost the entire crypt and endothelium were strongly immunopositive in the control group. Treatment with MCPA, AP probiotic, and MCP, reduced the expression of GAL-3 in AOM-treated mice in comparison to the control group.

The immunoexpression of VEGF in the MCPA- and AP probiotic-treated groups was weakly positive. Although the staining intensity of the endothelial cells in the MCP-treated group was barely positive, the percentage of positive cells was less than 10% in both AP probiotic- and MCP-treated groups. VEGF expression in the control group was intense.

**Discussion**

The optimal dose of AOM required to induce colonic carcinogenesis in mice is dependent on the genetic constitution of the rodent strain, as well as environmental factors (20, 23-24, 26). Previous studies have reported the histological progression of colonic cancer in AOM-treated rodents (27). There is however, an urgent need to standardize the dosage of AOM used to induce colonic cancer. This novel study utilised GAL-3 and VEGF markers to determine the optimal AOM dose required to initiate colonic carcinogenesis in a mouse model.

In experiment I of our study, intense immunostaining of GAL-3 and VEGF in the colon of Balb/c mice was observed after 16 weeks of AOM treatment. This optimal and tolerable dosage was equivalent to 15 mg/kg AOM administered i.p. once every 4 weeks. However, in Wistar rats, a similar dose of
1,2- dimethylhydrazine administered for 8 weeks led to strong immunoeexpression of GAL-3 and development of dysplastic ACF at week 32 of colonic carcinogenesis (27). The survival of all animals in our model indicates that both dose regimens (10 mg/kg and 15 mg/kg) were non-toxic to the Balb/c mice. In our study, the strong expression of GAL-3 in both the mucosal crypts and blood vessels within the lamina propria of AOM-treated mice implies the absorption and elevated free circulation of the protein marker associated with disease progression. Evidently, the distribution and immunoexpression of GAL-3 in colonic carcinogenesis is dependent on the stage of cancer and metastasis. Thus, our results corroborate the association of immunoeexpression of GAL-3 with the severity of the colonic lesion.

Whilst some studies report a decline in GAL-3 expression during the development of colonic cancer, others show an increase in GAL-3 expression (28, 29). Our study found that there was a significant increase of GAL-3 in the AOM-treated groups at weeks 9 and 16 (p<0.05). However, there was no significant difference between weeks 9 and 16. This advocates a prognostic significance of GAL-3 as a reliable biomarker for colorectal cancer detection. A limitation to this, however, is the need for in vivo sampling at different time intervals in order to confirm disease prognosis. Hence, the prognostic value of GAL-3 as an indicator of colorectal cancer remains uncertain.

Similarly, in the present study, VEGF immunoexpression was up-regulated with tumor progression. The incidence of tumor formation has been associated with elevated expression of GAL-3 and VEGF; consequently they are both good indicators of cancer cell proliferation and angiogenesis (27, 30). VEGF expression varies in that some studies show the presence of VEGF after tumor development, whilst others show VEGF expression before detection of ACF (20). Our study demonstrates an association between GAL-3 and VEGF expression at the early stage of colonic carcinogenesis.

An increased concentration of free GAL-3 in the circulating blood of patients with colonic cancer has been...
reported, thereby contributing to the rapid metastatic spread of cancerous cells (31). Circulating GAL-3 has the ability to promote metastatic cell proliferation (31). Cell surface GAL-3 can interact with laminin and promote tumor cell release from the primary site (32). The overexpression of cell surface-associated extracellular GAL-3 in epithelial cells can trigger cancer cell interaction by binding glycoconjugates to integrins, thereby activating intracellular VEGF signaling.

Table II. Expression of immunohistochemical markers galectin-3 (GAL-3) and vascular endothelial growth factor (VEGF) in the Balb/c mouse model of colonic carcinogenesis at 16 weeks. Data are mean values.

<table>
<thead>
<tr>
<th>Group</th>
<th>GAL-3</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crypt</td>
<td>Endothelium</td>
</tr>
<tr>
<td>MCPA (N=10)</td>
<td>0.4</td>
<td>1.3</td>
</tr>
<tr>
<td>AP (N=10)</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>MCP (N=10)</td>
<td>0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Control (N=10)</td>
<td>2.6</td>
<td>2.6</td>
</tr>
</tbody>
</table>

MCPA: Modified pectin alginate + probiotic, AP: alginate + probiotic, MCP: modified citrus pectin. Score: Percentage of positively stained cells (0: 0-10%, 1: 10-39%, 2: 40-69% and 3: >70%), SI: staining intensity (0: no staining, 1: weak staining, 2: moderate staining and 3: strong staining).

Figure 5. Vascular endothelial growth factor (VEGF) immunoeexpression in the Balb/c mouse model of colonic carcinogenesis at week 12 (A, B) and week 16 (C, D). The arrows indicate stained endothelial cells located at the submucosa muscularis.
In the present study, 12 weeks after the initiation of AOM in the mice, there was an elevated expression of GAL-3 together with an up-regulation of VEGF in the colonic tissue compared to the control group.

With regards to experiment II, the administration of the MCPA probiotic microbeads to our mouse model demonstrated a reduction of GAL-3 and VEGF immunoexpression with lower tumor incidence. Likewise, the AP probiotic microbeads also inhibited GAL-3 expression during carcinogenesis, together with a low incidence of precancerous lesions. The combination of the probiotic and MCP was more effective in reducing GAL-3 immunoreactivity in colonic carcinogenesis than both the single therapy of MCP and AP probiotic alone. Furthermore, in all three treatment regimens, immunoreexpression of GAL-3 was lower in the mucosal cells of the crypts compared to the endothelial cells. The immunoreexpression of VEGF ranged from low in the colon of mice receiving the MCPA treatment to low/absent in the AP- and the MCP-treated groups. This is indicative of MCP bioactivity regulating cell proliferation and angiogenesis in cancer progression. Azémar et al. demonstrated the inhibition of tumour-associated angiogenesis by MCP in an animal model (33).

In the present study, the staining pattern of GAL-3 was cytoplasmic. GAL-3 is synthesized as a cytosolic protein that is translocated to the mitochondria and acts as an inhibitor of apoptosis (programmed cell death) (34-35). Both reactive oxygen species and antioxidant properties of probiotics play a key role in the prevention of colonic cancer (36). The reduction of GAL-3 expression in the MCPA- and AP-treated groups may be a function of their apoptotic activity. The availability of orally-ingested MCP and resistant starch in the MCPA may influence the glycolytic and metabolic activities of the *L. acidophilus* ATCC 4356 in the gastrointestinal tract. This leads to increased production of ATP, which stabilizes the mitochondria by reducing oxidative damage and the release of apoptotic molecules, thereby maintaining colonic mucosal integrity.

GAL-3 inhibits the inflammatory response of the intestinal system via gut-associated lymphoid tissue (GALT). GALT
modulates macrophage signalling recruitment (37). Additionally, GAL-3 can bind to cell-surface receptors creating a clustering effect (32). Consequentially, a higher concentration of GAL-3 at this binding site activates T-cells, with possible evasion of the immune surveillance system. GAL-3 has three domains namely the -NH2 and -COOH terminals and Asp-Trp-Gly-Arg (NWGR) anti-death motif. The β-1,4-galactan of the neutral sugar chain in MCP binds specifically to the COOH terminal carbohydrate-recognition domain of cytoplasmic GAL-3 (11). However, the S-glycoprotein layer of *L. acidophilus* ATCC 4356 also has both amino and carboxyl terminal domains (38), which would compete to bind to the GAL-3 -COOH terminal. The initiation of colonization/adhesion of the probiotic bacteria to specific receptors on the epithelial cell surface of the colon may competitively inhibit extracellular matrix interactions of GAL-3 in addition to the MCP–GAL-3 binding. Extracellular macromolecules or exopolysaccharides synthesized by the bacteria may also contribute to modulating the immune response (39, 40). Biopolymers and prebiotic agents' such as the modified pectin, alginate and resistant starch, may also modulate intestinal homeostasis (41). MCPA ensures the viability of the probiotic bacteria to the point of target delivery (colon), thus its efficiency is significant. It is plausible to assume that in addition to the protective role of MCPA as a biopolymer, it also modifies the functionality and physiological properties of the probiotic during gastric transit or adhesion/colonization (personal communication, Mellem and Reddy, 2015). The total number of faecal lactobacilli detected after oral administration of the MCPA *L. acidophilus* ATCC 4356 microbeads in AOM-treated Balb/c mice increased significantly by 10% (0.8±0.08 log10 cfu/ g) (p<0.05) from the initial faecal count after 4 weeks of daily MCPA (42).

Tumors produce proteins or hormones that circulate and spread to other tissues or organs away from the initial site, thus initiating a paraneoplastic syndrome. The blurred vision or the loss of vision acuity, as was observed in our Balb/c
mouse model, has also been noted in human studies and is indicative of an early sign of cancer development (43). In our study, the uptake and bioavailability of MCPA reduced the blurred vision, probably via an autoimmune response. However, the bioavailability of MCPA was higher than MCP. The uptake of β-glycan by macrophages has been suggested as the proposed mechanism of MCP absorption (11, 44). Orally ingested MCPA modifies the functional properties of L. acidophilus ATCC 4356 cell envelope. The L. acidophilus ATCC 4356 surface-associated proteins interacting with MCPA may easily be internalised by the intestinal epithelial cell or GALT (macrophages), thus, improving the bioavailability and the anticancer effect of MCP.

In conclusion, this novel study utilised the combination of a probiotic L. acidophilus ATCC 4356 and MCP with alginate as a chemopreventative cancer therapy. The chemopreventative ability of MCPA is significantly demonstrated in this study via the inhibition of GAL-3 and VEGF immunoexpression in the Balb/c mouse model of AOM-induced colonic cancer. MCPA probiotic dramatically inhibits precancerous lesions. This inhibition is associated with reduced cell proliferation and angiogenesis. MCPA has a good biodegradable ability, is inexpensive, non-toxic, has a proven efficiency, is easy to use and is stable at low temperatures, warranting its use as a drug carrier by pharmaceuticals. Further studies will clarify the mode of action of MCPA probiotic in vivo cancer and preclinical models.

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References