

Oral inoculation of probiotics *Lactobacillus acidophilus* NCFM suppresses tumour growth both in segmental orthotopic colon cancer and extra-intestinal tissue

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Abstract

Modulation of the cellular response by the administration of probiotic bacteria may be an effective strategy for preventing or inhibiting tumour growth. We orally pre-inoculated mice with probiotics *Lactobacillus acidophilus* NCFM (*La*) for 14 d. Subcutaneous dorsal-flank tumours and segmental orthotopic colon cancers were implanted into mice using CT-26 murine colon adenocarcinoma cells. On day 28 after tumour initiation, the lamina propria of the colon, mesenteric lymph nodes (MLN) and spleen were harvested and purified for flow cytometry and mRNA analyses. We demonstrated that *La* pre-inoculation reduced tumour volume growth by 50.3%, compared with untreated mice at 28 d after tumour implants (2465.5 (SEM 1290.4) v. 4950.9 (SEM 1689.3) mm³, $P < 0.001$). Inoculation with *La* reduced the severity of colonic carcinogenesis caused by CT-26 cells, such as level of colonic involvement and structural abnormality of epithelial/crypt damage. Moreover, *La* enhanced apoptosis of CT-26 cells both in dorsal-flank tumour and segmental orthotopic colon cancer, and the mean counts of apoptotic body were higher in mice pre-inoculated with *La* ($P < 0.05$) compared with untreated mice. *La* pre-inoculation down-regulated the CXCR4 mRNA expressions in the colon, MLN and extra-intestinal tissue, compared with untreated mice ($P < 0.05$). In addition, *La* pre-inoculation reduced the mean fluorescence index of MHC class I (H-2Dd, -Kd and -Ld) in flow cytometry analysis. Taken together, these findings suggest that probiotics *La* may play a role in attenuating tumour growth during CT-26 cell carcinogenesis. The down-regulated expression of CXCR4 mRNA and MHC class I, as well as increasing apoptosis in tumour tissue, indicated that *La* may be associated with modulating the cellular response triggered by colon carcinogenesis.

Key words: Probiotics: *Lactobacillus acidophilus* NCFM: CT-26 cells: Colon carcinogenesis: Apoptosis

Colon carcinoma is a leading cause of digestive system neoplasm. The colon cancer mortality rate is second only to that of lung cancer in men and breast cancer in women, and colon cancer rates have increased over the past 20–30 years⁽¹⁾. Diet contributes to colon cancer risk. In fact, up to

75% of cases are thought to be associated with diet⁽²⁾, indicating that a person can reduce his or her colon cancer risk simply via diet modification.

Probiotics consist of a preparation of viable micro-organisms that alter the existing microflora of the intestine, thereby

Abbreviations: CT-26 alone, mice were implanted with CT-26 cells after 14 d of PBS inoculation; CT-26 + *Ec*, mice were pre-inoculated with *Escherichia coli* K12 1×10^8 colony-forming units/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells; CT-26 + *La*, mice were pre-inoculated with *Lactobacillus acidophilus* NCFM 1×10^8 colony-forming units/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells; *Ec*, *Escherichia coli* K12; *La*, *Lactobacillus acidophilus* NCFM; LP, lamina propria; MLN, mesenteric lymph node.

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exerting beneficial health effects on the host by modulating one or several components of humoral, cellular or non-specific immunity⁽³⁾. Studies have suggested that utilisation of lactobacilli in foodstuffs and medicines prevents infection by pathogenic bacteria^(4,5) as well as cancer formation^(6,7). Among the common bacteria that reside in the colon, bifidobacteria and lactobacilli, in particular, are thought to have beneficial effects in humans⁽⁸⁾; however, the precise mechanisms by which these organisms exert anti-tumorigenic effects are uncertain. Probiotics may retard colon carcinogenesis by influencing metabolic, immunological and protective functions within the colon, and it is possible that they may stimulate tumour cell apoptosis. Apoptosis is an active cellular process in which individual cells are triggered to undergo self-destruction. It has been well documented that tumour cell apoptosis blocks tumour progression⁽⁹⁾. However, whether and how probiotics affect tumour cell apoptosis remains unclear; supplementation with probiotics may be an effective approach to preventing colon carcinogenesis⁽¹⁰⁾.

To determine whether probiotics influence colon carcinogenesis, we utilised a CT-26 colon carcinoma animal model. CT-26 cells are *N*-nitroso-*N*-methylurethane-induced murine colon adenocarcinoma cells derived from BALB/cByJ mice. CT-26 cells are ideal for modelling colon cancer both *in vivo* and *in vitro*^(11,12). We hypothesised that *Lactobacillus acidophilus* NCFM (*La*) enhances apoptosis in colon tumour cells to inhibit colon carcinoma growth. Thus, we analysed the effect of probiotics on tumour volume and CT-26 apoptosis. The present study demonstrated that pre-inoculation with *La* can retard tumour growth and promote apoptosis in CT-26-derived adenocarcinomas *in vivo* via the modulation of anti- and pro-apoptotic protein expression. The present data indicate that probiotic supplementation may represent an effective approach to preventing or inhibiting colon carcinogenesis.

CT-26 colon carcinoma is a metastatic murine tumour that, at late stages of tumour development, metastasises to other tissues, such as spleen, liver and kidneys. Chemokine receptors are not only expressed by leucocytes, but also by epithelial cells and several types of carcinomas⁽¹³⁾. CXCR4, the chemokine receptor for CXCL12, has recently been shown to be involved in the metastatic processes of several neoplasms. CXCR4 is overexpressed in human colon cancer tissue and murine cancer cells (such as CT-26), compared with normal mucosa and benign lesions^(14,15). Here, we determined the role of CXCR4 in metastatic formation by CT-26 colon carcinoma cells, and examined whether the expression of CXCR4 can be suppressed by probiotics.

Most tumour-associated antigens known today were identified by their ability to induce cellular responses, predominantly those mediated by cytotoxic T-lymphocytes. Cytotoxic T-lymphocytes recognise short peptides encased in a designated pocket formed by MHC class I molecules, which are expressed by most nucleated cells in the body, including tumour cells^(16–18). In the present study, we explored whether there was a change in the surface expression of MHC class I molecules in colon carcinogenesis caused by CT-26 cells, and whether the expression may be suppressed by probiotics.

The overall objective of the present study was to determine whether probiotics are a beneficial supplement during colon carcinogenesis. Specifically, we evaluated the role of probiotics in the reduction of tumour volume and determined the preventive effects of probiotics on intestinal neoplasm in an *in vivo* animal model. We observed that repeated oral administration of probiotics *La* before CT-26 cell implantation showed an attenuated effect on carcinogenesis, which is associated with enhanced apoptosis in tumour tissues.

Materials and methods

Mice and diet

Female BALB/cByJ mice, 4–6 weeks old, were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and maintained at Chang Gung Memorial Hospital (Taoyuan, Taiwan). Mice were fed autoclaved food (the standard diet containing 53% of carbohydrate, 20.5% of protein, 18.5% of fat, 4.8% of mineral and 2.7% of fibre, Picolab Mouse Diet 20; PMIEnter National Nutrition, Taipei, Taiwan) and water *ad libitum*. The study was approved by the Chang Gung Memorial Hospital Institutional Animal Care and Use Committee.

In vivo experimental design

Mice were initiated into the study when they were 6 weeks old and body weight was over 20 g. Female mice were randomised into four groups (the number of animals per group is given in the Results section and figure legends). Experimental mouse groups included: BALB/c mice implanted with 5×10^6 CT-26 cells (ATCC CRL-2638; Manassas, VA, USA) after 14 consecutive days of inoculation with PBS ingestion (mice were implanted with CT-26 cells after 14 d of PBS inoculation, CT-26 alone); BALB/c mice pre-inoculated with *La*, a probiotic bacteria for 14 consecutive days before implantation with 5×10^6 CT-26 cells (mice were pre-inoculated with *L. acidophilus* NCFM 1×10^8 colony-forming units/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells, CT-26 + *La*); BALB/c mice pre-inoculated with *Escherichia coli* K12 (*Ec*), a commensal intestinal bacteria for 14 consecutive days before implantation with 5×10^6 CT-26 cells (mice were pre-inoculated with *Escherichia coli* K12 1×10^8 colony-forming units/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells, CT-26 + *Ec*); untreated BALB/c mice (untreated control).

Colon carcinoma cells

CT-26 colon cancer cell lines were purchased from American Type Culture Collection (ATCC CRL-2638; Manassas, VA, USA). The cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Bioind, Kibbutz, Beit Haemek, Israel) at 37°C in 5% CO₂. The cells were used at 75–85% confluence.

Bacterial preparation and inoculation

La (Rhodia Inc., Madison, WI, USA) was inoculated in deMan, Rogosa and Sharpe broth (Difco, Sparks, MD, USA), and *Ec* (BCRC 12 238, ATCC 27 325; Hsinchu, Taiwan) was inoculated in Luria Bertani broth (Bio Basic, Markham, ON, Canada). Bacterial cultures were grown at 37°C for 20 h, after which they were resuspended in PBS before inoculation into mice. *La* and *Ec* were administered intragastrically using a ball-tipped feeding needle at 1×10^8 colony-forming units/mouse. Mice were inoculated daily for 14 consecutive days before cancer cell implantation, after which they were inoculated at 1×10^9 colony-forming units/mouse weekly for 3 weeks. The equal volume of PBS was administered intragastrically into the mice (CT-26-alone group) using a ball-tipped feeding needle during the experimental period.

Tumour models

Subcutaneous primary tumours. Subcutaneous tumours were initiated by injecting 5×10^6 viable CT-26 cells suspended in 100 μ l of serum-free medium into the right flank of female BALB/cByJ mice. Injections were performed at a slow rate with a 30-gauge needle. Tumour growth was assessed every 3 d. Tumour volume was measured with vernier calipers and calculated using the equation: tumour volume (mm^3) = $0.52 \times \text{length} \times \text{width}^2$.

Segmental orthotopic colon cancer. Female BALB/cByJ mice were anaesthetised by the intraperitoneal injection of ketamine (50 mg/kg for mouse; NK, Tainan, Taiwan). Then, a midline incision was made and a non-traumatic clamp (Fine Science Tools, Foster City, CA, USA) was positioned on the colon. A polyethylene catheter was inserted rectally and the isolated portion of the colon was washed twice with 500 μ l PBS to remove bowel contents. With the catheter in place, a second clamp was applied to the colon 1 cm distal to the first one. The second clamp encompassed both the colon wall and the tube, creating a 1 cm-long closed bowel loop⁽¹⁹⁾. CT-26 cells (5×10^6) suspended in 100 μ l Hank's balanced salt solution were injected into the colon lumen. After the catheter was removed, the cells were not disturbed for 10 min to permit implantation. Both clamps were then removed, and the abdominal wall was closed in a two-layer fashion. Total operation time, including incision, catheter placement, cell delivery and wall closure, was approximately 20 min/mouse. The remaining groups, including controls, were operated on using the same surgical technique with PBS used in the last 10 min incubation period instead of CT-26 cells. At 28 d after tumour initiation, mice were killed. Multiple tumours were measured in each mouse. To measure tumour volume, tumour diameter was obtained three times per tumour with a caliper and the values were summed to determine the overall volume.

Extra-intestinal metastasis model. CT-26 cells (5×10^6) suspended in 100 μ l Hank's balanced salt solution were injected into the female BALB/cByJ mice (for the groups CT-26, CT-26 + *La* and CT-26 + *Ec*) via the intravenous route. To establish spleen and liver metastasis, CT-26 cells were

injected into the spleen and portal vein of mice under diethyl ether anaesthesia after an abdominal incision was made.

Histological examination and terminal deoxynucleotidyl transferase dUTP nick end labelling staining

At 28 d after tumour initiation, mice were killed. Tumour and colon tissues were harvested and stained with haematoxylin and eosin. To visualise apoptosis, the terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling assay was carried out using the Apo-BrdU-IHC™ *in situ* DNA Fragmentation Assay Kit (BioVision, Mountain View, CA, USA), according to the manufacturer's instructions. The number of apoptotic bodies was calculated using at least ten different high-power fields per slide. Histological assessment was performed in a blinded fashion using a scoring system as described in the following: the level of colonic involvement (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural); increase in nuclear:cytoplasmic ratio in cells (0, 0–25%; 1, 25–50%; 2, >50%); structural abnormality of epithelial/crypt damage (0, none; 1, basal 1/3; 2, basal 2/3; 3, crypt loss; 4, crypt and surface epithelial destruction). The above category was summed to obtain the overall score.

Annexin-V/propidium iodide double-staining apoptosis assay

Tumour tissues were cut into small pieces and smashed with disposable pestles. The homogenised tissue was mixed with Dulbecco's modified Eagle's medium and passed through a 70 μ m strainer to produce the single-cell suspensions. The cell suspensions were washed and resuspended in PBS buffer. The cells were resuspended in pre-diluted binding buffer, adjusting to a cell density of $2-5 \times 10^5$ cells/ml. Apoptotic cells were then identified by double supravital staining with recombinant fluorescein isothiocyanate-conjugated Annexin-V and propidium iodide, using the Annexin V-fluorescein isothiocyanate apoptosis detection kit (AbD Serotec, Kidlington, Oxford, UK), according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Western blot analysis

Tumours were cut into small pieces and lysed in 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) buffer (0.5% CHAPS, 10 mM-Tris-HCl, pH 7.5, 1 mM-MgCl₂, 1 mM-ethylene glycol tetraacetic acid, 5 mM-mercaptoethanol, 10% glycerol and 0.1 mM-phenylmethanesulfonyl fluoride) for 30 min on ice. Then, 30 μ g of total protein from each treatment were prepared and separated on 10% SDS-polyacrylamide mini gels for Bcl-2, caspase-3, caspase-9 and β -actin detection. Proteins were transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) and incubated overnight with Bcl-2 (catalogue no. 04-436), caspase-3 (catalogue no. 06-735), caspase-9 (catalogue no. 04-444) and β -actin (catalogue no. MAB1501) antibodies (Millipore, Temecula, CA, USA).

Immunoreactive protein was visualised using the Fluorchem imaging system (Alpha Innotech, San Leandro, CA, USA).

Determination of chemokine mRNA expressions using RT-PCR

Total RNA was isolated from various organs (isolated from the mesenteric lymph nodes (MLN), spleen, liver and the lamina propria (LP) of the colon) using RNazol (Life Technologies, Carlsbad, CA, USA) and was used for complementary DNA synthesis. Complementary DNA were used as templates for PCR using specific primers (forward 5'-GGTCTGGAGACTA-TGACTCC-3', reverse 5'-CACAGATGTACCTGTCATCC-3') and probes (5'-(FAM)-TCTGGATCCCAGCCCTCCTCTG-(TAMRA)-3') for mouse CXCR4. Specific primers and probes for mouse β -actin were as follows: forward 5'-CGTGAAAAGATGACCCAG-ATCA-3', reverse 5'-TGGTACGACCAG-AGGCATACAG-3'; probe 5'-(FAM)-TCAACACCCCAGCCATGTACGTAGCC-(TAMRA)-3'. The reference of specific primers and probes was mouse CXCR4 (Mm01292123_m1) and β -actin (Mm00607939_s1; Applied Biosystems, Foster City, CA, USA). The results were normalised to β -actin expression.

Cell surface phenotypes

Lymphocyte suspensions were prepared from the MLN and spleen as described previously⁽²⁰⁾. Each colon of BALB/c mice was flushed with Hanks' balanced salt solution, cut longitudinally, and the gut epithelium removed from the LP as described previously⁽²¹⁾. Lymphocytes were isolated from the MLN, spleen and the LP of the colon in the various groups of mice, and were stained using a panel of monoclonal antibody directed against H-2Dd (catalogue no. 110607), -Kd (catalogue no. 116607) and -Ld (catalogue no. 114507) (all phycoerythrin-conjugated; Biolegend, San Diego, CA, USA). Cells were acquired (at least 10 000 events for the MLN and 30 000 events for the spleen and the LP) using a FACScan (Becton Dickinson, San Jose, CA, USA) and analysed with Cell Quest software.

Statistical analysis

All data are presented as means with their standard errors of the mean. Statistical comparisons were analysed using one-way ANOVA (GraphPad Prism software; La Jolla, CA, USA) and SPSS 16.0 (Chicago, IL, USA). Tumour volume data were statistically analysed using two-way ANOVA. *P* values <0.05 were considered significant.

Results

Probiotic Lactobacillus acidophilus pre-inoculation suppresses tumour volume growth of murine CT-26 colon adenocarcinoma

Subcutaneous tumour implantation of CT-26 cells in BALB/c mice resulted in rapid tumour development. Thus, we hypothesised that inoculation with probiotics after tumour initiation would not suppress tumour growth, while inoculation with *La* before tumour initiation would lead to the establishment of

adequate intestinal colonisation that is required for the anti-carcinogenic effect. Consistent with our hypothesis, we observed significantly smaller tumour size in animals pre-inoculated with *L. acidophilus* (Fig. 1). At 21 d post-tumour implantation, we saw a 35.5% reduction in mean tumour volume in mice that received 14 d of oral inoculation with *La* before implantation of CT-26 cells (CT-26 + *La* group) compared with untreated mice (CT-26 group) (1350.5 *v.* 2210.9 mm³, respectively; *P*<0.05). Furthermore, by 24 d post-tumour implantation, pre-inoculation with *La* restrained tumour growth by 41.8% compared with mice implanted with CT-26 cells alone (2320.3 *v.* 3984.9 mm³, respectively; *P*<0.05). Strikingly, an even more pronounced decrement (about 50.3%) in mean tumour volume in *La*-treated mice was detected at 28 d post-tumour implantation (2465.5 (*La* pre-inoculation) *v.* 4950.9 mm³ (CT-26 cells alone); *P*<0.001; Fig. 1). The above data shown are pooled from three independent experiments with a total of ten to fifteen animals per group.

Lactobacillus acidophilus pre-inoculation results in smaller macroscopic tumour size and apoptosis of some tumour cells

At 28 d after CT-26 cell implantation, tumours were resected and histological analysis was performed on dorso-lateral flank tumours (Fig. 2(a)–(c)). We observed increased tumour cell apoptosis in the CT-26 + *La* group compared with CT-26 and CT-26 + *Ec* tumours (Fig. 2(b)). Tumour cell apoptosis was also assessed using terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling staining (Fig. 2(e)–(g)). The number of apoptotic bodies was significantly higher in CT-26 + *La* tumours compared with CT-26 and CT-26 + *Ec* tumours (Fig. 2(d) and (h)), suggesting that pre-inoculation with *La* induces apoptosis in CT-26 cell-derived tumours. The number of apoptotic bodies was calculated for at least ten different high-power fields. We found that the mean counts were higher in mice pre-inoculated with *L. acidophilus* and implanted with CT-26 cells compared with the other groups. We suppose that probiotics *La* showed its effect on cell apoptosis and anti-proliferation in CT-26 cells, and so results in smaller macroscopic tumour size.

Lactobacillus acidophilus pre-inoculation may retard tumour growth in a mouse model of segmental orthotopic colon cancer

To explore the effect of probiotics on the tumour growth of intestinal tissue, we performed a surgical technique to establish a mouse model of segmental orthotopic colon cancer. The tumours in the orthotopic model were multiple colonic tumours with mesentery tissue involvement, especially in mice implanted with CT-26 cells alone. The number of tumours per mouse was ranged from one to sixteen sites, and all tumours were measured except that the diameter was less than 5 mm. The overall tumour size of multiple sites was determined by measuring three tumour diameters of each tumour with a caliper and summing to obtain the overall volume of each mouse. CT-26 + *La* tumour volume was significantly reduced (2654.5



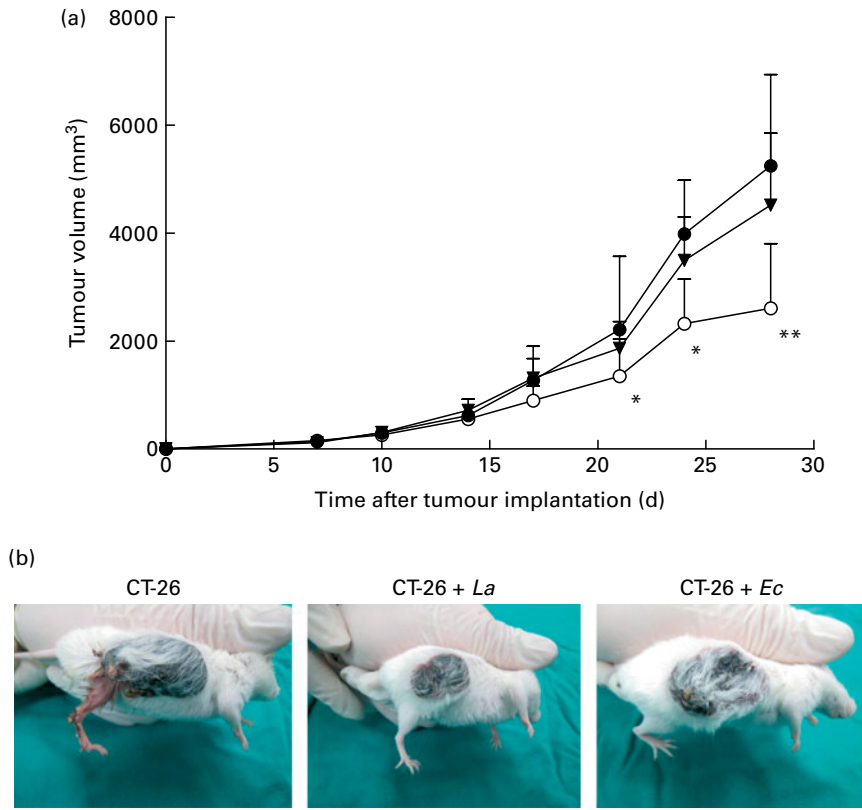


Fig. 1. *Lactobacillus acidophilus* NCFM (*La*) pre-inoculation decreased subcutaneous CT-26 tumour growth. Tumour size was measured every 3 d using calipers. (a) Tumour volumes were determined for CT-26 (●—), CT-26 + *La* (○—) and CT-26 + *Ec* (▼—) mice. Values are means of data pooled from three independent experiments with a total of ten to fifteen animals per group, with standard errors of the mean represented by vertical bars. Mean values were significantly different from those of CT-26 tumours: * $P < 0.05$, ** $P < 0.001$. (b) Representative CT-26, CT-26 + *La* and CT-26 + *Ec* tumours are shown. CT-26 alone, mice were implanted with CT-26 cells after 14 d of PBS inoculation; CT-26 + *La*, mice were pre-inoculated with 1×10^8 colony-forming units (cfu)/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells; CT-26 + *Ec*, mice were pre-inoculated with *Escherichia coli* K12 1×10^8 cfu/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells.

(SEM 154.9) mm³) compared with CT-26 + *Ec* (3918.7 (SEM 264.8) mm³) or CT-26 alone (4198.6 (SEM 361.5) mm³, $P < 0.05$; Fig. 3(a)–(c)), suggesting that pre-inoculation with *La* can retard the tumour growth of murine CT-26 adenocarcinoma. Histological pathology of the tumour on segmental colonic tissue with epithelial/crypt involvement was examined for haematoxylin and eosin staining (Fig. 3(d)–(g)). Further analysis of the structural abnormality of epithelial/crypt damage, and the nuclear:cytoplasmic ratio in cells revealed lower histopathology scores in CT-26 + *La*, compared with CT-26 + *Ec* or CT-26 alone (Fig. 3(h)). The above data shown are pooled from three independent experiments with a total of ten to fifteen animals per group.

***Lactobacillus acidophilus* pre-inoculation enhances apoptosis of some tumour cells**

Histological pathology of the tumours of segmental colon tissues of BALB/c mice was examined for terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling staining (Fig. 3(j)–(m)). The number of apoptotic bodies was calculated for at least ten different high-power fields. We found that the mean counts were higher in mice

pre-inoculated with *L. acidophilus* and implanted with CT-26 cells compared with the other groups (Fig. 3(i)).

***Lactobacillus acidophilus* NCFM treatment regulates the expression of apoptosis-regulating proteins in CT-26 adenocarcinomas**

To determine how the *La* treatment leads to CT-26 cell apoptosis, we examined annexin-V and propidium iodide staining in colon carcinomas (Fig. 4(a)). We found that CT-26 + *La* tumours displayed increased apoptosis compared with CT-26 + *Ec* and CT-26 tumours (10.8 (SEM 2.3) v. 3.7 (SEM 1.8) and 2.8 (SEM 1.4) %, respectively).

To examine the protein levels of Bcl-2, caspase-3, caspase-9 and β -actin, Western blot analyses were performed (Fig. 4(b) and (c)). Bcl-2 is an oncoprotein that inhibits apoptosis of tumour cells. Caspase-9 is thought to be an initiator caspase, whereas caspase-3 is considered to be a death effector of tumour cells. Bcl-2 protein levels were lower in CT-26 + *La* tumours compared with CT-26 + *Ec* and CT-26 tumours (Fig. 4(b)). In addition, caspase-9 and caspase-3 levels were higher in CT-26 + *La* tumours compared with CT-26 + *Ec* and CT-26 tumours (Fig. 4(c)). Taken together, these data

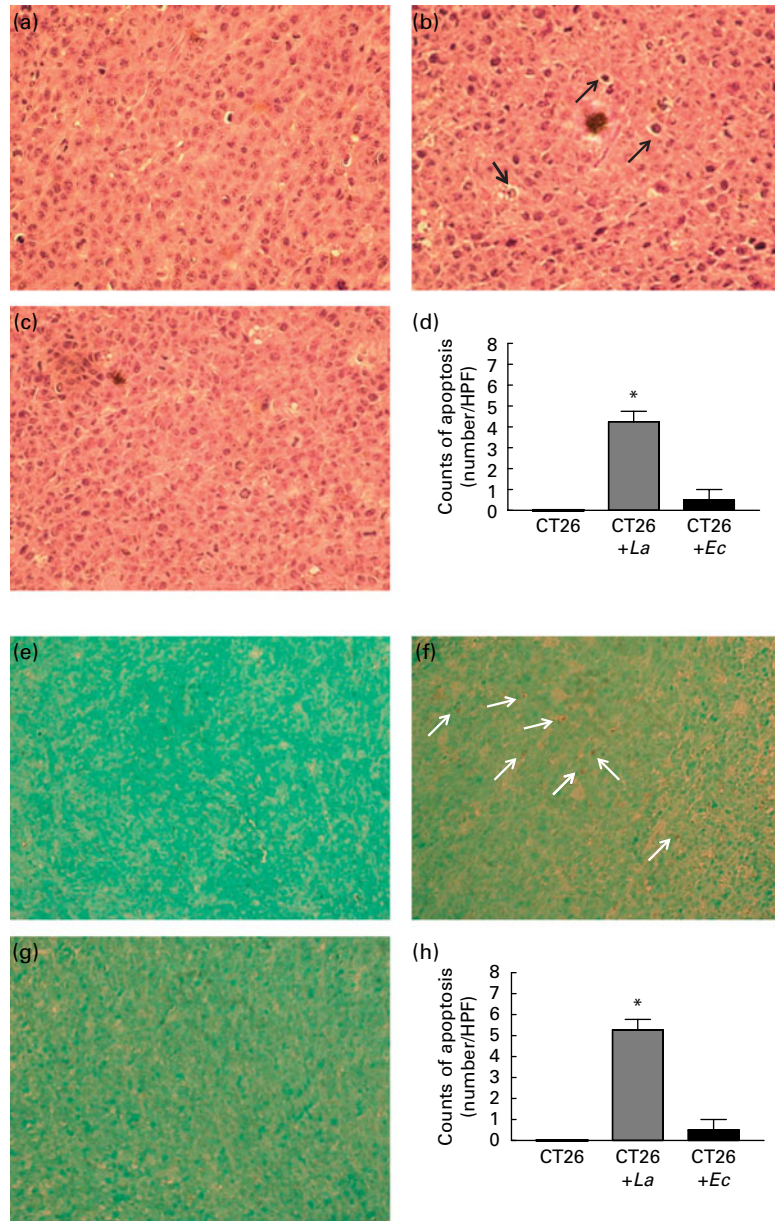


Fig. 2. *Lactobacillus acidophilus* NCFM (*La*) pre-inoculation promotes apoptosis in subcutaneous (s.c.) CT-26 tumours. Histological analysis of dorso-lateral flank tumours using (a–c) haematoxylin and eosin (H&E) and (e–g) terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling (TUNEL) staining. (a and e) CT-26(s.c.) alone, (b and f) CT-26(s.c.) + *La* and (c and g) CT-26(s.c.) + *Ec* tumours were analysed for apoptosis (black arrows in H&E staining) and brown staining (white arrows in TUNEL staining) with magnification $400\times$. (d and h) The number of apoptotic bodies was also calculated using at least ten different high-power fields (HPF) per slide. Values are means, with standard errors of the mean represented by vertical bars ($n\ 10$). * Mean value was significantly different from that for CT-26 alone ($P < 0.05$). CT-26 alone, mice were implanted with CT-26 cells after 14 d of PBS inoculation; CT-26 + *La*, mice were pre-inoculated with *La* 1×10^8 colony-forming units (cfu)/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells; CT-26 + *Ec*, mice were pre-inoculated with *Escherichia coli* K12 1×10^8 cfu/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells.

indicate that *La* promotes apoptosis in CT-26-derived adenocarcinomas *in vivo* via the modulation of anti- and pro-apoptotic protein expression.

Lactobacillus acidophilus pre-inoculation may down-regulate the CXCR4 mRNA expressions in the colon, mesenteric lymph nodes and extra-intestinal tissue

To determine the expressions of chemokine receptor CXCR4 under the influence of colon carcinogenesis, we isolated and

homogenised colon tissues, MLN and extra-intestinal metastatic tissue, including spleen and liver. Briefly, the surgical technique for segmental orthotopic colon cancer established colon carcinogenesis and mesentery tissue (including MLN) involvement. Intrasplenic and intravenous injection established extra-intestinal metastasis such as spleen and liver. When CT-26 cells were implanted, pre-inoculation with probiotics *La* may down-regulate the expressions of CXCR4 mRNA in the colon, MLN and extra-intestinal metastatic tissue compared with the CT-26 alone and CT-26 + *Ec* groups (Fig. 5).

Lactobacillus acidophilus pre-inoculation may reduce the mean fluorescence index of MHC class I

To determine the expressions of MHC class I molecules under the influence of colon carcinogenesis, we isolated cells from the MLN, spleens and the LP of colon tissues. Cells were stained using a panel of monoclonal antibodies directed against MHC class I (H-2Dd, -Kd and -Ld). When CT-26 cells were implanted, pre-inoculation with *La* resulted in the down-regulation of the expressions of MHC class I (H-2Dd, -Kd and -Ld) in cells isolated from the LP of the colon, MLN and spleen compared with the groups CT-26 alone and CT-26 + *Ec* (Fig. 6).

Discussion

The frequency of colon carcinoma patients is increasing in developed countries⁽¹⁾. Importantly, probiotics have been shown to reduce the incidence of colon cancer in animal models⁽²²⁾; however, the mechanisms responsible for this anti-cancer activity are uncharacterised. In the present study, the efficacy of probiotic *La* was analysed using the colon carcinoma cell line CT-26. Oral administration of *La* effectively reduced colon carcinoma tumour growth and the extent of affected tissues, suggesting that pre-inoculation with *La* was associated with suppressed tumour growth.

Previous studies using animal colon cancer models concluded that colon microflora are involved in the aetiology of

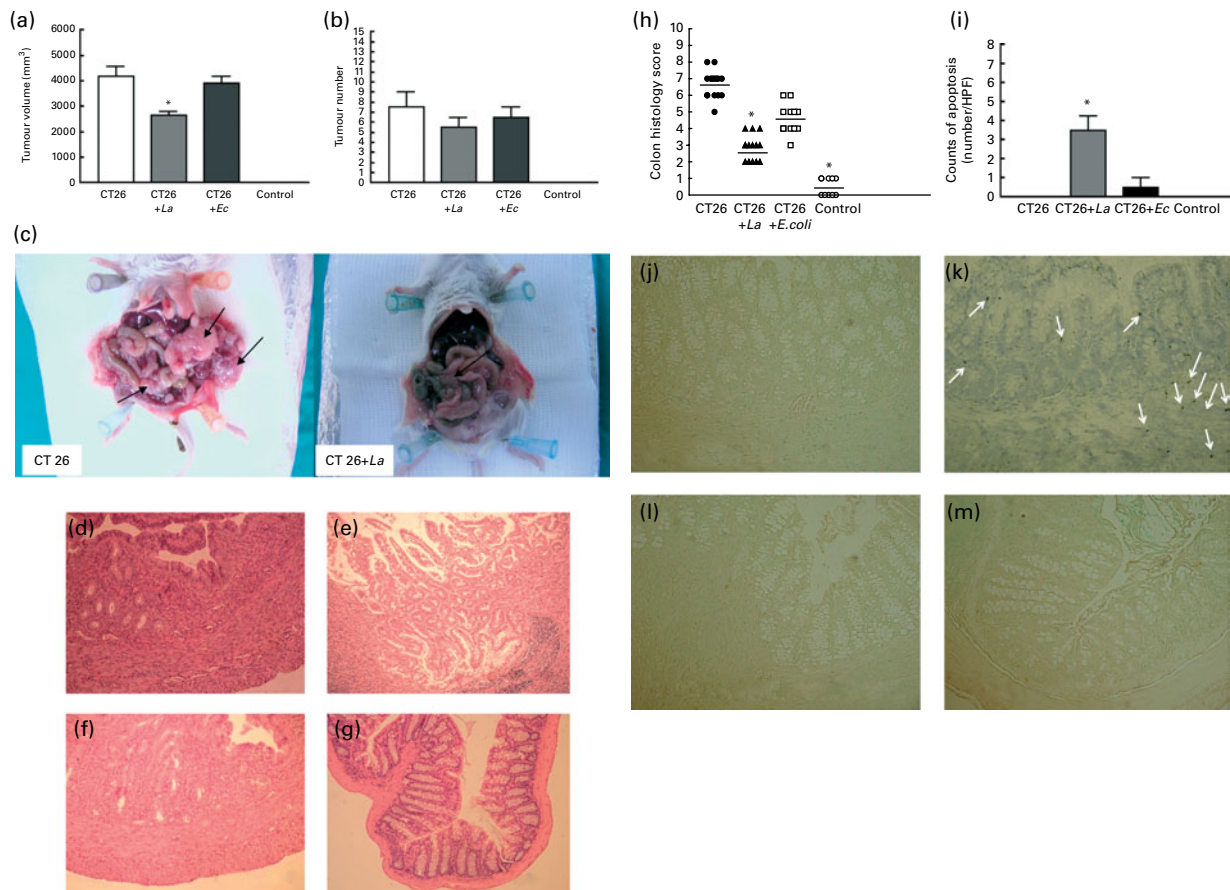


Fig. 3. *Lactobacillus acidophilus* NCFM (*La*) inhibits tumour growth and promotes apoptosis in segmental orthotopic colon cancers. (a) Abdominal tumour size and (b) number of tumours were measured after mice had been killed on day 28 of tumour development. (c) Two representative images show these intra-abdominal tumours (black arrows) in CT-26 alone and CT-26 + *La*. Histological pathology of the tumour on segmental colonic tissue of female BALB/c mice, haematoxylin and eosin stain with magnification 200 ×. The different groups are (d) CT-26 alone, (e) CT-26 + *La*, (f) CT-26 + *Ec* and (g) control. The (h) colonic histology scores of different groups of mice at 28 d after CT-26 cell implantation were assessed by the determination of colonic involvement and tissue damage. ●, CT-26 alone; ▲, CT-26 + *La*; □, CT-26 + *Ec*; ○, control. Values are measurements of individual mice pooled from three independent experiments. The horizontal line represents the mean score of different groups. Data from the colonic histology scores were analysed using one-way ANOVA (non-parametric). The mean score of mice CT-26 + *La* is lower than those of mice CT-26 alone and CT-26 + *Ec* (n 10–15 female BALB/c mice). * Mean values were significantly different from those of the CT-26-alone group (P < 0.05). Segmental colon tumours from (j) CT-26 (intracolonic implantation) alone, (k) CT-26 (intracolonic implantation) + *La*, (l) CT-26 (intracolonic implantation) + *Ec* and (m) control-colon mice were analysed for apoptosis (brown staining, white arrow) using terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling staining, with magnification 200 ×. The (i) number of apoptotic bodies was also calculated using at least ten different high-power fields (HPF) per slide. Values are means of data pooled from three independent experiments with a total of ten to fifteen animals per group, with standard errors of the mean represented by vertical bars. * Mean value was significantly different from that for CT-26 alone (P < 0.05). CT-26 alone, mice were implanted with CT-26 cells after 14 d of PBS inoculation; CT-26 + *La*, mice were pre-inoculated with *La* 1×10^8 colony-forming units (cfu)/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells; CT-26 + *Ec*, mice were pre-inoculated with *Escherichia coli* K12 1×10^8 cfu/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells.

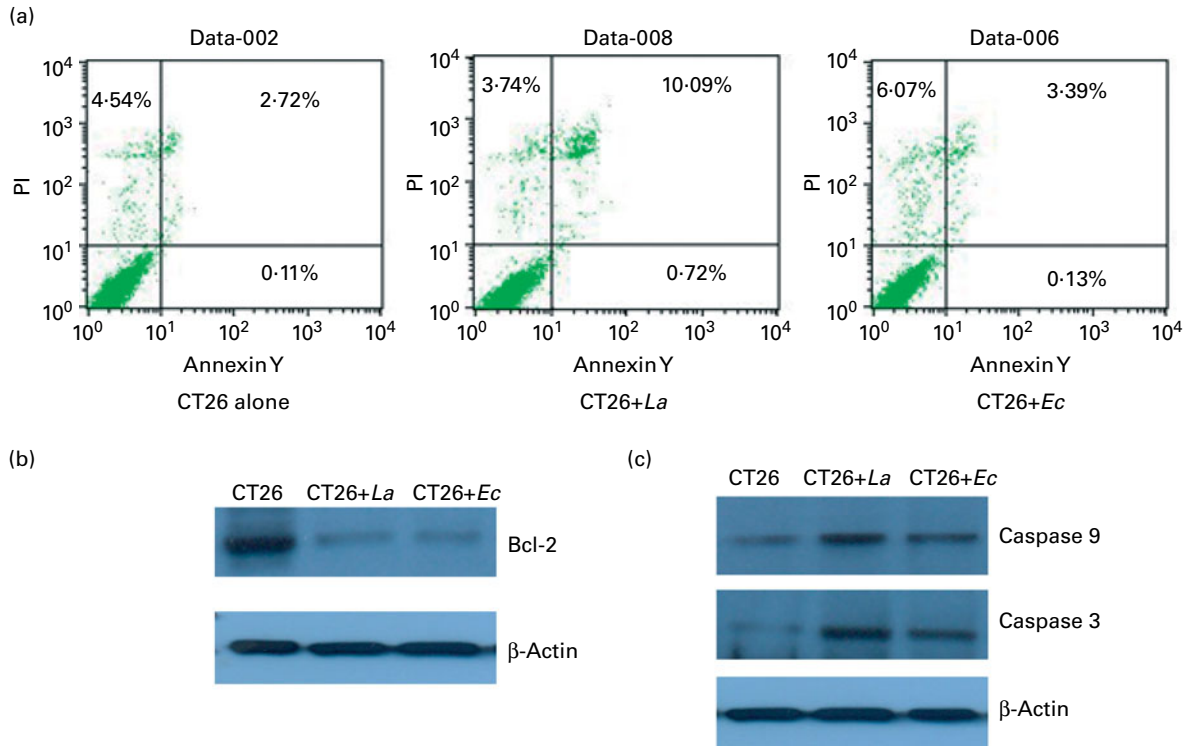


Fig. 4. Effect of probiotics *Lactobacillus acidophilus* NCFM (*La*) on the apoptosis of tumour cells caused by CT-26 cell carcinogenesis in mice. (a) The single-cell suspension was prepared and apoptosis was detected by annexin-V fluorescein isothiocyanate through flow cytometry. Mice CT-26 + *La* showed enhancement of apoptosis. (b and c) The expressions of some proteins associated with cell apoptosis were detected by Western blotting. Protein was extracted and analysed by SDS-PAGE (10%), followed by Western blot analysis using Bcl-2, caspase-3, caspase-9 antibody or β-actin as a protein loading control. Lane 1, CT-26 alone; lane 2, CT-26 + *La*; lane 3, CT-26 + *Ec*. The expression of CT-26 + *La* is higher in caspase-3 and caspase-9, but lower in Bcl-2 (*n* 10 female BALB/c mice). CT-26 alone, mice were implanted with CT-26 cells after 14 d of PBS inoculation; CT-26 + *La*, mice were pre-inoculated with *La* 1×10^8 colony-forming units (cfu)/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells; CT-26 + *Ec*, mice were pre-inoculated with *Escherichia coli* K12 1×10^8 cfu/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells.

carcinogenesis⁽²³⁾. For example, a number of studies indicate that specific bacteria, such as *Streptococcus bovis*, *Bacteroides*⁽²⁴⁾ and *Clostridia*⁽²⁵⁾ may promote colon cancer, while probiotic strains of bacteria inhibit tumour growth^(26–28). In animal models, *La* and *Bifidobacterium longum* are capable of reducing the incidence of colon tumours and aberrant crypt foci, respectively⁽²²⁾. In the present study, *La*, a probiotic bacteria strain that accumulates in the intestinal tract after oral administration, inhibited tumour growth. In comparison, *Ec*, a commensal intestinal bacteria strain, showed some influence but no significant anti-carcinogenic effect. These results suggest that colon microflora may play a critical role in human health and disease, especially with respect to colon carcinoma.

According to the previous literature⁽²⁹⁾ and the present pilot study, the majority of animal models (such as mice and rats) used 2 weeks or more than 2 weeks treatment of probiotics in colon cancer, pancreatic neoplasm or breast cancer models; only a few studies used 1 week or less than 14 days. We chose a 14 d treatment protocol based on the above reasons and we tried to establish adequate colonisation of probiotics before tumour implantation. The reason of using *Ec* as control over other control (e.g. heat-killed *La* bacteria) was that we tried to mimic a live commensal bacteria strain that has a similar effect to live physiological condition in the host.

The underlying mechanisms by which probiotics inhibit colon carcinoma remain unclear. Several models have been suggested⁽³⁰⁾, including binding of potential mutagens⁽³¹⁾ and reduced enzymatic activities involved in carcinogen formation⁽³²⁾. In addition, some probiotic strains increase colon carcinoma cell apoptosis in rats^(33–35), but not in mice. Furthermore, *in vitro* studies suggest that a cocktail of probiotics (VSL#3; containing four strains of *Lactobacilli*, three strains of *Bifidobacteria* and *Streptococcus thermophilus*) induce apoptosis in HT-29 and Caco-2 cells, and yield conjugated linoleic acid, which may indirectly alter tumour metabolism⁽³⁶⁾. The present study showed that pre-inoculation with *La* in BALB/c mice resulted in retarding the growth of tumour volume, enhancing apoptosis of tumour cells and down-regulating the expression of surface protein, which may be associated with the immune response. The potential mechanisms responsible for the anti-tumour activity of probiotics *La* might be (1) altering intestinal micro-ecosystem and lower intestinal pH, (2) altering tumour metabolism (e.g. producing SCFA, conjugated fatty acid)⁽³⁶⁾, (3) enhancing host immune responses (induced by peptidoglycan of the cell wall⁽³⁷⁾ or secretory protein of probiotic bacteria), (4) relating to apoptosis or proliferation of tumour cells (e.g. the molecule such as polyamine in hyperproliferation and cell migration involved in almost all steps of colorectal tumorigenesis⁽³⁸⁾, and probiotics may

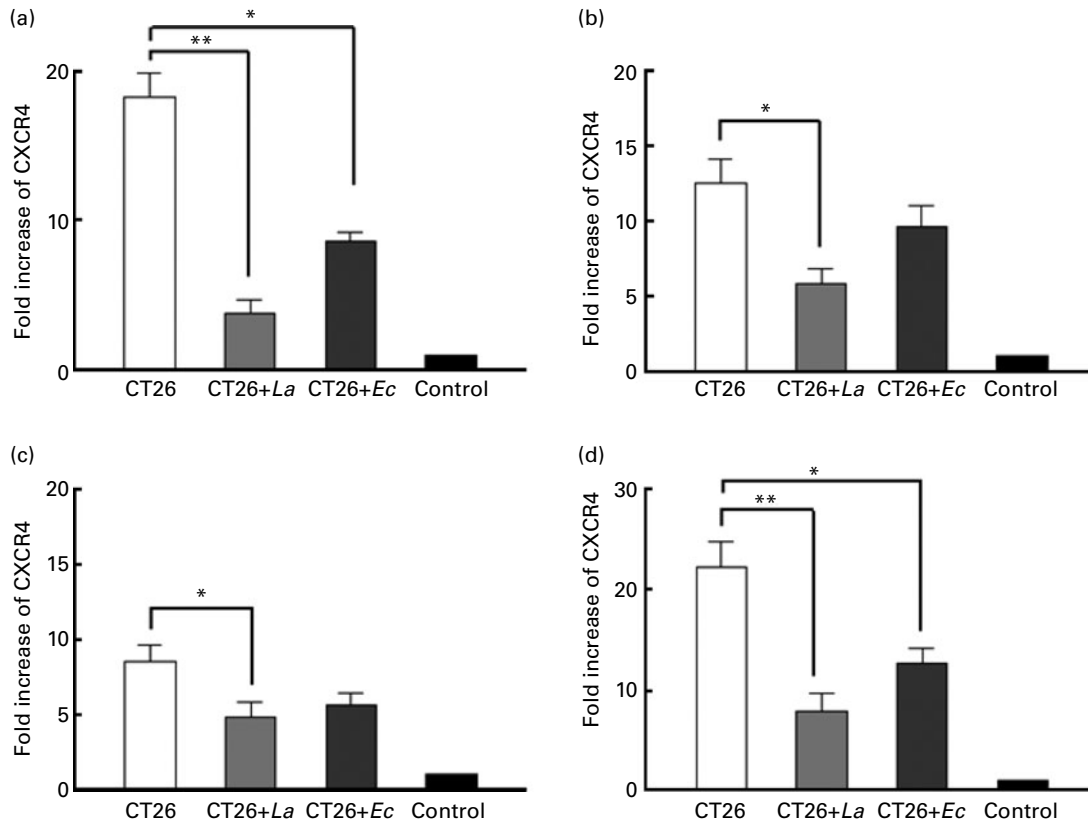


Fig. 5. To determine the expression of the chemokine receptor CXCR4 under a colon carcinoma cell line, we isolated and homogenised the colon, mesenteric lymph nodes (MLN) and extra-intestinal tissue. (a) The above samples were performed with real-time PCR to analyse the expression of CXCR4 mRNA in CT-26 alone (□), CT-26 + *La* (■), CT-26 + *Ec* (■) and control (■) mice. CT-26 + *La* may down-regulate the expression of CXCR4 mRNA in the (a) colon, (b) MLN, (c) spleen and (d) liver, compared with the other groups such as CT-26 alone and CT-26 + *Ec*. Statistical analyses were performed using one-way ANOVA. Values are means, with standard errors of the mean represented by vertical bars (n 10–15 animals). Mean value was significantly different from that for CT-26-alone: * P <0.05, ** P <0.001. CT-26 alone, mice were implanted with CT-26 cells after 14 d of PBS inoculation; CT-26 + *Lactobacillus acidophilus* NCFM (*La*), mice were pre-inoculated with *La* 1×10^8 colony-forming units (cfu)/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells; CT-26 + *Ec*, mice were pre-inoculated with *Escherichia coli* K12 1×10^8 cfu/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells.

decrease polyamine levels in the colon). The role of probiotics was only one part of anti-tumour activity, and the detail mechanisms should be much more complex. However, further study is necessary to understand the mechanisms of anti-tumour effect exerted by probiotics.

To our knowledge, a role for probiotics in promoting apoptosis in colon cancer cells is seldom explored. We explored the effects of probiotics on apoptosis in CT-26 cell-induced tumours. Using a novel *in vivo* murine model, we demonstrated that pre-inoculation of *La* enhanced apoptosis in both subcutaneous dorsal-flank tumours and segmental orthotopic colon cancers. These findings are consistent with previous studies. Specifically, increased apoptosis in fish cell lines was observed following treatment with probiotics⁽³⁹⁾. In addition, *in vitro* studies have revealed that the human probiotic, *Propionibacterium freudenreichii*, induced apoptosis of colorectal adenocarcinoma cells via its metabolites, the SCFA acetate and propionate⁽⁴⁰⁾.

Caspases are synthesised as inactive proenzymes that are processed proteolytically to active forms. Caspase-9 can be activated by a number of proteins. Caspase-9 activation in turn may lead to the activation of caspase-3, which promotes apoptosis⁽⁴¹⁾. Levels of Bcl-2, an oncoprotein, are used to

measure cell survival because Bcl-2 inhibits apoptosis⁽⁴²⁾ and, as a result, stimulates tumour growth^(42,43). We observed lower Bcl-2 expression in CT-26 cells isolated from mice that were pre-inoculated with *La*. Moreover, caspase-3 and caspase-9 expression was higher in *La*-treated cells compared with *Ec*-inoculated or untreated mice. Furthermore, *La* pre-inoculation resulted in a higher percentage of annexin-V staining compared with the other groups, indicating that *La* may enhance apoptosis in CT-26 cell-derived carcinomas. We observed increased CT-26 cell apoptosis following *La* pre-inoculation.

In the present study, we hypothesised that *La* exerted its anti-tumour effect through modulation of the immune response of the host, for example, by reducing CXCR4 expression which is related to the anti-metastatic effect and reducing the expression of MHC class I which is related to colon carcinogenesis. CXCR4, the chemokine receptor for CXCL12, has been shown to be involved in the metastatic processes of several neoplasms⁽⁴⁴⁾. CXCR4 antibody treatment reduced the metastasis of a breast carcinoma cell line, supporting that CXCR4 is essential for invasion into tissues⁽⁴⁵⁾. Inhibition of CXCR4 may be used therapeutically to suppress the outgrowth of micro-metastases⁽¹⁵⁾ and reduce the metastatic

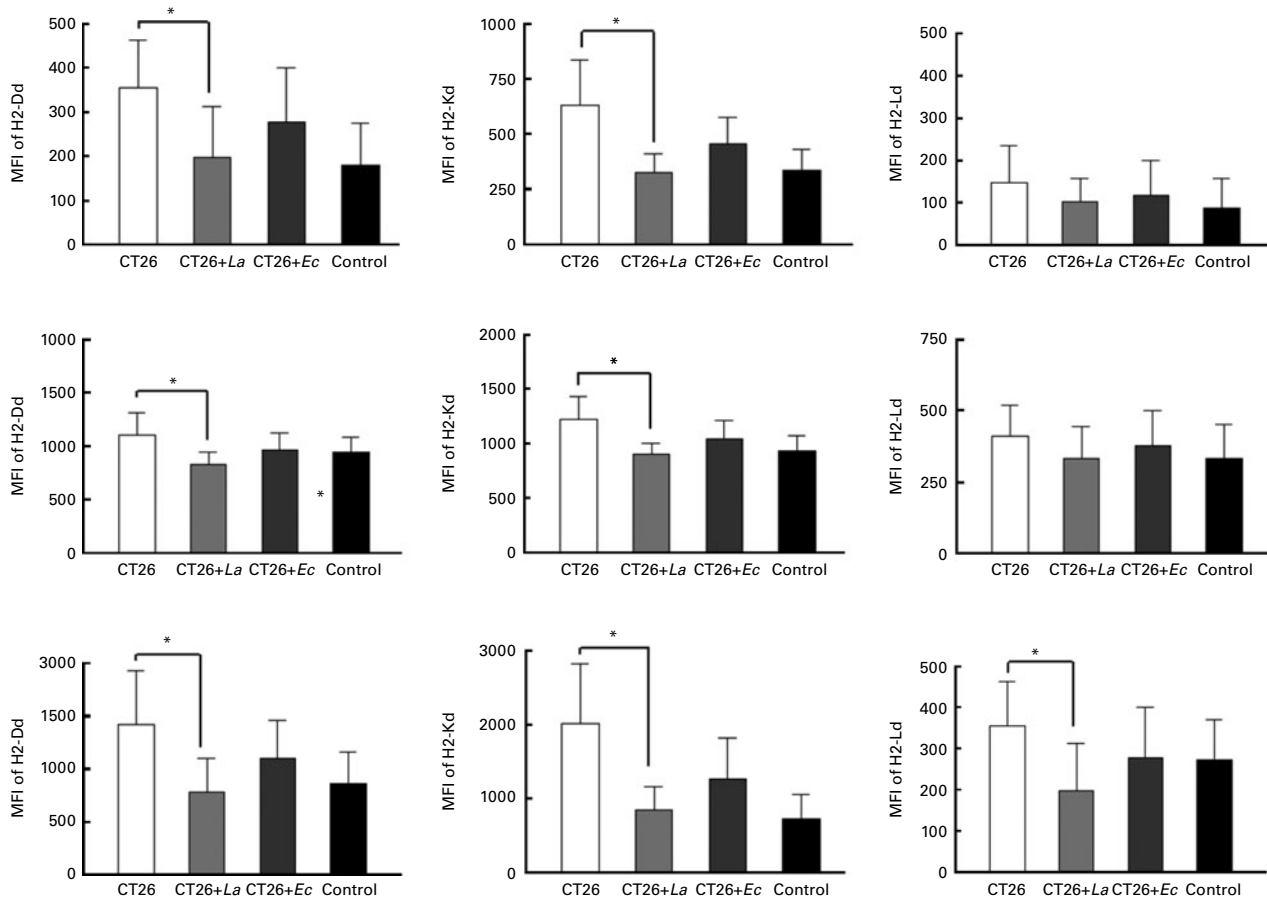


Fig. 6. To determine the expression of the MHC class I molecule under a colon carcinoma cell line, we isolated cells from the mesenteric lymph nodes (MLN), spleen and the lamina propria (LP) of colon tissue. Mean fluorescence indices (MFI) of the MHC class I molecule (H-2Dd, -Kd and -Ld) in flow cytometry analysis shown in CT-26 alone (□), CT-26 + *La* (■), CT-26 + *Ec* (■) and control (■) mice. Under CT-26 cells implanted, pre-inoculation with *La* may down-regulate the expression of MHC class I (H-2Dd, -Kd and -Ld) in the (a) LP of the colon, (b) MLN and (c) spleen, which compared with the other groups such as CT-26 alone and CT-26 + *Ec*. Statistical analyses were performed using one-way ANOVA. Values are means, with standard errors of the mean represented by vertical bars (n 10–15 animals). * Mean value was significantly different from that for CT-26-alone ($P < 0.05$). CT-26 alone, mice were implanted with CT-26 cells after 14 d of PBS inoculation; CT-26 + *Lactobacillus acidophilus* NCFM (*La*), mice were pre-inoculated with La 1×10^8 colony-forming units (cfu)/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells; CT-26 + *Ec*, mice were pre-inoculated with *Escherichia coli* K12 1×10^9 cfu/mouse per d for 14 consecutive days and were implanted with 5×10^6 CT-26 cells.

potential of cancer cells⁽⁴⁶⁾. In the present study, we showed that inoculation with probiotics *La* results in a down-regulation of the expressions of CXCR4 mRNA in the colon, MLN and extra-intestinal tissues. The results suggested that the lower expression of CXCR4 might be associated with reducing cancer carcinogenesis and metastatic potential.

We tried to analyse the cell surface phenotypes associated with the immune reaction in CT-26 carcinogenesis. The expression of MHC-I (H-2Dd, -Kd and -Ld) might be affected by probiotics *La* inoculation. Previous studies on antigenic peptides covalently linked to either the MHC-I H chain^(16,47) or β_2m ^(48,49) have collectively shown that the C-terminal protrusion imposed by the synthetic linker is tolerated with respect to both MHC-I binding and T-cell recognition when used with H-2Dd, -Kd, -Ld and HLA-A2. The anticipated structural distortion is adjacent to the C-terminal anchor residue and, as such, is potentially detrimental to both proper positioning within the MHC-I binding groove and subsequent T-cell recognition. The present study demonstrated that inoculation with probiotics *La* may down-regulate mean

fluorescence indices of MHC class I expression (H-2Dd, -Kd and -Ld) in the LP of the colon, MLN and spleen, which might be expressed by tumour cells. The above results suggest that inoculation with probiotics *La* can reduce the expression of MHC class I, which is associated with subsequent T-cell recognition and carcinogenesis in mice.

Urbanska *et al.*⁽⁵⁰⁾ reported that daily oral administration of *L. acidophilus* in a yogurt formulation in *Apc* (Min/+) mice resulted in minimising intestinal inflammation, and delaying overall polyp progression, fewer gastrointestinal intra-epithelial neoplasias with a lower grade of dysplasia in detected tumours. The present study showed that pre-inoculation with *L. acidophilus* in BALB/c mice resulted in retarding the growth of tumour volume, lower histopathology scores (lesser colonic tissue involvement and fewer structural abnormalities of epithelial/crypt damage), enhancing apoptosis of tumour cells and down-regulating the expression of surface protein, which may be associated with the immune response. Both these two studies could be potentially useful in designing future probiotic formulations containing *L. acidophilus* in the

prophylaxis or management supplements for colon cancer, polyposis and other gastrointestinal diseases.

In conclusion, we demonstrated that probiotic *La* retarded the growth of tumour volume and enhanced the apoptosis of tumour cells. In addition, probiotics down-regulated the expression of CXCR4 mRNA, and lessened the mean fluorescence index of MHC class I (H-2Dd, -Kd and -Ld) in the colon, MLN, and spleen tissue of BALB/c mice. These findings suggest that probiotics may play a role in attenuating the tumour growth of CT-26 colon carcinogenesis. Increasing apoptosis in tumour tissues indicated that inoculation with probiotics may be associated with modulating the cellular response during colon carcinogenesis caused by CT-26 cells.

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