
Anti-tumour immune effect of oral administration of *Lactobacillus plantarum* to CT26 tumour-bearing mice

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Colorectal cancer (CRC) is one of the most prevalent forms of cancer that shows a high mortality and increasing incidence. There are numerous successful treatment options for CRC, including surgery, chemotherapy, radiotherapy and immunotherapy; however, their side effects and limitations are considerable. Probiotics may be an effective strategy for preventing and inhibiting tumour growth through stimulation of host innate and adaptive immunity. We investigated and compared potential anti-tumour immune responses induced by two isolated *Lactobacillus* strains, *Lactobacillus plantarum* A and *Lactobacillus rhamnosus* b, by pre-inoculating mice with lactobacilli for 14 days. Subsequently, subcutaneous and orthotopic intestinal tumours were generated in the pre-inoculated mice using CT26 murine adenocarcinoma cells and were assessed for response against the tumour. Our results indicated that oral administration with *L. plantarum* inhibited CT26 cell growth in BALB/c mice and prolonged the survival time of tumour-bearing mice compared with mice administered *L. rhamnosus*. *L. plantarum* produced protective immunity against the challenge with CT26 cells by increasing the effector functions of CD8⁺ and natural killer (NK) cell infiltration into tumour tissue, up-regulation of IFN- γ (but not IL-4 or IL-17) production, and promotion of Th1-type CD4⁺ T differentiation. Consequently, our results suggest that *L. plantarum* can enhance the anti-tumour immune response and delay tumour formation.

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1. Introduction

Colorectal cancer (CRC) is one of the most prevalent forms of cancer with an increasing incidence and high mortality in China (Jemal *et al.* 2010). Many factors contribute to colon

cancer risk, including genetics, diet and lifestyle (Marshall 2008; Chien *et al.* 2012). According to epidemiological and micro-ecology studies, accumulating evidence indicates that gut microbes are involved in the etiology of colon cancer (Azcárate-Peril *et al.* 2011). Although chemotherapy,

Keywords. Colon cancer; CT26 cells; immunity; *Lactobacillus*

Abbreviations used: APC, allophycocyanin; CFU, colony forming unit; CRC, colorectal cancer; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; MRS, deMan, Rogosa and Sharpe; NK, natural killer cells; PE, phycoerythrin

radiotherapy and surgery remain the pre-dominant treatment methods for colon cancer, their toxicity and side effects are considerable (Osterlund *et al.* 2007). Therefore, enhancing the immune response and improving the gut microbiota are intriguing strategies for the prevention of CRC (Geier *et al.* 2006). Numerous studies have proposed that shifting of immune responses from a T-helper 1 (Th1) to a T-helper 2 (Th2) pattern is the cause of an impaired response to malignancies (Evans *et al.* 2006).

Probiotics are defined as 'live micro-organisms which, when ingested in sufficient quantities, exert health benefits on the host' (Erickson and Hubbrad 2000). Growing evidence suggests that probiotics can reinforce natural defences, protect against gastrointestinal disorders, and enhance innate or systemic immunity (Hirayama and Rafter 2000; Rafer 2003; Liong 2008). In addition, probiotics induce dendritic cell (DC) maturation, elevate natural killer cell (NK) cytotoxicity, and up-regulate cytokine secretion, including promoting IFN- γ production (Takagi *et al.* 2001; Delcenserie *et al.* 2008; Azcárate-Peril *et al.* 2011). The genera *Lactobacillus* are prominent probiotics members, and their beneficial effects are always outlined in various ailments such as food allergies (Kalliomäki and Isolauri 2003; Schiavi *et al.* 2011), inflammatory bowel disease (IBD) and ulcerative colitis (UC) (Bibiloni *et al.* 2005). *Lactobacillus* exerts beneficial health effects on the host by modulating humoral, cellular, or non-specific immunity, which may require interactions among microflora, epithelial cells, and immune cells (Smyth *et al.* 2001; Christensen *et al.* 2002; Seow *et al.* 2010). It has also been reported that *Lactobacillus* can induce DC maturation and differentiation of Th1 immune response, which is important for tumour inhibition (Sun *et al.* 2005; Ewaschuk *et al.* 2006).

Although the majority of studies have suggested that the variety of effects lactobacilli have on the host's health includes anti-tumour effects (Linsalata and Russo 2008), the precise mechanisms of the anti-tumour effects remain unclear. One possibility is that lactobacilli may retard colon carcinogenesis by enhancing the host immune response and altering the intestinal micro-ecosystem, changing tumour metabolism and affecting tumour cell apoptosis and proliferation (Chien *et al.* 2012; Abou-Elkacem *et al.* 2013; Wang *et al.* 2014). In this study, we focus on identifying specific strains that have a beneficial immune effect on tumour growth suppression. We hypothesized that lactobacilli can activate anti-tumour immunity and retard tumour growth. Therefore, *Lactobacillus plantarum* (*L. plantarum*) and *Lactobacillus rhamnosus* (*L. rhamnosus*) from traditional fermented milk products were isolated and evaluated for their anti-tumour effects. We compared their ability to induce immune responses, including maturation of DCs, secretion of cytokines, polarization of CD4⁺ T cells, and activation of NK cell activity.

2. Materials and methods

2.1 Mice and ethics statement

Female BALB/c mice (aged 6 to 8 weeks) were purchased from Beijing HFK Bioscience Co., Ltd., China (SCXK-Jing-20090004), and raised in the Animal Center of Jilin University. All animal experiments were performed in strict accordance with the guidelines of the Animal Care and Ethics Committee of the National Association of Laboratory Animal Care.

2.2 Bacterial and colon carcinoma cells

L. plantarum and *L. rhamnosus* were isolated from traditional fermented milk products (from Lhasa of Tibet, China) and identified through comparisons of conserved and polymorphic 16s rRNA, following standard PCR conditions using universal primers (AGAGTTTGATCTGGCTC AGG and ACGGCAACCTTGTTACGAGTT) (Wang *et al.* 2014). The sequences were subjected to similarity searches in NCBI public databases to infer possible phylogenetic classifications. Using BLAST, the nucleotide sequence similarity of isolated strains (termed *L. rhamnosus* b and *L. plantarum* A, and abbreviated *L. rhamnosus* and *L. plantarum* in this study) was 99% and 100%, respectively, compared with *L. rhamnosus* (JF414108.1) and *L. plantarum* (KC836552.1) (data not shown).

CT26 colon cancer cell lines were purchased from the American Type Culture Collection (ATCC CRL-2638; Manassas, VA, USA). Cell lines were routinely cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco) at 37°C in 5% CO₂. Cells were used at 75–80% confluence.

2.3 Immunity and experimental design

Mice (body weight >20 g) were randomly divided into three groups (n=10/group). *L. plantarum* or *L. rhamnosus* strains were routinely cultured in deMan, Rogosa, and Sharpe broth (MRS; Difco, Detroit, MI, USA) and administered via oral gavage for 14 consecutive days at a concentration of 1×10^8 colony-forming units (CFU)/mouse. Next, a tumour model was constructed by implanting CT26 tumour cells into mice. Bacterial administration continued once a week for three weeks at a concentration of 1×10^9 CFU. An equal volume (100 μ L) of phosphate buffered saline (PBS) was administered to control mice using the same method (figure 1a). The groups were designated as CT26+*L. plantarum* (CT26+*L.p*), CT26+*L. rhamnosus* (CT26+*L.r*), and CT26 alone (CT26). All the experiments were independently repeated three times

as the same methodology and same experimental design (3 groups, 10 mice/ group).

2.4 CT26 challenge to construct implantation tumour model

2.4.1 Primary subcutaneous implantation tumour model: Serum-free media (100 μ L) containing 2×10^6 CT26 cells (cell viability $\geq 95\%$) were slowly injected subcutaneously into the right flank of mice using a 30-gauge needle. Tumour growth rates were preliminarily evaluated at intervals of 5 days after tumour initiation by monitoring the volume of tumours using the formula $V = 0.52 \times L \times W^2$ where L is the length of the tumour and W is the width of the tumour (Chien *et al.* 2012). Survival end points were determined by either spontaneous death of the mice or the presence of moribund signs. The percent survival evaluation was performed according to Kaplan-Meier analyses (Chen *et al.* 2011) for the primary subcutaneous tumour model.

2.4.2 Histopathological examination: To observe tumour histopathological changes after CT26 challenge into mice pre-inoculated with lactobacilli, tumours were isolated from each group and placed in 10% neutral-buffered formalin for fixation prior to hematoxylin-eosin (HE) staining using a standard protocol (Dwivedi *et al.* 2012).

2.4.3 Orthotopic implantation tumour model: Once the subcutaneous xenograft measured approximately 500 mm³, we selected an intact tumour block from one of the ten subcutaneous tumour-bearing mice, which did not do not ulcerate through the skin. Tumour block was selected if it was growing well, light red, fish-like, and had mild necrosis after removing the fibrous capsule. The selected tumour was cut into 1-mm³-sized fragments for use in orthotopic implantation as previously described (Abou-Elkacem *et al.* 2013). Briefly, mice were anesthetized via an intraperitoneal injection of ketamine (50 mg/kg; NK, Shanghai, China). Then, a laparotomy of 0.5 to 1 cm was conducted and the cecum was exteriorized. Subsequently, the serosa of the cecum was scraped slightly with two forceps and 1-mm³ fragment was implanted onto the serosa of the cecum, followed by the addition of one drop of α -amino-acrylate medical biological glue (Guangzhou Baiyun Medical Glue Co., LTD, Guangzhou, China) for several seconds. The cecum was then returned to the peritoneal cavity and the abdominal wound was closed with a 6.0 suture. Finally, tumours sizes were analysed every week after surgical treatment using a digital color Doppler ultrasonic diagnostic imaging (DC-6, Mindary, Shenzhen, China) and the above-mentioned formula. Twenty-one days after tumour implantation, orthotopic tumour-bearing mice were sacrificed by cervical dislocation. The orthotopic tumour was separated and weighed using an electronic balance, and immune responses were analysed.

2.5 Evaluation of the protective immune responses induced by lactobacilli

2.5.1 Preparation of single-cell suspensions of lymphocytes from orthotopic implantation tumour mice: Single-cell suspensions of spleen were prepared as described previously (Wykes *et al.* 2007; Qiao *et al.* 2008; Chen *et al.* 2011). Tumour-infiltrating lymphocytes were obtained by tumour processing and enzymatic digestion using 2.5 mg/mL collagenase type IV (Worthington, Lakewood, NJ, USA) and 50 U/mL DNase I (Roche, Indianapolis, IN, USA) in RPMI-1640 at 37°C for 60 min as described previously (Chen *et al.* 2011; Ni *et al.* 2011). The same methodology was adopted for subcutaneous tumour as well.

2.5.2 Flow cytometry: The following antibodies and corresponding isotype controls (all purchased from BD Pharmingen, USA) were used for staining as previously described (Shaohua *et al.* 2014): CD16/CD32 (clone 2.4G2), CD3-PE (clone 145-2C11), CD4-FITC (clone H129.19), CD8 α -APC (clone RPA-T8), CD3-FITC (clone 145-2C11), CD49b-PE (clone DX5), CD11c-APC (clone HL3), CD80-FITC (clone 16-10A1), and CD86-PE (clone GL1). To stain intracellular granzyme B in CD8⁺ or NK cells, cells were first stained for CD8 α -APC or CD49b-PE, followed by fixation and permeabilization using the Cytofix/Cytoperm™ Plus Fixation/Permeabilization kit (BD Biosciences). Cells were then stained with granzyme B-FITC (clone NGZB). All stained cells were washed twice with FACS buffer (0.1% BSA and 0.05% sodium azide in PBS) before examination by flow cytometry.

2.5.3 Cytokine analyses: Total IFN- γ production from splenocytes was detected by flow cytometry and ELISA as previously described (Ni *et al.* 2011). Cytokine production from CD4⁺ T cells was analysed after induction of activated DCs according to the protocol for intracellular cytokine staining by flow cytometry (Kandasamy *et al.* 2011). Briefly, CD4⁺ T lymphocytes from naïve BALB/c mice spleens were purified using a CD4⁺ MicroBead kit. Then, DCs in spleens derived from the CT26+*L. plantarum* or CT26 alone groups were isolated by positive selection using the CD11c MicroBead kit (all kits were purchased from Miltenyi Biotech, Bergisch Gladbach, Germany). After 6 h of co-culture with stimulated CD4⁺ T lymphocytes and purified DCs, cells were stained with PE-IFN- γ (clone XMG1.2), PE-IL-4 (clone 11B11), or PE-IL-17 (clone TC11-18H10), and analysed by flow cytometry.

2.6 Statistical analyses

All data are expressed as the mean \pm standard error of the mean (SEM). Statistical significance was determined using unpaired two-tailed *t*-tests and $p < 0.05$ was considered

statistically significant. Statistical analyses were conducted using GraphPad Prism 5.0 software.

3. Results

3.1 Administration of *L. plantarum* suppresses CT26 growth in subcutaneous tumour-bearing mice

To estimate the effect of *Lactobacillus* administration on CT26 growth, we evaluated tumour size (figure 1b). Results showed that *L. plantarum* (CT26+*L. plantarum* group) significantly inhibited CT26 growth at 25 day (213.4 ± 47.56 vs. 598.1

± 154.6 mm³, $p = 0.0365$) and 30 day post-inoculation (487.1 ± 132.8 vs. 1122 ± 139.8 mm³, $p = 0.0045$) compared with PBS (CT26 alone group). However, *L. rhamnosus* (CT26+*L. rhamnosus* group) did not significantly affect CT26 growth at 25 day (391.8 ± 78.79 vs. 598.1 ± 154.6 mm³, $p = 0.2499$) or at 30 day (843 ± 130.3 vs. 1122 ± 139.8 mm³, $p = 0.1617$) compared with the PBS (CT26 alone group). The subcutaneous tumour-bearing mice from the CT26+*L. plantarum* group survived much longer than mice from other groups ($p = 0.0396$) (figure 1c).

Although histopathology showed obvious evidence of mitosis and cell polymorphisms in all groups, prominent lymphocytic infiltration was observed only in the

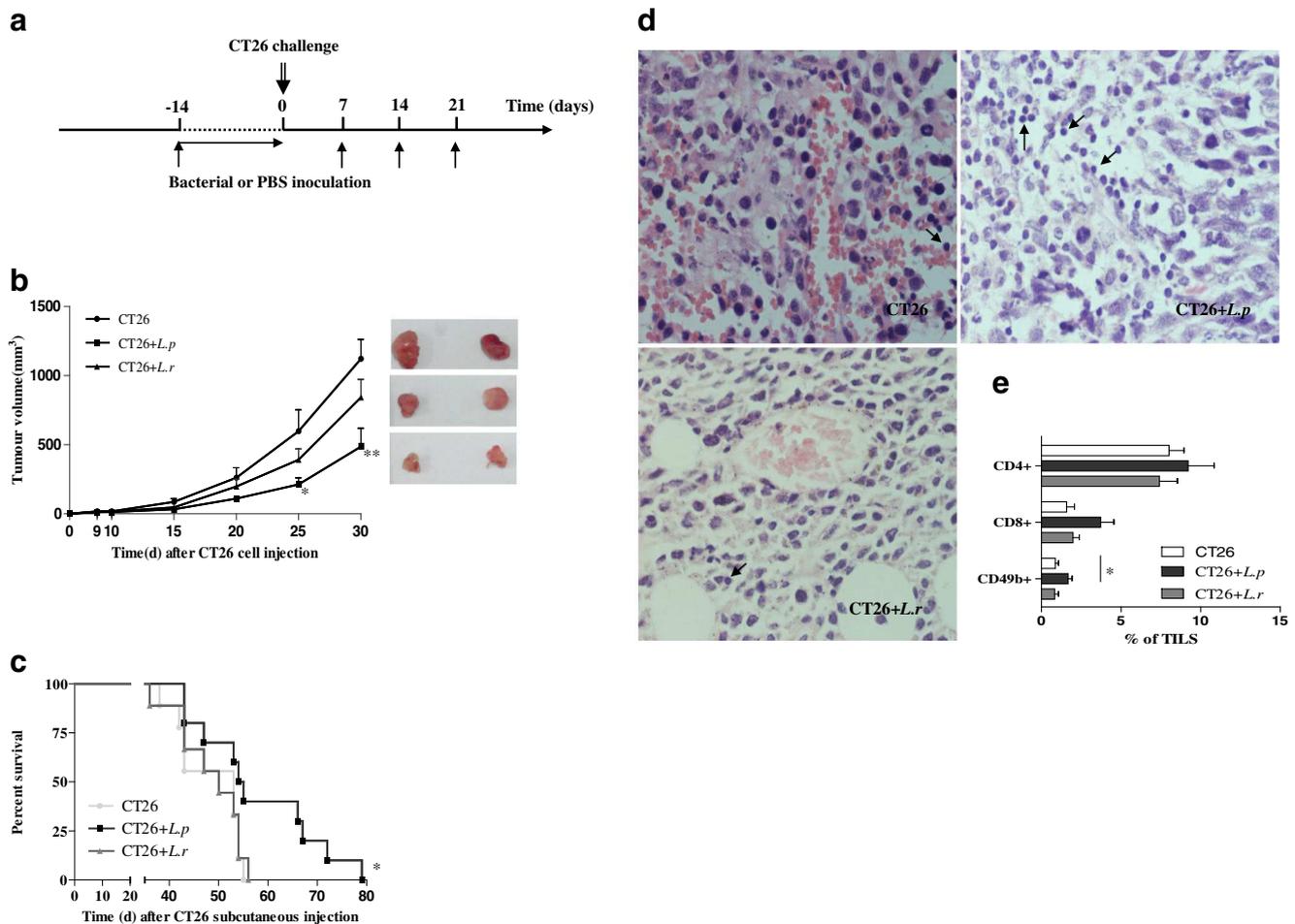


Figure 1. Oral administration of *L. plantarum* suppressed subcutaneous tumour growth in BALB/c mice *in vivo*. (a) 2×10^6 CT26 cells were subcutaneously injected into the right flank of BALB/c mice prior to administration of various *Lactobacillus* strains intragastrically. (b) The tumour volumes were determined from the histograms for CT26, CT26+*L.p* or CT26+*L.r* at 5 day intervals after CT26 subcutaneously injection. Representatives of primary tumours from the CT26 or CT26+*L.p* or CT26+*L.r* groups are shown. (c) The percentage survival curves of *L. plantarum* pre-inoculated mice that survived much longer than CT26-alone group ($P = 0.0396$). (d) Histopathological assessment with hematoxylin and eosin for tumours from the CT26, CT26+*L.p* or CT26+*L.r* groups. The black arrows indicate tumour-infiltrating lymphocytes (TILs). (e) Percentage of the lymphocyte populations of CD3⁺CD4⁺, CD3⁺CD8⁺ or CD3⁺CD49b⁺ cells in the tumour of mice from the CT26, CT26+*L.p* or CT26+*L.r* groups by flow cytometry. All data are presented as the mean \pm SEM. All experiments were performed three times with the similar results. Statistical differences between groups are indicated by the P -values * $P < 0.05$, ** $P < 0.01$.

CT26+*L. plantarum* group (figure 1d). To further explore the effect of *L. plantarum* administration on the tumour microenvironment, single-cell suspensions from tumours were quantitatively analysed using flow cytometry (figure 1e). These data showed an increasing trend towards CD4⁺ and CD8⁺ T cell infiltration upon *L. plantarum* pre-inoculation compared to PBS treatment (9.22±1.622 vs. 8.02±0.9313%, $p=0.5391$; 3.72±0.8285 vs. 1.58±0.4974%, $p=0.0577$, respectively). Importantly, the NK cell subpopulation defined as CD3⁺CD49b⁺ was significantly increased in the subcutaneous tumour upon pre-inoculation with *L. plantarum* compared to the PBS treatment (1.68±0.2498 vs. 0.88±0.1985%, $p=0.0365$).

3.2 Administration of *L. plantarum* retards CT26 development in orthotopic tumour-bearing mice

To further estimate the anti-tumour effect of probiotics, we implanted CT26 subcutaneous tumour pieces into the cecum to generate orthotopic CRC xenografts. After 7 day of post-implantation, we observed the xenograft in intestinal using a digital color Doppler ultrasonic diagnostic imaging, and saw it growing on the cecum with diameter variation of 0.3–0.6 cm for individuals (figure 2a). No metastases were observed in the orthotopic implantation model, but a significant decrease in tumour volume was observed in the CT26+*L. plantarum* group compared to the CT26 alone group at 7, 14, and 21 day (44.28±10.25 vs. 95.67±16.79 mm³, $p=0.0176$ at 7 day; 188.5±44.29 vs. 344.5±59.11 mm³, $p=0.0489$ at 14 day; 430.7±86.44 vs. 708.6±96.75 mm³, $p=0.046$ at 21 day) (figure 2b). The tumour weight in the CT26+*L. plantarum* group at 21 day post-implantation was reduced by 45.1% compared to the CT26 alone group (0.8248±0.1385 vs. 1.503±0.2898 g, respectively, $p=0.049$) (figure 2c). The tumour size in the CT26+*L. rhamnosus* group did not significantly differ from the CT26 alone group ($p>0.05$). These results suggested that *L. plantarum* was also efficient in suppressing CT26 in the intestinal tract.

Histopathology results showed obvious necrosis and lymphocyte infiltration in the CT26+*L. plantarum* group. However, necrosis was also observed in the CT26+*L. rhamnosus* and CT26 groups with less lymphocyte infiltration (figure 2d). Quantitative flow cytometry analyses on the tumour-infiltrating lymphocytes demonstrated that the percentage of CD4⁺ and CD8⁺ T cells in tumour tissue from the CT26+*L. plantarum* group were increased compared to the CT26 alone group; however, the difference was not significant ($p>0.05$). Moreover, *L. plantarum* pre-inoculation increased granzyme B-secreting CD8⁺ T cell infiltration compared to the PBS treatment (2.75±0.4622 vs. 1.4±0.3486%, $p=0.0315$). We also observed a remarkable increase in CD49b⁺ cell percentages upon pre-inoculation with *L. plantarum* compared to CT26 alone

(2.13±0.284 vs. 1.35±0.2197%, respectively, $p=0.0435$). In particular, significant enhancement of granzyme B-secretion from CD49b⁺ cells was observed in tumours of the CT26+*L. plantarum* group (1.98±0.3047 vs. 0.90±0.2820%, respectively, $p=0.018$). There was a trend towards granzyme B-secreting CD49b⁺ or CD8⁺ T cells upon CT26+*L. rhamnosus* treatment ($p>0.05$). These data indicated that *L. plantarum* promoted the accumulation of CD8⁺ T and NK cells, and particularly elevated granzyme B-secreting cells in the tumour microenvironment.

3.3 Effect of lactobacilli on spleen T-lymphocyte, NK subpopulations and IFN- γ production

To determine whether *Lactobacillus* influenced immune cell subpopulations capable of retarding tumour growth, we evaluated CD3⁺ CD4⁺ and CD3⁺ CD8⁺ cells from spleens of orthotopic tumour-bearing mice using flow cytometry (figure 3a). Subpopulations of CD4⁺ T cells showed no significant changes in the CT26+*L. plantarum* or CT26+*L. rhamnosus* groups compared with CT26 alone group (66.09±2.05 vs. 60.7±1.542%, $p=0.05$; 59.81±1.332 vs. 60.7±1.542%, $p=0.6675$, respectively); however, the percentage of CD8⁺ T cells were significantly decreased in the CT26+*L. plantarum* group (19.08±0.9394 vs. 26.55±0.9643%, respectively, $p<0.01$). The percentage of CD8⁺ T cells in the CT26+*L. rhamnosus* group were similar to those in the CT26 alone group ($p>0.05$). Consequently, a significant increase in CD4⁺/CD8⁺ T cell ratio was observed following treatment with *L. plantarum* compared with PBS (3.557±0.2295 vs. 2.323±0.1158, respectively, $p<0.01$). However, we did not observe a significant increase in the CT26+*L. rhamnosus* group ($p>0.05$).

To determine the percentage of NK cells after the treatment with lactobacilli, we analysed CD3⁺ CD49b⁺ expression on spleen cells using flow cytometry (figure 3b). A significant increase in NK cells was observed in the CT26+*L. plantarum* group compared with the CT26 alone group (18.57±2.211 vs. 12.09±1.705%, respectively, $p=0.0316$). An increasing trend in NK cells, which was not significant, was observed in the CT26+*L. rhamnosus* group ($p>0.05$).

A major source of IFN- γ secretion is associated with NK cell activation. If NK cells had been activated, we would expect increased IFN- γ production from splenocytes. A significant increase in the percentage of IFN- γ production was observed in the CT26+*L. plantarum* group compared to the CT26 alone group (4.28±0.5401 vs. 2.483±0.5088%, $p=0.0262$) (figure 3c). ELISA results also revealed that *L. plantarum* up-regulated IFN- γ secretion from splenocytes compared with PBS (14.17±1.792 vs. 9.17±0.7194 pg/mL, $p=0.0185$) (figure 3d).

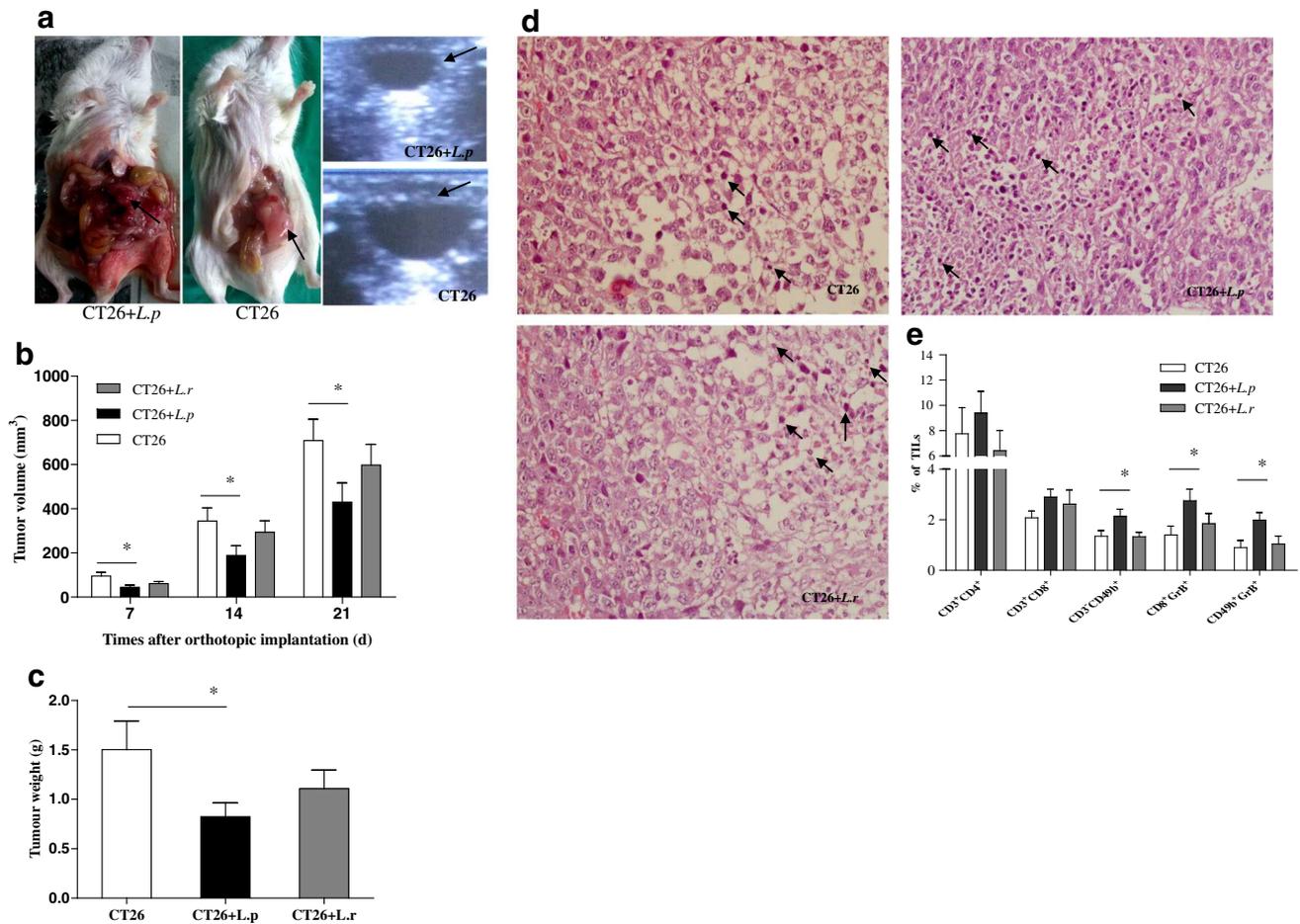


Figure 2. Oral administration of *L. plantarum* inhibits CT26 tumour growth in the orthotopic implantation tumour mouse model. (a) Left: two representative images show orthotopic implantation tumours on the serosa of the cecum (black arrows) from the CT26+*L.p* or CT26 groups. Right: representative abdominal ultrasound images display the tumour sizes in mice from the CT26+*L.p* or CT26 groups. (b) The tumour volumes were determined from the histograms for the CT26, CT26+*L.p* or CT26+*L.r* groups at 7 day intervals after abdominal surgical tumour implantation using abdominal ultrasound images. (c) The tumour weights were measured 21 day after tumour surgical implantation. (d) Histopathological assessment with hematoxylin and eosin for intestinal tumours from the CT26, CT26+*L.p* or CT26+*L.r* groups. The black arrows indicate tumour-infiltrating lymphocytes. (e) Effect on the number of tumour-infiltrating lymphocytes for *Lactobacillus* pre-inoculation. After 21 days of tumour inoculation, the percentages of CD3⁺CD4⁺, CD3⁺CD8⁺ or CD3⁺CD49b⁺ (NK) cells, or granzyme B-secretion from CD8⁺ and NK (CD49b⁺) cells from tumour tissues, were determined using flow cytometry.

3.4 Induction of DC maturation and Th1 cytokine production by *L. plantarum*

The orthotopic tumour-bearing mice were used to assay the effect of inoculation with lactobacilli on DC maturation in spleen. Cells were stained for CD11c⁺CD80⁺ or CD11c⁺CD86⁺ (figure 4a). To exclude the possibility that tumour-related elements could enhance DC activation, we added one group of normal mice treated with PBS (instead of lactobacilli) as control. The percentage of CD11c⁺ DCs from the CT26+*L. plantarum* group mice was significantly increased compared to that from the CT26 group (4.321±0.4535 vs. 3.039±0.3309%, $p=0.0348$). In addition, both CD80⁺ and CD86⁺

expression in the CT26+*L. plantarum* group were up-regulated compared to that of the CT26 alone group (7.333±0.5406 vs. 3.989±0.3447%, $p<0.0001$; 36.79±1.449 vs. 29.35±1.559%, $p=0.0026$, respectively) (figure 4b). When CT26 was compared with control, data of CD80⁺ and CD86⁺ expression were similar (3.989±0.3447 vs. 3.547±0.3153%, $p=0.3566$; 29.35±1.559 vs. 29.22±2.155%, $p=0.9618$, respectively). These results suggested that *L. plantarum* induced DC maturation in the tumour environment.

The potent anti-tumour activity of *L. plantarum* encouraged us to further explore whether activated DC could induce CD4⁺ T cells to produce protective cytokines to resist tumour growth. IFN- γ , IL-4 and IL-17 production was

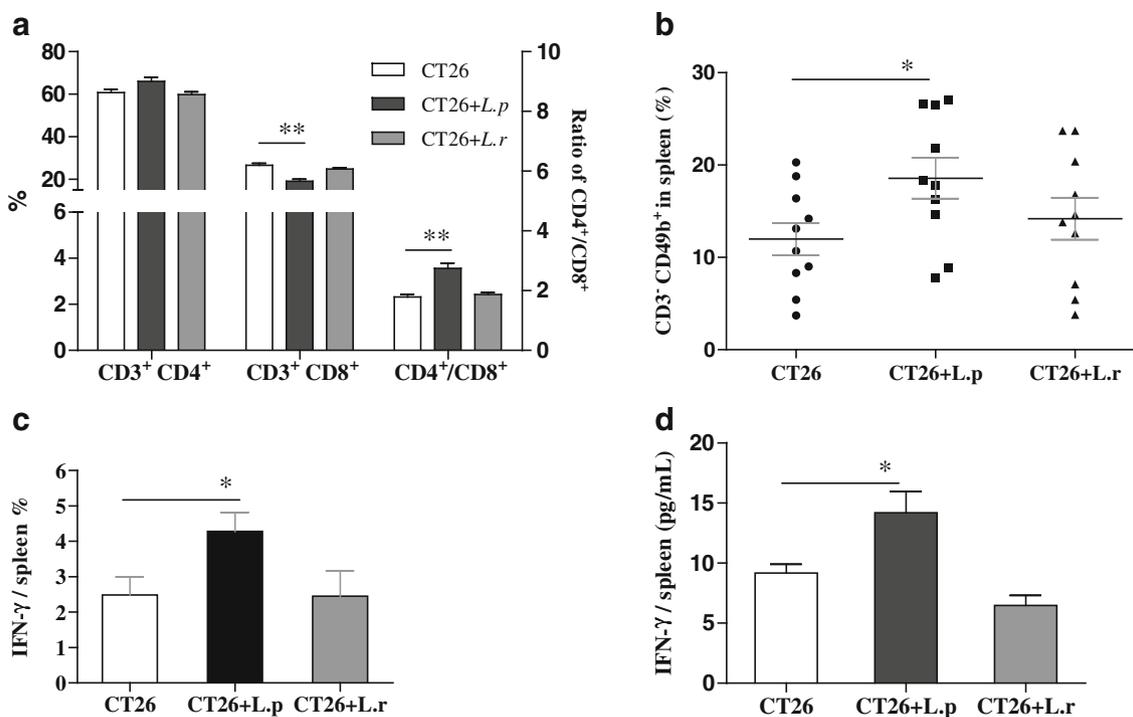


Figure 3. Effect of lactobacilli on T-lymphocyte or NK subpopulations and IFN- γ production of spleen from orthotopic tumour-bearing mice. (a) The percentages of CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells in the quadrants and their ratio are shown in histograms. (b) The percentages of CD3⁺CD49b⁺ cell displayed as histograms. (c) The histograms show the concentrations of IFN- γ from splenocytes by flow cytometry. (d) IFN- γ from splenocyte was determined by ELISA.

examined using flow cytometry (figure 4c). The results demonstrated increased IFN- γ production after induction with *L. plantarum*-activated DCs compared with non-induction (1.947 ± 0.1701 vs. $1.38 \pm 0.1869\%$, $p=0.0377$), whereas IL-4 and IL-17 secretion was not affected by treatment with *L. plantarum* (figure 4d). These data demonstrated that *L. plantarum* induced DCs to promote CD4⁺ T cell production of Th1-type cytokines, thereby contributing to the inhibition of tumourigenesis.

4. Discussion

CRC is one of the most preventable forms of cancer in the world (Wang *et al.* 2014). Epidemiological studies have reported that consumption of fermented dairy products may elicit anti-tumour effects and are associated with a lower incidence of CRC (Geier *et al.* 2006; Narushima *et al.* 2010). However, there are also inconsistent statements regarding the anti-tumour effects, which may be associated with different bacterial strains, culture conditions, and different experimental protocols. Nevertheless, probiotics are still being investigated for cancer prevention (Chien *et al.* 2012). Before a specific probiotic strain can be considered part of a cancer treatment regimen, its

function needs to be properly assessed. Studies have demonstrated that *Lactobacillus acidophilus*, *L. casei*, *L. plantarum*, and *Bifidobacterium longum* can elevate anti-tumour immunity (Lee *et al.* 2004). However, some probiotics, such as baker's yeast, VSL#3, may not effectively reduce cancer cell development (Ghoneum and Gollapudi 2004; Arthur *et al.* 2013). Therefore, more effective probiotic strains should be selected to test for the possibility of anti-tumour immunity activation and CRC prevention.

In this study, two isolated strains of *L. plantarum* and *L. rhamnosus* were used as versatile lactic acid bacteria in traditional fermented products (Yu *et al.* 2011; Qureshi *et al.* 2014) and some species of these strains potentially regressed CRC (Geier *et al.* 2006; Seow *et al.* 2010; Li *et al.* 2012).

Most relevant studies orally administer *Lactobacillus* to mice prior to tumour challenge for approximately 2 weeks (at a concentration of 2×10^8 CFU) or for three consecutive weeks using a seven days on/three days off regimen (at a concentration of 2×10^8 CFU) (Chien *et al.* 2012, Yazdi *et al.* 2013). Based on previous reports, lactobacilli may require time to establish adequate colonization. Therefore, we chose a 2 week treatment protocol prior to tumour implantation to establish adequate probiotic colonization.

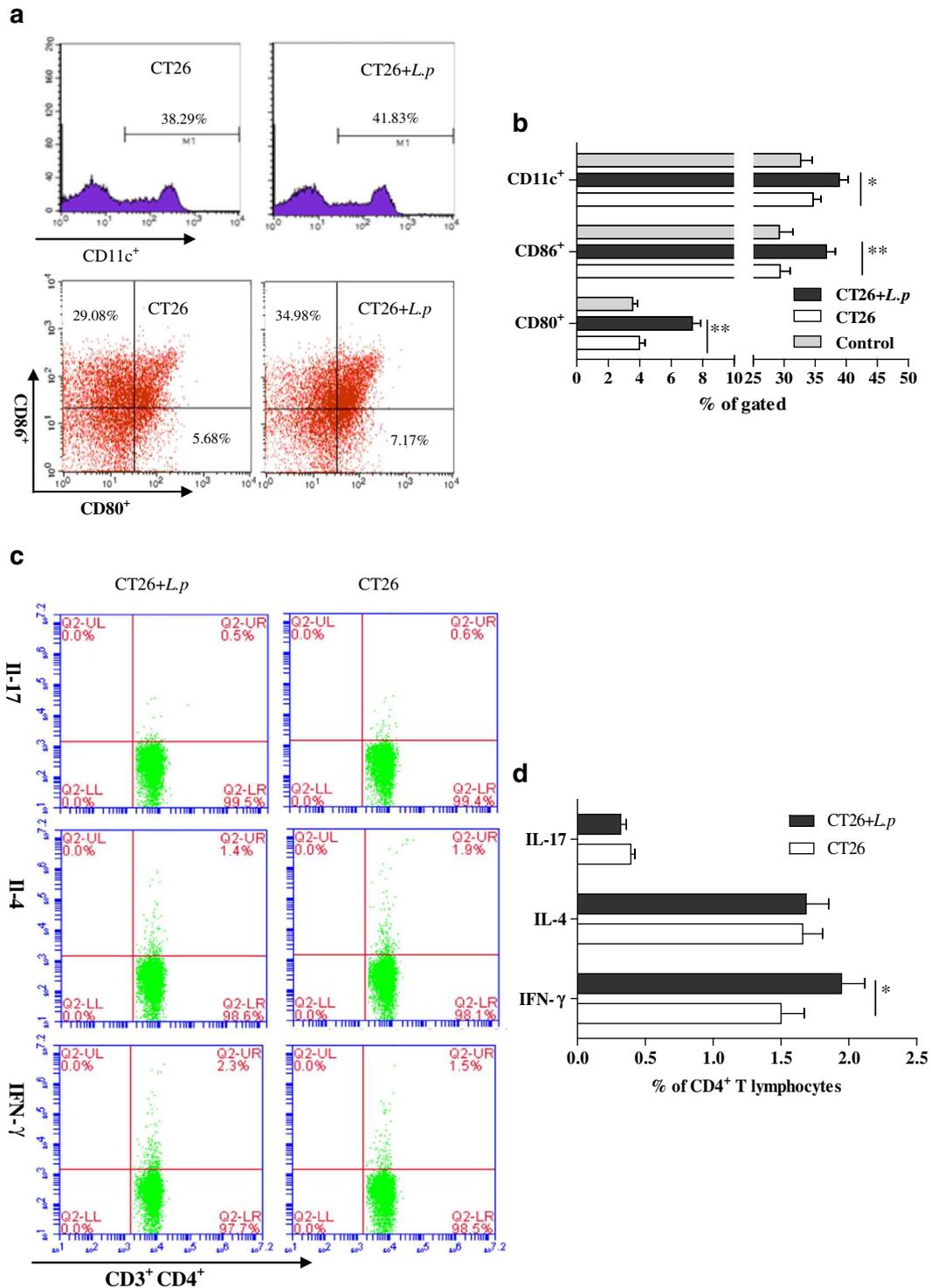


Figure 4. *L. plantarum* pre-inoculation induced DCs maturation and Th1 cytokines production. Ten normal mice treated with PBS (in place of lactobacilli) without implanting CT26 as control. 21 days after CT26 tumour inoculation, DC percentages and maturation profiles were determined in the spleen. (a) Representative flow cytometric analysis of CD11c⁺ (above) and CD80⁺ or CD86⁺ among CD11c⁺ cells (below) in the spleen. (b) The histograms show the percentages of CD11c⁺, CD11c⁺CD80⁺ or CD11c⁺CD86⁺ cells in the quadrants. *L. plantarum*-activated DCs could induce Th1-type cytokines production. (c) Representative flow cytometric analysis for determining the production of IFN-γ, IL-4 or IL-17 from CD4⁺ T cells 21 days after tumour initiation. (d) The histograms show the percentages of IFN-γ, IL-4 or IL-17 production in each quadrant.

In our experiments, we observed that pre-inoculation with *L. plantarum* A significantly reduced tumour growth, prolonged survival time, and activated innate immunity. We also observed that *L. rhamnosus* b affected tumour volume within 25 days after the CT26 challenge, with a slight decrease of tumour volume occurring after 25 days. However, *L. rhamnosus* had poor extended protective effects according to survival analyses, which may be associated with strain diversity and niche adaptation. This isolate may be more susceptible to alterations in consecutive batches or loss of its biological functions (Francois *et al.* 2013).

Infiltration of immune effector cells (such as T cells and NK cells) into tumours suppresses tumour cell growth (Hu *et al.* 2014). We observed that *L. plantarum* administration led to the modification of tumour microenvironment. Specifically, we found increased intratumoural levels of NK cells and granzyme B-secreting CD8⁺ T cells. Therefore, *L. plantarum* might facilitate accumulation and elevate effector functions for CD8⁺ T and NK cells in the tumour microenvironment. We also observed this increase in the CT26+ *L. plantarum* group from histopathology results.

Flow cytometric analyses showed that *L. plantarum* treatment elevated the percentage of CD4⁺ T cells and decreased CD8⁺ T cell populations in spleens from orthotopic tumour-bearing mice. Accordingly, *L. plantarum* increased the ratio of CD4⁺/CD8⁺ cells significantly, suggesting that *L. plantarum* might activate Th cells more (Huan Shen *et al.* 2013); this is consistent with previous study (Maroof *et al.* 2012). Additionally, *L. plantarum* enhances NK activity through endogenous tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) production, which might be beneficial for malignant tumour prevention (Mano Horinaka *et al.* 2010). In our study, we found that the number of NK cells was significantly increased after administering *L. plantarum* to orthotopic tumour-bearing mice. IFN- γ production was mainly associated with activated NK cells, which can further promote DC maturation and CD4⁺ T cells differentiation towards Th1 type (Gao and Xiong 2011). The result of an increase of IFN- γ from spleen in the CT26+ *L. plantarum* group encouraged us to study the effect further.

Previous reports showed that *Lactobacillus* were able to activate myeloid dendritic cells (MDCs) to induce strong T cell immune responses (Mohamadzadeh *et al.* 2005) and also could increase CD40 and CD80 expression to treat abnormal immature phenotype of colonic DC for ulcerative colitis (Al-Hassi *et al.* 2014). In this study, we found that *L. plantarum* significantly induced DC activation and maturation ($p < 0.05$). Although the CT26 tumour challenge slightly increased CD11C⁺ subpopulations, activation of DC was not observed compared to control. This result was also consistent with our previous study that *L. plantarum* A activate the

maturation of DC for BALB/c mice without tumour challenge (in press).

Previous studies have demonstrated that lactobacilli can activate innate immunity cells (e.g. DC), thereby affecting Th1 and Th2 type pathways and reconstructing the balance in the local and systemic immune environment (Maassen *et al.* 2000; Mohamadzadeh *et al.* 2005, 2008). Moreover, different species of lactobacilli can elicit a differential cytokine production to drive the polarization of T cells towards Th1, Th2, Th17 or regulatory T cells (Bäuerl *et al.* 2013). We found that *L. plantarum*-activated DCs promoted Th1-type cytokine secretion with a significant increase in IFN- γ production, whereas IL-4 and IL-17 production was not affected. These results demonstrated that *L. plantarum*-activated DCs facilitate polarization of T cells towards the Th1 response to promote humoral or cell-mediated immunity. Therefore, *L. plantarum*, as a strain of probiotics, has a potential benefit on activating host's immunity to resist tumour growth in this study, which is consistent with reports on the immunity effect of *L. plantarum*. However, further studies on the anti-tumour mechanism of *L. plantarum* in vitro are also needed.

In conclusion, *L. plantarum* retarded the growth of CT26 tumours and enhanced the innate immune response in BALB/c mice. *L. plantarum* treatment resulted in reduced tumour size, prolonged survival, and promoted CD8⁺ and NK cell migration into tumour tissues. Moreover, *L. plantarum* promoted DC maturation to induce polarization of CD4⁺ T cells towards Th1 response, thereby contributing to protection against colon carcinogenesis in tumour-bearing mouse model. However, *L. rhamnosus* did not elicit significant anti-tumour response.

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