L.reuteri JMR-01 adjuvant 12C6+ irradiation exert anti-colon carcinoma effects by modulating the gut microbiota in mice

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Abstract

Probiotics such as Lactobacillus can modulate the intestinal microbiota and have been considered as an effective strategy for ameliorating colon carcinoma; nevertheless, its efficiency remains the biggest challenge. We investigated the efficacy of JMR-01 adjuvant $^{12}C^{6+}$ irradiation on the treatment of mice bearing CT-26 and clarified the underlying mechanisms including modulating immune cells and intestinal flora. Anti-proliferation effect of live probiotic JMR-01 and inactived probiotic (LP + IP) on CT-26 reached a maximum of 39.55% among other experiment groups at 24 h when the ratio of cell to CFU was 1:1 in vitro, which was at a high level to our knowledge. These activities have been fully validated in vivo, mice treated by $^{12}C^{6+}$ irradiation + LP + IP (IR + LP + IP) for fifty-day held the highest survival rate (71.4%) and the cure rate (14.3%), which were higher than that in the reported literatures. Additionally, we found that the strongest stimulation index of splenocyte (2.47) and the phagocytosis index peritoneal macrophage (3.68) were achieved by LP + IP compared with single LP and IP. We demonstrated a significantly decreased abundance of *Bacteroides fragilis*, *Clostridium perfringens* and the increased abundance of *Lactobacillus* and *Bifidobacterium* in mice administrated of LP + IP via plate counting of microbial in fecal. Moreover, fecal enzyme activities were dramatically decreasing and short chain fatty acids (SCFA) concentrations were significantly increasing in IR + LP + IP group compared with tumor control group, which was consistent with the trend of plate counting results. JMR-01 LP + IP adjuvant $^{12}C^{6+}$ irradiation could mitigate cancer progression by modulating immune cells and intestinal flora.

1. Introduction

Colon cancer is the third most common cancer, being surpassed by only lung and breast cancers, and the leading cause of death worldwide[1]. Despite numerous therapeutic options including surgery, chemotherapy, radiotherapy and immunotherapy, the prognosis of colon cancer remains poor and there is a high frequency of recurrence. Therefore, the safe, economic and effective treatments are urgently needed for colon carcinoma. Over the past few decades, studies have demonstrated the association of colon cancer with dysbiosis of the gut microbiota[2-4]. Therefore, modulating gut microbiota has been viewed as a promising target for treating digestive diseases, especially colon cancer[5].

Probiotics, especially Lactobacillus, have been proven to exert immunomodulatory, anti-inflammatory and anticarcinogenic activities when administered in proper dose [6, 7]. Furthermore, probiotics supplements not only enhanced the actions of chemotherapy and immunotherapy but also reduced the side effects of these medications[8, 9]. In a randomized study of 150 patients with colorectal cancer receiving 5-FU-based chemotherapy, L. rhamnosus GG supplementation reduced episodes of severe diarrhoea and abdominal discomfort[10]. In addiation, multiple publications have reported a role for probiotics in enhancing responses to immune checkpoint blockade used to treat colon cancer[11-13]. Lukas isolated Bifidobacterium pseudolongum, Lactobacillus johnsonii, and Olsenella, which could significantly enhance efficacy of immune checkpoint inhibitors[14]. Therefore, the combination of traditional therapeutics and probiotics may be a potential method for the treatment of colon cancer.

It is well known that radiotherapy is essential in the treatment of primary or recurrent colon carcinoma, but is often limited by the tolerance doses of the organs at risk. However, charged particle beams such as carbon ions exhibit substantial physical and biological advantages compared to conventional photon irradiation[15]. Carbon ion exhibit a “Bragg Peak,” a characteristic energy deposition curve where there is very little energy deposited proximal or distal to a target. Instead, the majority of energy is released at a specific depth[16]. Carbon ions distribute more energy during their travel through the tissue. This property is called linear-energy-transfer (LET) and carbon ions have a higher LET than protons[15]. The biological advantage of a higher LET is the increased likeliness of double-strand DNA breaks resulting in a higher biological effectiveness[17]. Thus, carbon ion radiotherapy with less toxic, more effective and higher cure rate due to its physio-biological characteristics is another selection for tumors that are not suitable for surgical and have poor or tolerable effects of conventional radiotherapy[18, 19]. Although the function of respective probiotic and irradiation had been investigated by a number of studies in vivo and in vitro, whether probiotics supplementation adjuvant 12C6+ irradiation could produce sufficient therapeutic effects on colon carcinoma was still not clear.

The aim of this study was to investigate the efficacy of JMR-01 against CT-26 cells in vitro and JMR-01 adjuvant 12C6+ irradiation on the treatment of mice bearing CT-26. To clarify the suggested mechanisms, we assessed the immunomodulatory effects of JMR-01 including the index of macrophage as well as lymphocyte proliferation index. In addition, the abundance of specific gut microbiota in fecal of experiment mice, the activity of the fecal enzymes and the concentrations of fecal SCFA metabolites were measured. It might provide a new safe and effective therapeutic intervention to alleviate colon carcinoma.

2. Material And Methods

2.1 Preparation of cells and probiotics

CT-26 cells were purchased from Shanghai Cell Bank and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. CT-26 cells were maintained in a 5% CO2 humidified chamber at 37℃.

L.reuteri JMR-01（NCBI Accession No. MT362007）was isolated from the fecal of cured mice bearing breast tumor. JMR-01 cultured in deMan, Rogosa and Sharpe (MRS) broth at 37℃ for 18 h; living probiotic JMR-01 (LP) was the precipitate harvested by centrifuging the fermentation solution at 8000 r/min after discarding supernatant, and the precipitate was then resuspending in a volume of phosphate-buffered solution (PBS) equal to supernatant and mixing evenly; living probiotic cell （LP-C）was obtained from the precipitate by centrifuging LP（living probiotic supernatant (LP-S) was obtained from the supernatant by centrifuging LP. Inactived probiotics (IP) was prepared by autoclaving LP at 105℃ for 30 minutes; inactived probiotic cell（IP-C） was obtained from the precipitate by centrifuging IP（inactived probiotic supernatant (IP-S) was obtained from the supernatant by centrifuging IP. Living probiotic combined with inactived probiotics (LP+IP) was the mixture of LP-C and IP; LP+IP cell （LP+IP-C）was

obtained from the precipitate by centrifuging LP+IP; LP+IP supernatant (LP+IP-S) was obtained from the supernatant by centrifuging LP+IP.

2.2 Cell viability assay

The anti-proliferation effect of JMR-01 on CT-26 cells was examined by using MTT assay[20]. 100 μL of CT-26 cells were seeded into 96-well plates at a concentration of 106 cells/mL and incubated overnight for stabilization. Next 100 μL LP, IP, LP+IP, IP-S and LP+IP-S were added to each well for 24 h to 48 h in air supplemented with 5% CO2 at 37 ℃. The negative control group was treated with RPMI-1640. After 24 h and 48 h, 10 μl of MTT solution (5 mg/ml) was added to each well and incubated for another 4 h. At the end of experiments, the medium was removed and the blue formazan crystals were solubilized with 200 μl of dimethyl-sulfoxide (DMSO, Solarbio), then the absorbance values were measured at 540 nm.

The optimization experiment was divided into 6 groups, which were LP: IP-B = 1:1, 1:2, 1:1/2; LP: IP =1:1, 1:2, 1:1/2cells to CFU. The negative control group was CT-26 cells without adding bacteria. The following experimental steps were abided by MTT assay described above. Inhibition rate = (1 – Atreatment/Acontrol) ×100% (A=absorbance).

2.3 Animal experiment

Male BALB/c mice (6-8-week-old) were obtained from Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. They were maintained in polycarbonate cages under controlled environment conditions at 23±2℃ and 60%–75% relative humidity in a 12 h light/dark cycle. All mice had free access to water and standard commercial rat chow. After one week adaptation period, mice were subcutaneously injected with CT-26 cells (106 CFU/mL, 100 μL/per mouse). When the volume of tumor reached 900 mm3, the mice were then randomly divided into 6 groups including normal group, tumor control group, IR, IR+LP, IR+IP and IR+LP+IP group. Except for normal group, IR and tumor control group were conducted gavage with 100 μL PBS buffer, the other groups were semidiurnal gavaged with 100 μL LP, IP, LP+IP (109 CFU/mL) respectively for 7 days before irradiation to realize the colonization of probiotics in mice and the above treatment was continued after irradiation until the end of the experiment. 12C6+ irradiation was conducted when the tumor volumes in each group reached nearly 1400 mm3. Then, mice in all groups except for normal and tumor control group were put into the mouse fixator, and the body except for tumor was covered with aluminum plate. The mice were irradiated at a dose of 4 Gy and a dose rate of 4 Gy/min with 12C6+ ion beam provided by Heavy Ion Research Facility at Lanzhou (HIRFL). Tumor sizes were monitored every other day and volumes were calculated using the following formula: Volume (mm3) = longest diameter × (shortest diameter)2 /2. The tumor inhibition rate was calculated when there was significantly difference between tumor control group and other treatment group ($P<0.05$). The tumor growth inhibition rates= (Vcontrol - Vtreatment)/ Vcontrol (V= Volume). All animal experiments were approved by the Institute of Modern Physics Animal Ethics Committee.

2.4 Detection the immunomodulatory function of JMR-01

2.4.1 Lymphocytes proliferation assay

Spleen cells were prepared as reported by Li[24]. Briefly, mice were euthanized by euthanasia. The spleens were minced in RPMI-1640 media and filtered through a fine nylon mesh. Mononuclear cells were isolated after lysis of red blood cells. Subsequently, splenocytes of 105 cells/mL were distributed into 96-well plates. Then 100 μL of JMR-01 LP, IP, LP+IP, LP-S, IP-S, (LP+IP)-S (the ratio with cells was 1:1, 1:10, 1:100), 100 μL peptidoglycan (50 μg/ mL, 100 μg/ mL) (Peptidoglycan was obtained by the following steps: First, JMR-01 was boiled for 20 min in 10% TCA. Suspensions were centrifuged at 10000 r/min for 15 min to collect cell wall precipitate without lipoteichoic acid. The precipitate was dissolved in trypsin-phosphate buffer (1 mg / ml, trypsin; 0.1 mo1/L PBS) and incubated at 37℃ overnight. Then centrifuged at 4000 r/min for 20 min and discarded the insoluble protease precipitate. By centrifuging the supernatant at 15000 r/min for 20 min, the crude extract of peptidoglycan was obtained) were added to 96-well plates and incubated at 37℃ for 48 h. Then 10μL CCK8 was added to each well, and the plates were incubated for 4 h. Splenocytes proliferation was measured by determining the absorbance at 570 nm. Proliferation index was calculated according to the following equation: Proliferation index= Test OD570/Control OD570.

2.4.2 phagocytosis of macrophages assay

Cervical dislocation was performed to sacrifice mice. The peritoneal macrophages were collected by lavage with 5 mL physiological saline solution. Macrophages of 105 cells/mL were distributed into 96-well plates. Then 100 μL of JMR-01 LP, IP, LP+IP, LP-S, IP-S, (LP+IP)-S (the ratio with cells was 1:1, 1:10, 1:100), 100 μL peptidoglycan (50 μg/ mL, 100 μg/ mL) were added to 96-well plates and incubated at 37℃ for 48 h. 100 μL 0.075% neutral red solution was added to each well at 48 h and the macrophages were washed with PBS to remove remanent neutral red after 0.5 h, then 100μL of cell lysis buffer (acetic acid:ethanol=1:1) was added to each well[25]. Phagocytosis was measured by determining the absorbance at 540 nm using an automated microplate reader, and calculated according to the following equation: Phagocytosis index= Test OD540/Control OD540.

2.5 The regulation of gut microbiota by JMR-01

2.5.1 Fecal bacterial counts

To detect the number of intestinal flora, the fecal samples were weighed, homogenized, serially diluted and plated on selective agar according to the implementation manual of technical specifications for inspection and evaluation of health food of the People's Republic of China (2003). After overnight incubation at 37℃, bacteria colonies were counted. Bifidobacterium BS Medium was used to analyze fecal Bifidobacterium. DeMan, Rogosa, and Sharpe agar (MRS) was used to assess Lactobacillus. In order to explore Enterobacterium, Enterococcus, Bacteroides fragilis, and Clostridium perfringens, fecal sample were plated on Eosin-Methylene Blue Agar, Bile Esculin Azide Agar, BBE agar and Clostridium Perfringens Medium respectively. Fecal bacteria count was expressed as log10 CFU/g feces.

2.5.2 Fecal enzyme assay

To determine fecal enzyme activities, fecal was collected at the beginning of treatment, at 16th day of treatment (tumor volumes of IR+IP group were significantly higher than PBS ($P<0.05$)), at 30th day of treatment (the tumor volumes of LP, IP and LP+IP were significantly higher than PBS ($P<0.05$)), at 40th day of treatment (the survival rate was 50% in tumor control group) and at 50th day of treatment (the end of experiment). The fecal samples were frozen immediately after collection and stored at −80°C. Preweighted fecal were homogenized in PBS buffer and then centrifuged at 8000 r/min for 5 min. Its supernatant placed into tubes containing 100 μL 15 mmol/L p-nitrobenzoic acid solution, 500 μL 0.2 mol/L PBS. The incubations were performed at 37℃ for 30 min in the water bath. The reaction was stopped via adding 1mL 20% trichloroacetic acid and read at 240 nm with microplate reader. The amount of decomposition of p-nitrobenzoic acid was calculated from the standard curve of p-aminobenzoic acid concentration, and the enzyme activity was calculated.

2.5.3 Determination of short chain fatty acids (SCFA) in mice fecal

Fecal samples (100 mg) were diluted in 1 mL 0.1% phosphoric acid. Samples were homogenized and centrifuged at 10000 rpm for 10 min at 4℃. Supernatants were passed through 0.45 μm-pore-size nitrocellulose filters. SCFA (acetate, propionate, butyrate) determination was performed by using high-performance liquid chromatography (HPLC) equipped with a C18 Column 300 × 7.8 mm and a UV detector at 210 nm. 20 μl of each sample was injected into the HPLC system and 0.1% Phosphoric acid was used as a mobile phase at a flow rate of 0.5 mL/min. The concentrations of SCFAs were estimated using the linear regression equations generated from acetic, propionic, and butyric acids standard curves. SCFAs concentrations were expressed in mM/g.

2.6 Statistical analysis

The statistical analysis were performed using the GraphPad PRISM 8.0.1 (GraphPad Inc., California, CA, USA), SPSS 20.0 (SPSS Inc., Chicago, IL, USA) and the Origin 9.0 (Origin Lab Corp., Northampton, MA, USA). The number of mice in each group was 14 (normal group=8), cell viability assay were carried out six replicates, and the other experiments were carried out three replicates. The data were presented as means ± standard deviations (SD).

3. Results

3.1 The effect of JMR-01 on CT-26 cells proliferation

The anti-proliferation effects of JMR-01 LP, IP, LP+IP were investigated in vitro using CT-26 cells. According to Fig.1, JMR-01 LP held higher inhibitory effects and the inhibition rate reached to 27.4% at 24 h ($P<0.05$), but its inhibitory effect decreased dramatically at 48 h. The most pronounced anti-proliferative effects were induced by JMR-01 IP-C (25.7%), IP-S (25.7%) and IP (23.8%) at 48 h. LP+IP-S had no

inhibitory effect on CT-26 at 24 and 48 h as shown in Fig.1. Thus, we combined LP with IP-C and IP to obtain the higher inhibition effect on CT-26 cells proliferation.

To screen the optimal combination of JMR-01, the inhibitory effects on CT-26 cells were performed by using JMR-01 LP and IP, LP and IP-C at a ratio of 1:1, 1:1/2, 1:2 at 24 h and 48 h. As shown in Fig.2, the strongest inhibition rate was achieved by LP: IP =1:1 at 48 h (39.55%). Therefore, we choosed LP: IP =1:1 as the choice used in animal model.

3.2 The anti-carcinoma effect of JMR-01 adjuvant $^{12}C^{6+}$ irradiation in vivo

The anti-tumor effect of JMR-01 was performed in colon carcinoma mice models. As shown in Fig. 3A, we observed smaller tumor size in mice treated with IR, IR+LP, IR+IP, IR+LP+IP compared with tumor control group during 10-50 d of treatment, indicating that $^{12}C^{6+}$ irradiation and JMR-01 adjuvant $^{12}C^{6+}$ irradiation could retard the tumor development. On day 34 of treatment, the tumor inhibitory rates in IR+LP+IP reached 54.2%, which was higher in comparsion with IR (37.9%), IR+LP (48.9%) and IR+IP (49.3%), indicating that JMR-01 especially LP+IP could improve the efficiency of $^{12}C^{6+}$ irradiation. The survival curves of each group were shown in Fig. 3B. At the end of experiment, the survival rates in tumor control group, IR and IR+LP groups were 35.7%, 50% and 42.9%, respectively. However, the survival rates of IR+LP+IP and IR+IP groups reached 71.4%. Surprisingly, the cure rate in IR+LP+IP group was 14.3% but no cured mice in other groups. These results indicated that JMR-01 LP+IP adjuvant $^{12}C^{6+}$ irradiation could extently block tumor progression, and LP played an important role in tumor recovery. The results suggested that JMR-01 especially LP+IP adjuvant $^{12}C^{6+}$ irradiation therapy had the ability to prolong the survival of bearing tumor mice, and enhance the tumor inhibitory effects.

3.3 Detection the immunomodulatory function of JMR-01

3.3.1 Effect of JMR-01 on splenocytes proliferation

To understand the immunomodulation function of JMR-01, their effects on splenocytes proliferation were performed. As shown in Fig. 4A, splenocytes proliferative index receiving high-dose LP+IP (2.47) and IP (1.82) were significantly higher than that in the high-dose LP groups (1.37) ($P<0.05$), suggesting JMR-01 LP, IP especially LP+IP could stimulate splenocytes proliferation. Due to probiotics bacterial extracts and supernatants maintaining their main probiotic properties at the intestinal level after heat treatment in most cases [21, 22], thus we subsequently measured the effects of supernatants on splenocytes. As shown in Fig. 4B, the supernatant of high-dose LP+IP (1.8) and IP (1.54) possessed higher lymphocytes proliferative indexes compared with high-dose LP（1.11）and there was significant difference between LP+IP and LP ($P<0.05$). Research showed that heat-treatment could lead to rupture of cell walls, with the release of cell wall components mainly peptidoglycans, which played key immunomodulating roles[23]. Thus, we extracted peptidoglycans from JMR-01 and determined its effects on splenocytes proliferation. According to Fig. 4C, peptidoglycans with concentration-dependent in promoting the splenocytes

proliferation. The results showed that all high dose JMR-01 LP, IP especially LP+IP had capacity to stimulate splenocytes proliferation.

3.3.2 Effect of JMR-01 on phagocytic activity of macrophage

To investigate the effects of JMR-01 LP, IP, LP+IP on phagocytic function of peritoneal macrophage, the phagocytosis index was measured. As shown in Fig. 5A, the phagocytosis index in high dose of LP +IP (3.68) and IP (3.27) group were higher than that in LP group (2.29), and there was significance between LP+IP and LP ($P<0.05$), suggesting JMR-01 LP, IP especially LP+IP could stimulate splenocytes proliferation. To further investigate whether the supernatants of JMR-01 IP and LP+IP could activate macrophage, we measured the phagocytic capacity of phagocyte absorbing neutral red. As shown in Fig. 5B, the phagocytosis index in the supernatant of high-dose LP+IP (3.48) and IP (2.72) were higher than that high-dose LP (1.49), and there was significance between LP+IP and LP ($P<0.05$). Furthermore, we obtained peptidoglycans from JMR-01 and determined its effects on the function of peritoneal macrophage. According to Fig. 5C, peptidoglycans extracted from JMR-01 LP had the ability of promoting the phagocytic function of peritoneal macrophage, and phagocytic function was enhanced by increasing concentrations. The results showed that JMR-01 LP, IP, especially LP+IP had capacity to promote the phagocytosis of peritoneal macrophage.

3.4 The regulation of gut microbiota by JMR-01

3.4.1 Fecal bacterial counts

To evaluate whether JMR-01 could regulate the intestinal microbiota in colon carcinoma mice. The amount of *Bacteroides fragilis, Clostridium perfringens, Enterobacter, Enterococcus, Lactobacillus* and *Bifidobacterium* were measured (Fig. 6). The results showed that the abundance of gut microbiota in experiment groups particular tumor control group were different from normal group. Compared with IR+LP, IR+IP, IR+LP+IP groups, the abundance of *Enterobacter*, *Clostridium perfringens* and *Enterococcus* were significantly increased in tumor control group ($P<0.05$). Meanwhile, the *Bacteroides fragilis* abundance in tumor control group were significantly higher than that in IR+LP and IR+LP+IP groups ($P<0.05$). However, the abundance of *Lactobacillus* in IR+LP+IP group was significantly higher than that in IR and tumor control groups ($P<0.05$) and the *Bifidobacterium* abundance in IR+LP+IP group came back to the normal level. These results suggested that the abundance of gut microbiota in tumor-bearing mice were markedly varied, while JMR-01 LP+LP+IP intervention can modulate intestinal flora imbalance induced by colon cancer and restore to the healthy level.

3.4.2 Fecal enzyme activities assay

As shown in Fig. 7, fecal enzyme activities in each group especially tumor control group were increased compared with normal control group, suggesting fecal enzymes were closely related to the occurence of colon carcinoma. The results indicated that IR, IR+LP, IR+IP, IR+LP+IP treatments reduced the activities of fecal enzymes compared with tumor control group. In particular, IR+LP+IP group showed a marked

decreasing nitroreductase activities in comparision with other experiment groups. At 0-16 d, IR+LP+IP group showed a slow growth rate compared with tumor control group and other treatment group. During 16-30 d, fecal enzyme activities were increasing with the tumor rapid growth except for IR+LP which may be owing to the death of half the mice in LP group. During 30-50 d, the survival rates of tumor mice were decreasing due to the death of mice with larger tumor volumes, which may led to decreased fecal enzyme activities. The results indicated that JMR-01 adjuvant $^{12}C^{6+}$ irradiation could decrease fecal enzyme activities.

3.4.3 Fecal short chain fatty acids (SCFAs) measurements

As shown in Fig. 8, the concentrations of acetic acid and propionic acid were markedly increased in IR, IR+LP, IR+IP, IR+LP+IP compared with tumor control group. Especially, the levels of the fecal acetic acid and propionic acid in the fecal of IR+LP+IP-treated mice markedly increased compared with tumor control group (acetic acid: 196.05 vs 53.93 mM/g, $P<0.05$; propionic acid: 665.77 vs 155.67 mM/g, $P<0.05$). These results suggested that the anticarcinogenic effect of IR+LP+IP might be partly due to alterations in microbial-derived metabolites.

Discussion

Currently, gut microbiota dysbiosis is closely related to the occurrence and development of colon cancer. Probiotics especially *Lactobacillus* supplementation provide a potential strategy for prevention or treatment of colon cancer, which could restore the altered microbiota by maintaining the homeostasis between the beneficial and harmful bacteria as well as activate immune system[24–26]. Yue et al[27] revealed that *Lactobacillus plantarum* YYC-3 could prevent the occurrence of early-stage CRC in $APC^{Min/+}$ mice, which was achieved by preventing alteration of the gut microbiota and downregulating inflammation. Oh et al[28] reported that *Lactobacillus gasseri* 505 and a new prebiotic, Cudrania tricuspidata leaf extract (CT) showed cancer-protective effects by recovering dysbiosis of gut microbiota, reducing inflammation and gut-damaged mice by AOM/DSS treatment. Zahran proved that *L.rhamnosus* ATCC 7469 along with low exposure of ionizing γ-R in a synergistic interactions were efficacious control against colon cancer progression through the modulation of key signaling growth factors associated with inflammation[29]. In the present study, we further demonstrated that another *Lactobacillus* strain, *Lactobacillus reuteri* JMR-01 adjuvant $^{12}C^{6+}$ irradiation could elevate the curative effect of mice model bearing colon carcinoma. This therapeutical effect was achieved by promoting the proliferation of splenocyte and phagocytosis of peritoneal macrophage, modulating gut microbiota composition along with decreasing fecal enzyme activities and increasing concentrations of the fecal metabolites SCFAs.

Researches demonstrated that probiotics or their components could exert anti-carcinoma effects in vitro and in vivo as shown in Table 1. According to Table 1, *Lactobacillus casei* ATCC 393 (the ratio of CFU to cells was 1:10) reduced 52% CT-26 progression at 24 h[6], and the anti-proliferation effect of five *Lactobacillus* strains on CT-26 was 45% at 72 h when the ratio of CFU to cells was 1:10 in vitro[30]. In this study, although the anti-proliferation effect of JMR-01 LP + IP on CT-26 cells was 39.55% at 24 h, we used

a lower dose of bacteria that the ratio of CFU to cell was 1:1 in vitro. The excellent anti-tumor effects of probiotics in vitro have also been demonstrated in vivo. Xu et al[31] demonstrated that mice with the CT-26 tumor volume of 100 mm^3 treated with living JY300-8 possessed the highest tumor inhibitory rate (83.48%) and survival rates (90%) compared with inactived JY300-8, DFUR and tumor control groups at 42 d. Chung et al demonstrated that the tumor growth inhibition rate of DLD-1 xenograft mouse model administrated of lactic acid bacterium *Pediococcus pentosaceus* was 50.8% at 42 d[32]. When the size of subcutaneous CT-26 tumors reached around 100 mm^3, or the orthotopic CT-26 tumors were implanted for about 7 days started treating. Drug-loaded spores-dex inhibited tumor growth up to 89% and 65%, respectively in subcutaneous and orthotopic tumor models in the 12-day treatment[5]. Tiptiri-Kourpeti[6] showed that *Lactobacillus casei* were daily administered to BALB/c mice for 13 days. At day 10, CT-26 cells were inoculated subcutaneously. Seven days later, tumor volume inhibition was 85% for *L. casei*-treated mice. It can be seen that a better therapeutic effect was achieved by probiotics in the early stage of colon cancer, thus in this study, the treatment was conducted when the tumor volumes reached nearly 1400 mm^3. CT-26 tumor-bearing mice were irradiated and gavaged with JMR-01 LP, IP, and LP + IP twice a day for 50 days. On day 32 of treatment, the tumor inhibitory rates in IR + LP + IP reached the highest value (54.2%) among other experiment groups. Furthermore, at the end of experiment, the survival rates in IR + LP + IP and IR + IP were 71.4% up to the highest. Surprisingly, we found cured mice in IR + LP + IP group and the cured rate was 14.3%, which was the highest as we knowledge.

Literatures showed that *Lactobacillus* possessed potent immunostimulatory activities, including increasing specific cellular and humoral immune responses against antigens by stimulating the proliferation of lymphocytes, improving the phagocytic activity of macrophages[33, 34]. In our study, although the level of enhancement depended on the component and dose of JMR-01 used, phagocytic activity of peritoneal macrophages and splenocytes proliferation index of the combination of LP and IP (3.68, 2.47) were higher than that in LP (3.27,1.82) and IP (3.29,1.37), indicating LP, IP especially LP + IP could stimulate immune cells, which is coincident with former report[35]. Our study speculated that this excellent immunomodulating property of LP + IP were achieved by means of combining the advantages of LP and release of peptidoglycans during heat-treatment. Our study demonstrated that peptidoglycans with concentration-dependent in promoting the splenocytes proliferation and phagocytic activity of peritoneal macrophages. *L.plantarum* JLK0142 showed a potent immunomodulatory including enhancement of phagocytosis and increment splenic lymphocyte proliferation activity owing to the production of exopolysaccharide [36]. However, *L.gasseri*[37] was demonstrated that could increase the proliferation of RAW264.7 macrophage cells via increasing S phase DNA synthesis, which was related to the up-regulation of proliferating cell nuclear antigen (PCNA) and cyclin A. For the inactivated bacteria, peptidoglycan, teichoic acid, cell surface protein and unmethylated DNA from LAB could perform immunomodulating effects, which is related to interacting with pattern recognition receptors (PRRs) of immune cells, because these components are considered as ligands of PRRs[38–40]. Taken together, live probiotics and inactivated probiotics could exert immunomodulating effects[41]. However, the underlying immunomodulatory mechanisms of LP combining with IP is not clear, which needs to be studied in

subsequent experiments. We also found tumor-bearing mice treated by IR + LP + IP possessed higher survival rate and cure rate than IR and tumor control group, which may be attributed to the immunostimulatory activities of LP + IP.

Accumulating evidence points to an important role of probiotics in regulating gut microbiota structure and composition correlated with the occurrence and development of colon cancer[42, 43].

Hence, we analyzed the abundance of specific microbiota in fecal samples from different treatment groups. The present study showed that the development of colon cancer resulted in an increased abundance of *fragile bacteroides, Clostridium perfringens*, *Enterobacter and Enterococcus* but JMR-01 LP + IP intervention significantly altered the gut microbiota dysbiosis caused by colon cancer. Kwong et al[44] demonstrated that *S.gallolyticus, B.fragilis, F.nucleatum, C.perfringens* and *G.morbillorum* might play a role in CRC development, which were enriched in the CRC microbiota. These pathogenic bacteria were significantly increasing in the intestinal microbiota of CRC host, especially increment of *fragile bacteroides* may results in the release of abundant amounts of nitroreductase enzymes that were involved in the conversion of pro-carcinogens to carcinogens (such as dimethylhydrazine, nitrite) that increased risk of colon cancer[25, 26, 45]. More than the decreased abundance of these pathogenic bacteria in mice administered of JMR-01 LP + IP compared with other groups, we also observed decreased activities and slower growth rate of nitroreductase. Chandel showed that supplementation of *L.rhamnosus* MD14 led to decreased activity of nitroreductase and had signifcantly high percentage of Aberrant crypt foci (ACF) reduction compared with dimethylhydrazine (DMH)-treated mice[46]. In another study, nitroreductase activity decreased by 30–40% in *Bifidobacterium longum* HY8001 and *Lactobacillus acidophilus* HY2104 treated group in comparison with tumor control rat model indicating that probiotics inhibited colon carcinogenesis by modulating the metabolic activity of intestinal microflora[47]. Taken together, the positive effect of protecting against colon carcinoma exerted by JMR-01 LP + IP contributed to reducing their fecal enzymatic activity by modulating the gastrointestinal microflora.

In our study, mice of tumor control group significantly reduced the abundance of *Lactobacillus* and *Bifidobacterium* in comparision to normal mice. However, JMR-01 LP + IP not only inhibited the increase in pathogenic bacteria but also recovered the level of probiotic bacteria including *Lactobacillus* and *Bifidobacterium* nearly to normal level, which was consistent with former findings. *Lactobacillus gasseri 505* (LG) and a new prebiotic, Cudrania tricuspidata leaf extract (CT) increased the compositions of *Lactobacillus, Akkermansia*, and *Bifidobacterium* in colitis-associated colorectal cancer [28]. The lactic acid bacterium *Pediococcus pentosaceus* as colorectal cancer therapy increased the abundance of Akkermansia, *Lachnospiraceae*, and *Bifidobacterium* associated with eubiosis[48]. It is known to that the host undergoing colon cancer showed the decreased production of SCFAs produced by *Lactobacillus*, *Bifidobacterium* and other intestinal microbiota, which played an important role in direct anticarcinogenic effects including causing antiproliferative and proapoptotic effects as well as maintaining healthy gut microbiota [49, 50]. In present study, IR + LP + IP with restorative effect of *Lactobacillus* and

Bifidobacterium was also evidenced that it could significantly increase the production of fecal SCFAs. Although the acetate, and propionate concentrations varied in different groups, the highest concentrations of SCFAs were observed in IR + LP + IP group. The levels of acetic acid and propionate in the fecal of IR + LP + IP increased by 3.6 and 4.3 folds compared with tumor control group (53.93 mM/g), which was consistent with the former report[51]. Furthermore, we observed an inverse correlation between SCFA concentrations and the progression of tumors in mice. Accordingly, the positive effect of anti-colon carcinoma exerted by JMR-01 LP + IP might attribute to increasing fecal SCFAs content, which might inhibit nuclear factor NF-kB activation associated with tumor development [34], and restoring gut microbiota dysbiosis.

To sum up, JMR-01 LP + IP adjuvant $^{12}C^{6+}$ radiation might exert anti-cancer effects on colon carcinoma by regulating of several colon cancer-related gut microbiota, thus decreasing activities of fecal enzymes and increasing contents of SCFA. In addition, they could enhance the function of immune cells supported by lymphocytes and peritoneal macrophages.

Declarations

Funding

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Competing Interests The authors declare no competing interests.

Author Contributions

All authors contributed to the study conception and design.Jin Bai, Shuyang Wang, Fuqiang Xu, Miaoyin Dong designed the research study; Jin Bai, Xisi Sun, Junkai Wang performed the experiments; Jin Bai, Shuyang Wang, Fuqiang Xu, Miaoyin Dong, Guoqing Xiao wrote the paper; and all authors approved the final manuscript.

Ethics Approval: All institutional and national guidelines for the care and use of laboratory animals were followed.

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Tables

Table 1
Probiotics were used for the treatment of colon cancer

Strains	Source	In vitro	In mice model	References
Lactobacillus casei ATCC 393	DSMZ, Braunschweig, Germany		The tumor volume was suppressed by 63.1% compared to the control group at 17 d.	[11]
Lactobacillus casei ATCC 393	DSMZ, Braunschweig, Germany	10^9 CFU/ml for 24 h, a 52% reduction in CT-26	Tumor inhibition rate was 81.7% for *L.casei*-treated mice as compared to control ones at 17 d.	[6]
lactic acid bacterium Pediococcus pentosaceus	Synthetic probiotic		Tumor inhibition rate was 81.7% for Pediococcus pentosaceus -treated mice at 42 d.	[32]
VSL#3	Sigma-Tau Pharmaceuticals Ltd		VSL#3 (65%, 0.25cm) administration significantly reduced both the tumor formation rate and the tumor load (control : 100%, 0.97cm)	[52]
Prebiotics-Encapsulated Probiotic	Synthetic		89% of tumor suppression rate in the 12-day treatment.	[5]
Lactobacillus gasseri 505 and a Cudrania tricuspidata leaf	fermented milk		All mice in the AOM/DSS group had colon tumors, the FCT group showed the highest suppressive effect in colon tumor cells.	[28]
Five *Lactobacillus* strains consisting of two *L.plantarum*, one *L.rhamnosus*, one *L.brevis*, and one *L. reuteri*		45% reduction in CT-26 at 1:10 ratio	About 60 and 40% of mice in the AOM/ DSS-induced group were in stage IA and IIIA, respectively, while only 40% of mice in the *Lactobacillus* group were in stage IA, and 60% were in neoplasia stage.	[30]
Bifidobacterium	fecal		Oral administration of *Bifidobacterium* combining with checkpoint blockade nearly abolished tumor outgrowth.	[53]

Figures

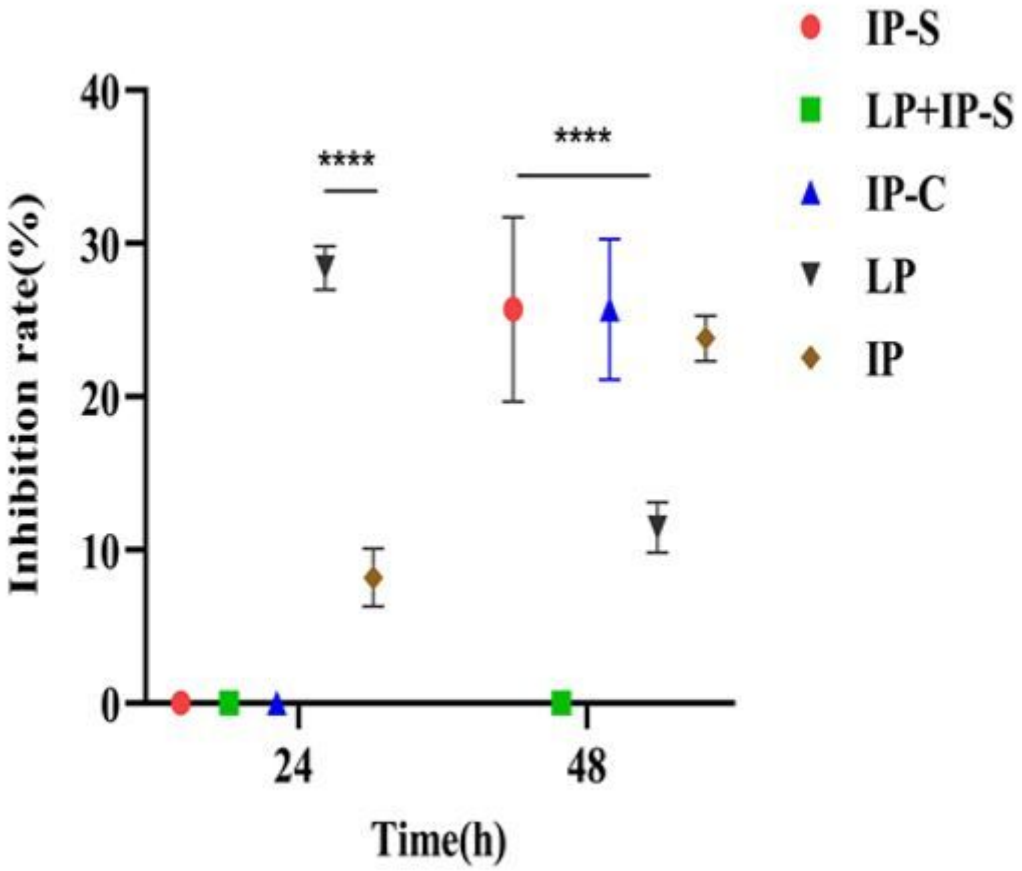

Figure 1

Inhibitory effect of JMR-01 on CT-26 cells.

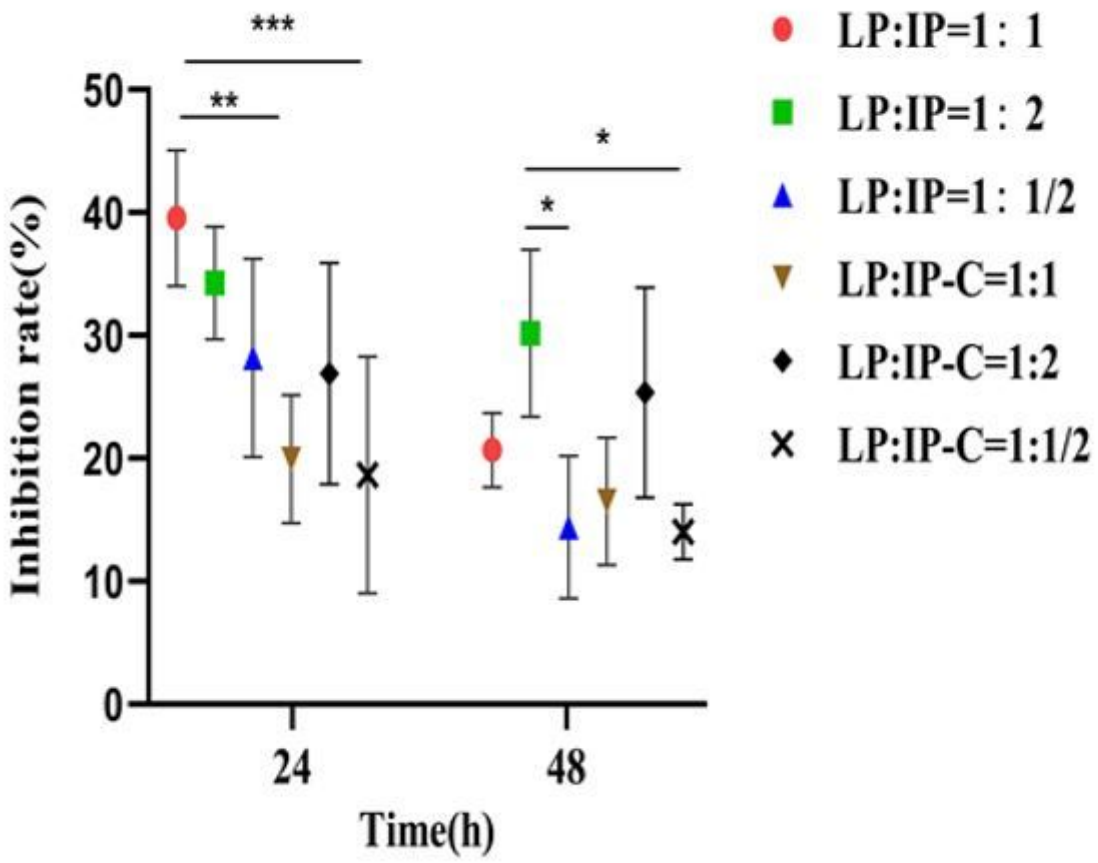

Figure 2

Determination the effect of different compositions of JMR-01 LP, IP and IP-B on CT-26 cells

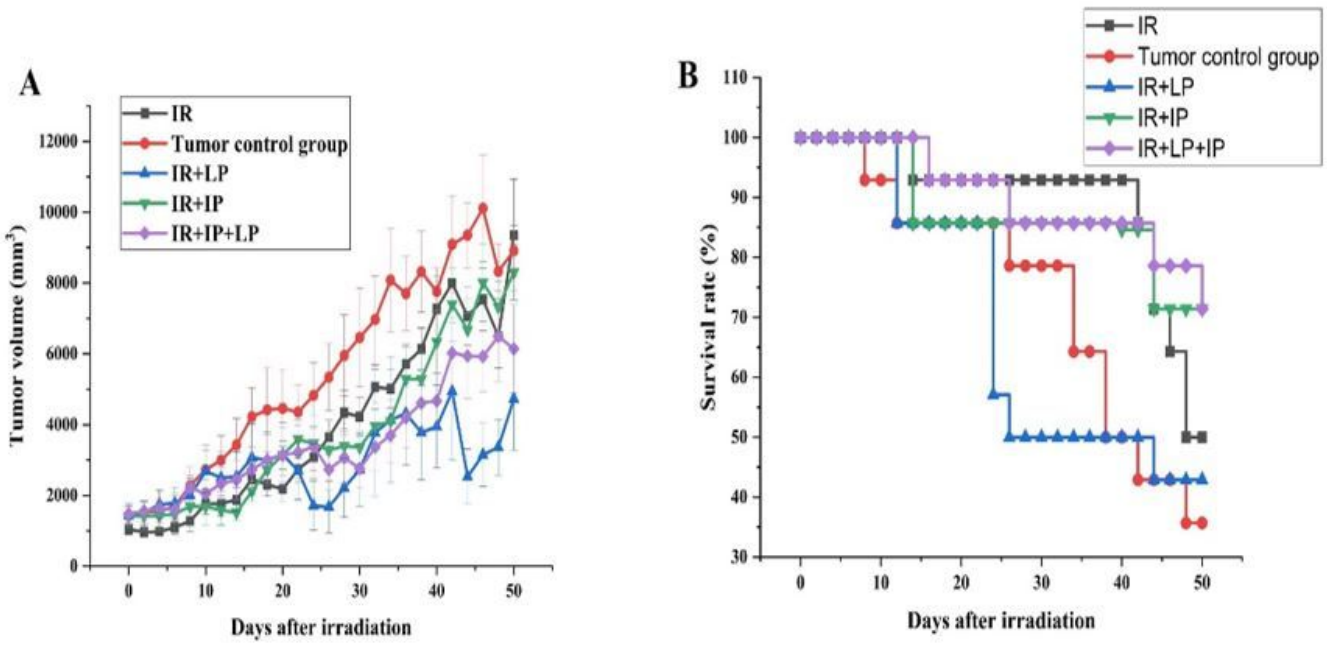

Figure 3

Effect of JMR-01 adjuvant $^{12}C^{6+}$ irradiation on colon carcinoma mice. (A) the tumor growth in tumor-bearing BABL/C mice after $^{12}C^{6+}$ irradiation (B) survival rates in tumor control group, IR, IR+LP, IR+IP, IR+LP+IP group.

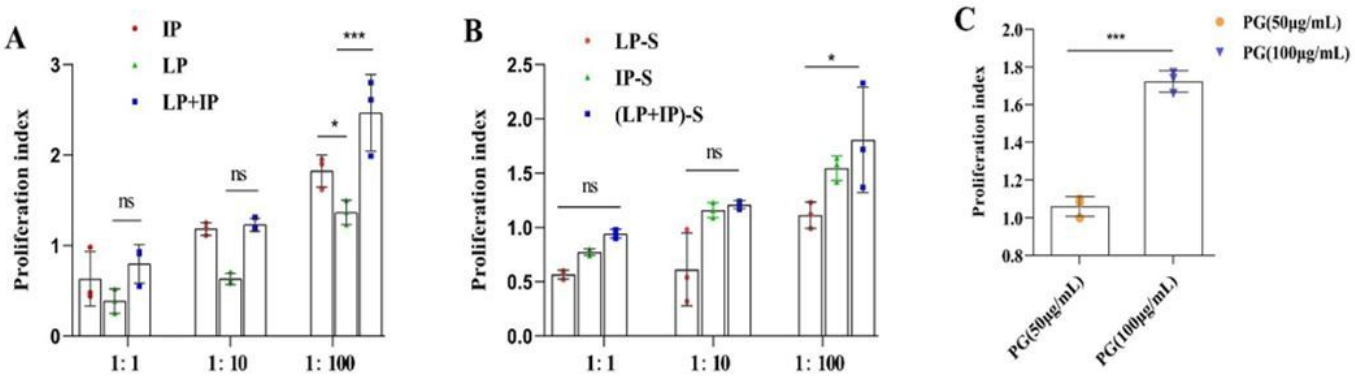

Figure 4

Determination of splenic lymphocyte proliferation index treated with JMR-01. (A) effects of JMR-01 LP, IP and LP+IP on splenic lymphocyte proliferation (JMR-01 LP, IP, LP+IP: lymphocyte=1:1(low dose), 10:1(mid dose), 100:1 (high dose)) (B) effects of supernatants of JMR-01 LP, IP, LP+IP on lymphocyte proliferation in different doses as described above. (C) effects of peptidoglycans extracted from JMR-01 on splenic lymphocyte proliferation.

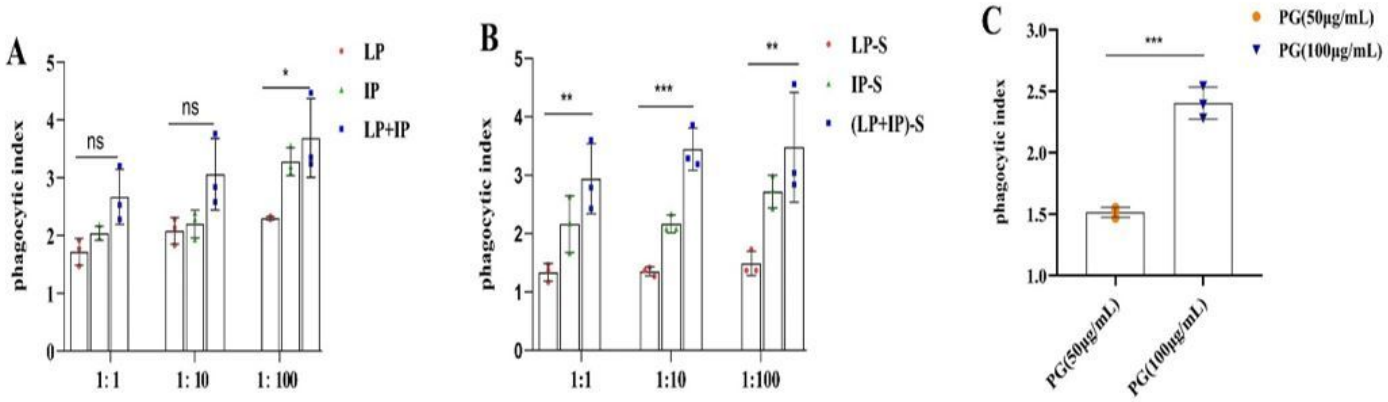

Figure 5

Determination of phagocytosis index treated with JMR-01. (A) effects of JMR-01 LP, IP and LP+IP on the macrophage phagocytosis (JMR-01 LP, IP, LP+IP: lymphocyte=1:1(low dose), 10:1(mid dose), 100:1 (high dose)) (B) effects of supernatants of JMR-01 LP, IP, LP+IP on on the macrophage phagocytosis in different doses as described above. (C) effects of peptidoglycans extracted from JMR-01 on macrophage phagocytosis.

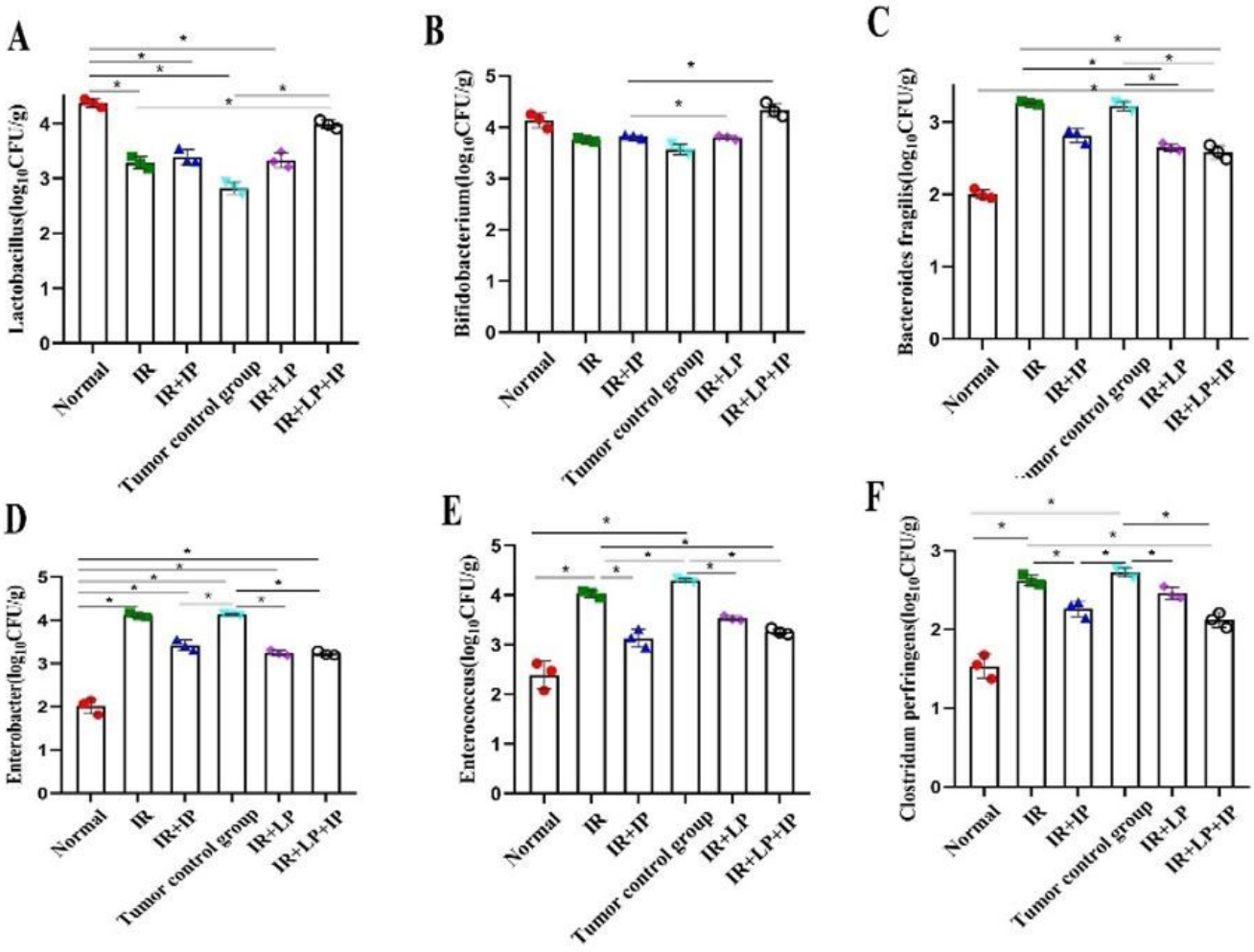

Figure 6

The abundance of *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Bifidobacterial*, *Clostridium perfringens* and *Bacteroides fragilis* of feces in each group. (A) *Lactobacillus* (B) *Bifidobacterial* (C) *Bacteroides fragilis* (D) *Enterobacter* (E) *Enterococcus* (F) *Clostridium perfringens*

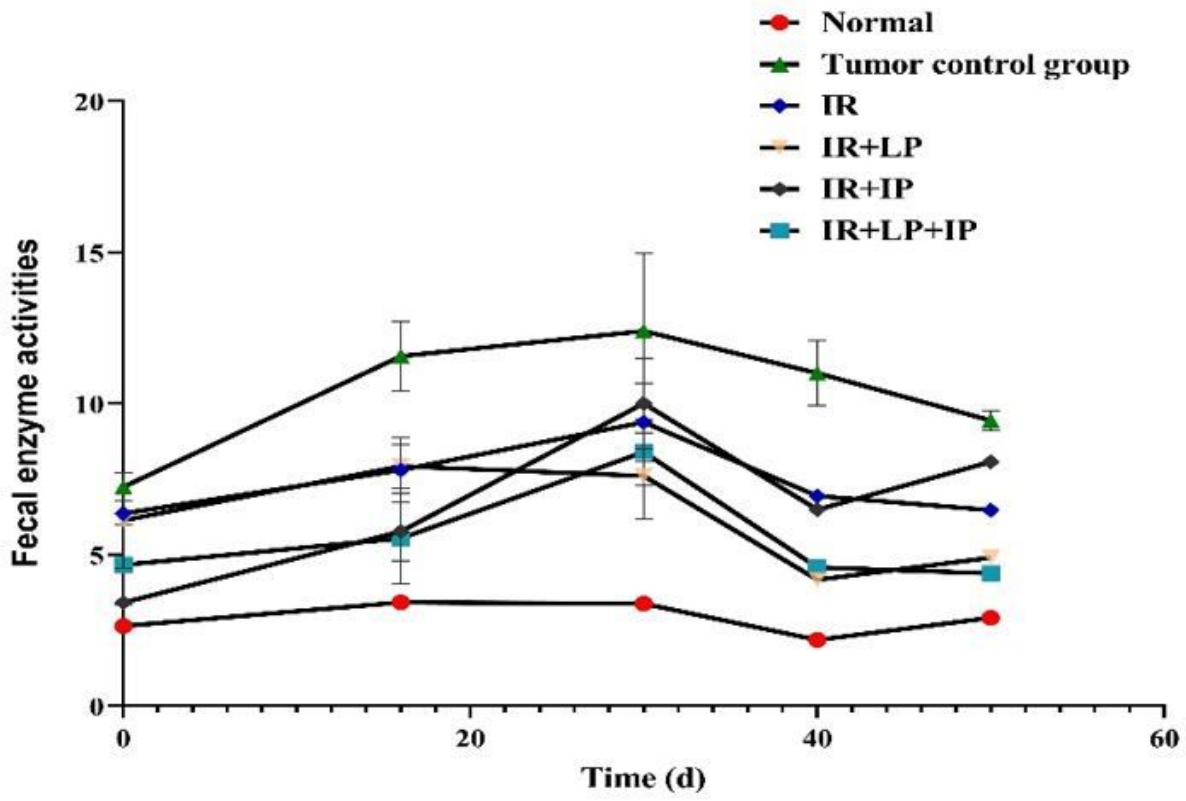

Figure 7

Effect of JMR-01 LP, IP, LP+IP adjuvant $^{12}C^{6+}$ irradiation on fecal enzyme activities

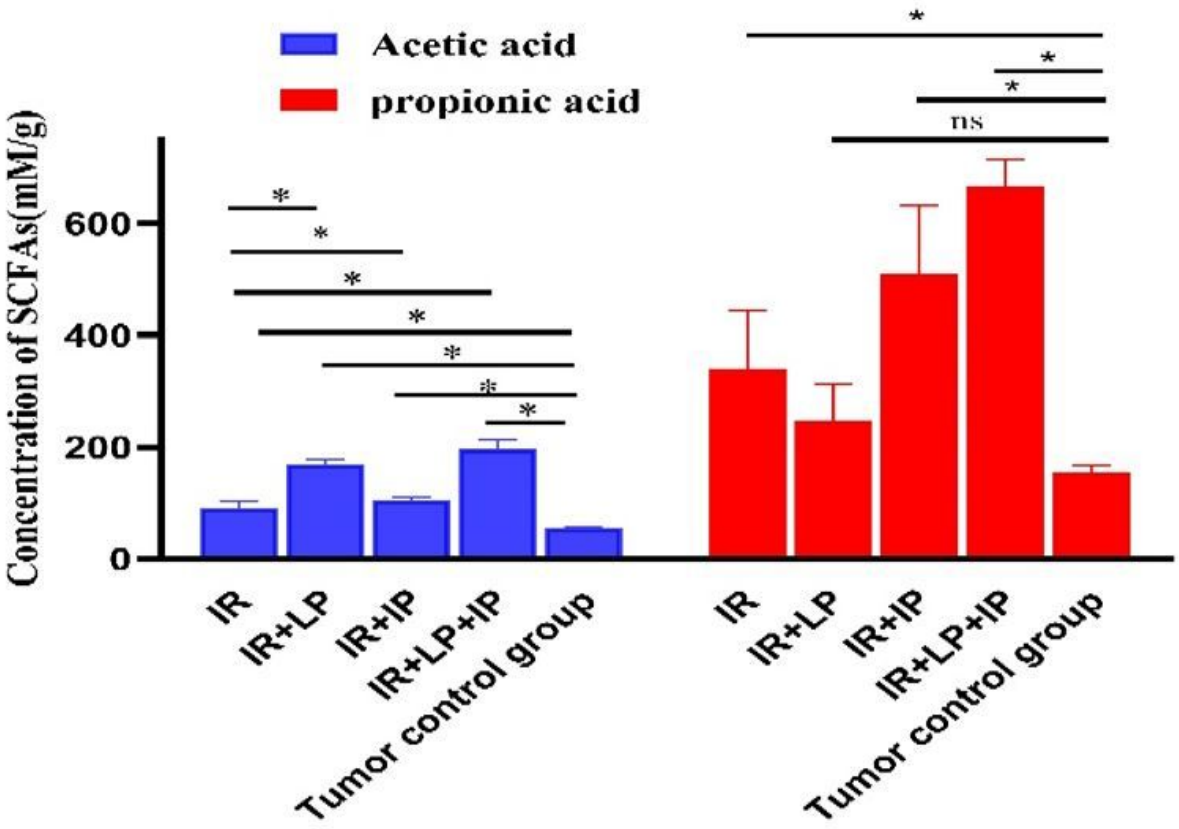

Figure 8

The concentrations of fecal short chain fatty acids