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FOOD MICROBIOLOGY - RESEARCH PAPER

Comparison of the effects of probiotic strains (*Lactobacillus gasseri*, *Lactiplantibacillus plantarum*, *Lactobacillus acidophilus*, and *Limosilactobacillus fermentum*) isolated from human and food products on the immune response of CT26 tumor–bearing mice

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Abstract

This study aimed to compare the effects of the probiotic bacteria, *L. gasseri* (52b), *L. plantarum* (M11), *L. acidophilus* (AC2), and *L. fermentum* (19SH), isolated from human source and traditional food products on the modulation of the immune system and inflammatory response on BALB/c mouse model bearing CT26 tumor. Five groups of female inbred BALB/c mice were orally administered with the probiotics and their mixes (MIX, at a 1:1 ratio) at varying dosages (1.5×10^8 cfu/ml and 1.2×10^9 cfu/ml) before and after the injection of a subcutaneous CT26 tumor over the course of 38 days via gavage. Finally, their effects on the tumor apoptosis and the cytokine levels in spleen cell cultures were analyzed and compared. M11, MIX, and 52b groups had the greatest levels of interleukin-12 (IL-12) and interferon gamma (IFN-γ) production. The highest production level of granzyme B (GrB) was related to the MIX and 52b groups. Moreover, these groups showed the lowest production level of (IL-4) and transforming growth factor beta (TGF-β). Furthermore, the groups of MIX and 52b demonstrated the greatest amount of lymphocyte proliferation of spleen cells in response to the tumor antigen. The delayed-type hypersensitivity (DTH) response significantly increased in the groups of MIX and 52b compared with the control ($p<0.05$). The findings demonstrated that the oral treatment of the human strain (52b) and the combination of these bacteria generated strong T helper type 1 (Th1) immune responses in the tumor tissue of the tumor-bearing mice, which led to the suppression of the tumor development.

Keywords Interleukin-12 · Immune system modulation · Colon cancer · Tumor apoptosis · Adherence

Introduction

Recent years have seen a rapid increase in the use of probiotic bacteria for health promotion on a global scale, creating its own industry. Numerous medical studies have demonstrated that probiotics can enhance gut and immune system health [1]. Recent global research has focused on the use of probiotics in the prevention and treatment of human diseases. It has been demonstrated that in some circumstances, elements like chemicals, radioactive rays, and even viruses can damage DNA, which ultimately causes cancer. Probiotic bacteria play an important role in binding and inactivating mutagens by their cell wall (CW) components and also by producing useful metabolites [2].

Apás et al. [3] evaluated the binding ability of the probiotics *Lactobacillus reuteri* DDL19 (*Limosilactobacillus reuteri*), *Enterococcus faecium* DDE39, and *Bifidobacterium bifidum* DDBA (isolated from human), as well as *Lactobacillus alimentarius* DDL48 (isolated from sourdough) mutagenic agents. They showed that oral administration of these probiotics reduced mutagens in feces and improved colorectal cancer in goats. They also mentioned that feeding a mixture of these bacteria with identical

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concentrations performed better in the treatment process than feeding them individually.

Other studies applied mutagenic agents such as 2-amino-3-methylimidazo[4,5-f] quinoline to rats and reported that it eventually led to colon cancer in the subjects; however, by regularly feeding *Bifidobacterium longum* bacteria of human origin to rats, the tumor was inhibited, and their disease improved [4].

According to Zmora et al. [5], people who have colorectal cancer consume fewer probiotics. The beneficial function of probiotics, which depends on support from the gut flora, is found to depend on colonization by the host gut microbiota. These findings suggest that a colonization barrier will have a significant therapeutic impact even if the probiotics used are beneficial. In other words, the therapeutic properties of probiotic bacteria depend on their probiotic properties, including their adhesion ability to epithelial cells, and the co-aggregation over competition for their placement in intestinal epithelial cells.

Lactobacilli have serious and significant differences with each other in their ability to modulate dendritic cell (DC) reactions and T cell activation which are probably related to differences in the structures of the bacterial CWs and their pattern receptors. The most well-known and famous family of innate receptors are Toll-like receptors (TLRs), among which, TLR2 recognizes bacterial CW compounds such as peptidoglycan (PGN) and is sometimes present as a heterodimer next to TLR6. It also recognizes the fatty acid chains on lipoteichoic acid (LTA) [6, 7].

Among these differences, we can point to variances in the amount of LTA produced per cell, the length of the chain, and the degree of substitution with di-alanine or glucose which, some researchers have mentioned, is due to the isolated source of the bacteria returns. In most cases, human strains, such as *L. gasseri*, increase the immune responses of Th1 cells in terms of the unique structure of LTA and high adhesion to epithelial cells. The level of probiotic production of inflammatory cytokines such as IL-12 and IFN-γ indicates that different types of probiotic bacteria stimulate immune cells in different ways; therefore, they can modulate the immune response and have an opposite effect on safety. Excessive production of inflammatory cytokines leads to chronic inflammation, which is associated with the occurrence of some diseases such as atherosclerosis and cancer [8].

Therefore, the objective of the present research is to evaluate and compare the effects of probiotic strains (*L. gasseri*, *L. plantarum*, *L. acidophilus*, and *L. fermentum*) isolated from human and food products on the immune response of CT26 tumor–bearing mice.

Materials and methods

Animals and ethics statement

The Pasteur Institute of Iran provided all of the female BALB/c mice used in this work, which was conducted under in vitro and in vivo settings. The animals were maintained at the animal house unit of the Bu Ali Research Institute at Mashhad University of Medical Sciences in Iran. The Biomedical Committee of Ferdowsi University, Mashhad gave the present study approval with the number IR.UM.REC.1400.004, and all ethical standards for practical work with mice were upheld. ARRIVE criteria are followed for reporting animal experiments [9–12].

Micro-organisms and cell line

The Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran, provided the native strains of *L. plantarum*, *L. gasseri*, *L. fermentum*, and *L. acidophilus* for this research (Table 1), which were then tested for the greatest levels of IL-12 (p70) induction in vitro. Additionally, murine CT26 colon cancer cells (RRID: CVCL 7254) were acquired from the Department of Immunology at the Bu Ali Research Institute in Mashhad, Iran.

Activation and fermentation steps of 25 strains of *L. plantarum*, *L. gasseri*, *L. fermentum*, and *L. acidophilus*

L. plantarum, *L. gasseri*, *L. fermentum*, and *L. acidophilus* strains were first incubated and activated in a de Man Rogosa Sharpe (MRS) broth (Merck Co., Germany) at 37 °C for 24 h. Then, the activated bacteria were inoculated to the MRS broth (pH = 6.4) in proportion 1% (V/V) and incubated at 37 °C for 18 h. For fermentation, they were then transferred to Erlenmeyer flasks containing 100 ml of MRS broth culture medium and incubated for 18 h at a speed of $4 \times g$ and temperature of 37 °C. The pH of the culture medium was continuously controlled during fermentation so as not to fall below pH = 6.4. After 18 h, the contents of the flasks were transferred to pre-weighed centrifuge tubes and centrifuged for 15 min at 4 °C and $10{,}000 \times g$. Afterward, the supernatant was removed, and the precipitate was washed twice with saline solution and once with distilled water (the weight of the pellets was measured each time). Finally, to heat kill the bacteria, the cells suspended in 2 ml of deionized water were placed in a hot water bath with a temperature of 75 °C for 60 min (the possible growth of bacteria in favorable conditions on the agar culture medium was re-evaluated to ensure

Table 1 Screening of tested strains for feeding to tumor-bearing mice out of the following 25 strains

N	Species	Strain number	Source
1	*L. plantarum*	M8	Milk KP212404
2		M11	Milk KP212405
3		S2G	Sourdough (wheat bran) NR104573.1
4		A7	Human feces KC355240
5		LF 48	Lighvan cheese [13]
6		LF 57	Lighvan cheese [13]
7		LF 55	Lighvan cheese [13]
8		D1	Sourdough [14]
9		21G	Fermented olives [15]
10		5G	Camel's milk KM495894.1
11		61G	Pitcher cheese KM495875.1
12	*L. plantarum* subsp. *plantarum*	8SH, standard strain	ATCC 14917
13	*L. plantarum*	10SH	Tarkhine (fermented seed) [16]
14		11SH	Human feces
15	*L. gasseri*	54C	Healthy human vagina KP090117
16		49A	Healthy human vagina KP090114
17		47 b	Healthy human vagina KP090116
18		52 b	Healthy human vagina KP090115
19		Standard Strain	ATCC 33323
20	*L. fermentum*	OF	Fermented food (chal) [14]
21	*L. fermentum* Beijerinck	Standard strain	ATCC 14931
22		Standard strain	ATCC 9338
23	*L. fermentum*	19SH	Horreh (fermented seed) [17]
24	*L. acidophilus*	AC2	Sourdough LC155899.1
25	*L. acidophilus* (Moro) Hansen and Mocquot	Standard strain	ATCC 4356

the killing of bacteria). The pellets were prepared in a freeze dryer (Labconco; USA and Canada) in lyophilized form and kept in a freezer (− 20 °C) until use [18, 19].

In vitro IL-12 (p70) production assay

Splenocyte cells were extracted from mice using the method of Sashihara et al. [18]. Five female inbred mice were procured from the Iranian Pasteur Institute. Then, splenocyte cells were isolated from mouse spleens under completely sterile conditions. For 10 min, the cells were centrifuged at 450 × *g*. Ammonium-chloride-potassium (ACK lysing buffer; pH = 8; NH4CL, KHCO3, and Na2EDTA; Merck Co., Germany) was used to remove erythrocytes. The ACK buffer was then completely removed from the cells by washing them several times in phosphate-buffered saline (PBS; Merck Co., Germany). Next, 10 ml Roswell Park Memorial Institute medium (RPMI-1640; Betacell BE25500, Iran) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Betacell BE31100, Iran), 100 µg/ml penicillin and streptomycin (Betacell BE28100, Iran), and 10% FBS-RPMI 1640 was added, and the cells were centrifuged at 450 × *g* for 5 min, counted (2.5×10^6 cells/ml), and cultured in 24-well tissue culture plates in 10% FBS-RPMI 1640 at 37 °C in the absence (negative control) or presence of 1 µg/ml lyophilized bacterial cells.

The negative control was a well that contained no bacteria. After 48 h, the tissue culture supernatants were collected and analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Mouse IL-12/P70 ELISA kit, Cat. No. E0020Mo, China) to determine the levels of IL-12 (p70) production [20].

CT26 colon carcinoma cell model and experimental design of probiotic bacteria administration

In each experiment, the native strains of *L. gasseri* (52b), *L. plantarum* (M11), *L. acidophilus* (AC2), and *L. fermentum* (19SH) and the mixture of these bacteria (MIX) with 1:1 ratio were fed to five groups of five mice (6–8 weeks old, 16–19 g). The control group received the same volume of PBS as the other groups [21]. The animals were housed in polycarbonate cages at room temperature with free access to commercial food and running water. Dietary pellets for mice contained 10% moisture, 0.5% sodium, 1% calcium, 0.65% phosphorus, 0.25% tryptophan, 0.33% methionine,

1.15% lysine, 0.7% threonine, a minimum of 4% raw fat, and a maximum of 4% raw fiber. The test strains were inoculated in 10 ml MRS broth and cultivated overnight at 37 °C under anaerobic conditions. After centrifuging them at 4000 × *g* for 30 min at 4 °C, they were collected, cleaned three times in sterile PBS, and then resuspended in PBS. After that, the suspensions were made for feeding at 0.5 McFarland (1.5×10^8 cfu/ml) and at 4 McFarland (1.2×10^9 cfu/ml) using spectrophotometry (WPA, S2000 UV–Vis, England) [22, 23]. Murine CT26 colon carcinoma cell lines were routinely cultured in 10% FBS-RPMI 1640 (Betacell, Iran) under sterile conditions at 37 °C in a humidified atmosphere of 5% CO_2 [22]. In total, 2×10^6 CT26 cell lines were given subcutaneously onto each mouse's right side on the 14th day of treatment. The mice were given 4 MacFarland via gavage daily from the 24th to the 38th day and 0.5 ml of the suspensions every other day from the 17th to the 24th day prior to tumor implantation. The concentration of mixtures of these bacteria during 38 days of feeding to the MIX group was exactly the same as other groups (Fig. 1) [24, 25].

Primary subcutaneous implantation tumor model

Serum-free media (100 µl) containing 2×10^6 CT26 cells (cell viability ≥ 95%) were slowly injected subcutaneously into the right flank of mice using a 30-G needle.

On the third day after injection, a CT26 cell line tumor was observed in all mice, and its hardness could be felt under their skin. With 1 day in between, the length and width of the tumor were measured with an engineering caliper, and its volume was obtained using the following equation:

$$V - \frac{1}{2}\mathrm{LW}^2 \quad (1)$$

where *L* is the length and *W* is the width.

Along with tumor volume, the weight and variations in tumor weight of the mice in each group were also noted [26]. Using the log-rank statistical test, the survival curves of mice experimentally infected with probiotic strains were compared [27].

Preparation of the tumor antigen

To create tumor antigens, mice with large tumors were used. This procedure involved removing the tumor from the mouse body and cutting it into small pieces (3 mm^3). The fragments were then ultrasonic sonicated (Bandelin SONOPULS HD 4400, Germany) and cleaned with sterile PBS. To excite splenocyte cells and detect the tested cytokines, 20 µg/ml of tumor antigen was employed (using the Bradford assay) [28].

Delayed type of hypersensitivity (DTH)

The method of Jin et al. [28] was used to perform the DTH test. In brief, 14 days after the subcutaneous injection of CT26 tumor cells, three mice from each group were randomly selected. The mice were then injected with 100 µl of tumor antigen at a concentration of 20 µg/ml into the left footpad and 100 µl of PBS into the right footpad. After 48 h, the thickness and swelling of the feetpads were measured using an engineering vernier caliper.

Cytokine analysis

After erythrocytes were deleted from the extracted spleen cells, cells were counted to 2.5×10^6 cells/ml and cultured in 24-well plates (SPL30024; South Korea) containing 10% FBS-RPMI 1640 (Betacell, Iran). Then, 20 µl of purified tumor antigen was added to each well, and 72 h after, plates were centrifuged (Eppendorf; Germany, 300 × *g*, 10 min), and supernatants were gathered and kept at − 80 °C until use. Tested cytokine levels in the spleen cell culture supernatants were identified using an ELISA kit IFN-γ (Mabtech AB, Sweden), IL-4 (E-0012; Parstoos, Iran), GrB (Bioassay Technology Laboratory, E0437 Mo Germany), TGF-β (KPG-MTGFK, 96 RXN, Iran), and IL-12 (Quantikine ELISA, USA R&D Systems, Inc.) as explained by the manufacturer (R&D kit instructions). The negative control was the well without tumor antigen [29].

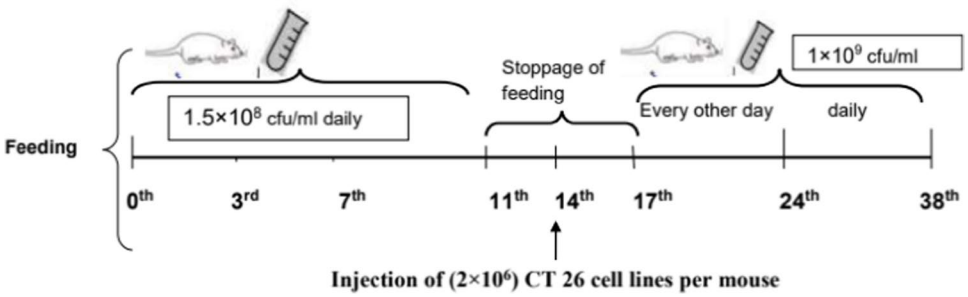


Fig. 1 Model of oral administration of tested bacteria to female inbred BALB/c mouse model control group received PBS. On day 14, 2×10^6 CT26 cells were injected subcutaneously. Growing tumors could be observed 3 days after inoculation of CT26 cells. The feeding process was carried out up to 24 days after the injection of the tumor

Lymphocyte proliferation assay

At a rate of 100 µl/well, the spleen cell suspensions with 1.5×10^6 viable cells/ml and no erythrocytes were added to 96-well tissue culture plates. Then, all sample wells other than the negative control and blank wells received a stimulator addition of 20 µl of tumor antigen (at a concentration of 20 µg/ml). At 37 °C, plates were incubated. Cells were labeled with 5 µl/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and re-incubated for an additional 4 h at 37 °C after 72 h at 37 °C, 90% humidity, and 5% CO_2. Using an Eppendorf centrifuge set at $300 \times g$ for 10 min, the labeling medium was removed. After being fixed with a 100 µl dimethyl sulfoxide (DMSO; Merck Co., Germany) solution, the cells were left to sit at room temperature for 5 min. The samples' absorbance was measured at 540 nm with an ELISA reader (Biotek ELX808, USA) [26]. Results are presented as stimulation index (SI) using the following formula:

$$\text{OD of splenocyte} + \frac{\text{tumor Ag}}{\text{OD of splenocyte} - \text{tumor Ag}} \quad (2)$$

Preparation of CW components (CW and PGN)

The CW and PGN of the tested bacteria were prepared using data from Hirose et al.'s study [30]. In a nutshell, the bacterial suspensions were first centrifuged (Sigma 3-30 K, Germany) at 4 °C for 20 min at a speed of $12{,}000 \times g$ and then washed with 0.1 M phosphate buffer (pH = 7.2). The cells were then broken down using an ultrasonic generator on ice. Centrifugation was used to separate healthy and undamaged cells for 30 min at a speed of $5000 \times g$ and a temperature of 4 °C. Re-introducing cell pellets into a 4% sodium dodecyl sulfate (SDS; Merck Co., Germany) solution, they were then boiled for 40 min. The mixture was centrifuged for 30 min at 25 °C and a speed of $40{,}000 \times g$ after being allowed to cool to room temperature. The leftover pellets were then thoroughly cleaned with sterile distilled water and lyophilized in a freeze dryer. PGN was created by mixing CW components (pellets) with a 5% trichloroacetic acid (TCA; Sumchun, China) solution, boiling the mixture for 20 min, and letting it cool to room temperature. It was recentrifuged, and the remaining pellets were cleaned using distilled water and chloroform (Merck co, Germany) in a 1:20 ratio. To get rid of TCA, the finished product was centrifuged at $16{,}000 \times g$ and 25 °C for 10 min. After several ethanol and ethyl ether washes on the remaining pellets (ROMIL, UK) [30, 31], cells were then lyophilized.

Measurement of cytokines induced by the CW and PGN of fed probiotics in the splenocyte cells in vitro

To measure the desired cytokines, the same procedure as in the earlier sections was used. In conclusion, 2.5×10^6 cells were cultured in each well in close proximity to the CWs and PGNs of the fed bacteria using female inbred BALB/c mice ($n = 3$). The spleen cells were cultured in 24-well plates using 10% FBS-RPMI 1640. Plates were incubated at 37 °C with or without (1 µg/ml lyophilized CW and PGN) the additive. Using a commercially available ELISA kit (Quantikine ELISA, USA R&D Systems, Inc.) and IFN-γ (Mabtech AB, Sweden), the tissue culture supernatants were collected after 48 h to determine the levels of IL-12 (p70) production [18].

Statistical evaluation

At least twice, each experiment was repeated. Results were displayed using means and standard deviation. Using Prism 8.0.2, the statistical analyses were carried out. One-way analysis of variance (ANOVA) and the Tukey-Kramer test were used to compare the means. Statistical significance was defined as a p-value of 0.05. Leven's test was used to determine whether the variance was equal for each group of mice, and the Shapiro-Wilk test was used to determine the normality distribution (in vivo). The six experimental groups (IFN-γ, IL-4, TGF-β, IL-12, and GrB) displayed an abnormal distribution of cytokine production. As a result, a non-parametric test was used to evaluate all groups of mice. The Kruskal–Wallis test was used to compare the amount of produced cytokines between the fed groups and the control group PBS as well as pairwise differences between fed groups. The median and SEM are used to express continuous variables (standard error of the median). Type 1 error in the multiple test was minimized by the Bonferroni correction. The data were evaluated using IBM SPSS software (Armonk, NY, version 23.0), and the significance level was set at ($p < 0.05$). The graphs were made using version 8 of GraphPad. Comparisons of time periods were made using survival analyses. The log-rank test was employed in this analysis to examine the mice's lifespan [12, 27].

Results

Screening of four strains fed to BALB/c mice out of 25 lactic acid strains

The level of the production of IL-12 by 25 strains of *L. plantarum*, *L. gasseri*, *L. fermentum*, and *L. acidophilus* from the sources of traditional fermented, dairy, and human foods was evaluated in the spleen cell culture (in vitro). As shown

in Fig. 2A, the highest production level of IL-12 among *L. plantarum* strains was related to the M11 strain isolated from milk (51.83 pg/ml), which had a significant difference compared to the negative control and other *L. plantarum* strains, except for the standard *L. plantarum* subsp. *plantarum* ATCC 14917 (43.66 pg/ml), ($p < 0.0001$). The highest production level of IL-12 among *L. gasseri* strains isolated from the human source was strain 52b (41.33 pg/ml), which was significantly different compared to the negative control, other strains of *L. gasseri*, and the standard strain (*L. gasseri* ATCC 33323) ($p < 0.0001$). Among the strains of *L. fermentum*, the 19SH strain (isolated from fermented food) and the *L. acidophilus* strain (isolated from sourdough) (AC2) were 20.975 pg/ml and 19.79 pg/ml, respectively, and they had the highest production levels of IL-12 compared to the negative control ($p = 0.031$; Fig. 2B).

Micro-organisms and feeding procedure

The mice were fed the bacteria with the greatest in vitro IL-12 production levels. We chose and screened the native strains *L. plantarum* (M11), *L. gasseri* (52b), *L. fermentum* (19SH), and *L. acidophilus* (AC2) (Fig. 2A, B). As is clear in Fig. 1, these bacteria were fed to mice as follows: 14 days before tumor injection, they were fed 0.5 ml with a concentration of 1.5×10^8 cfu/ml by gavage every day. Then, on the

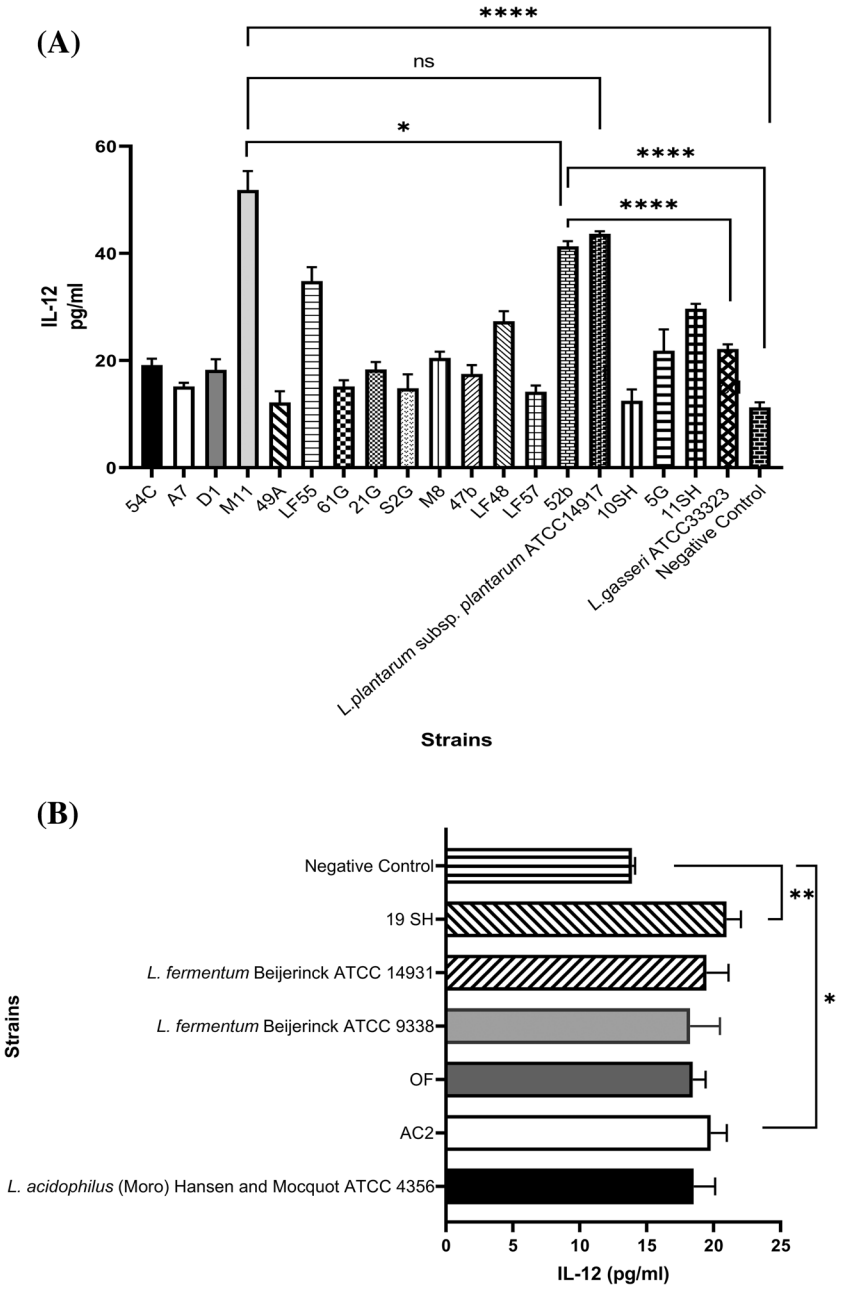


Fig. 2 A Heat-killed *L. plantarum* and *L. gasseri* from human samples and traditional foods IL-12 (p70) in spleen cell culture (in vitro). Data are presented as mean ± standard deviation, which were analyzed by one-way ANOVA and the Tukey test using Prism 8 software. A *p*-value of < 0.05 was considered statistically significant (****p-value < 0.0001 and *p-value < 0.0480). **B** IL-12 (p70) production by heat-killed *L. acidophilus* and *L. fermentum* from traditional foods in spleen cells culture (in vitro). 19 SH (**$p < 0.0092$) and AC2 (*$p < 0.0268$) compared with negative control, ns: not significant

14th day, 2×10^6 cell line CT26 was injected into the right side of the animal while adhering to all disciplinary and ethical rules. After the 3rd day, the tumor was palpable under the skin of all mice. Then, from the 17th to the 24th day, the mice were gavaged with a concentration of 1×10^9 cfu/ml and from the 24th to the 38th day, with the same concentration, in the amount of 0.5 ml daily.

Oral administration of live probiotic bacteria suppresses subcutaneous colon carcinoma growth in mice

Measurement of primary tumor growth

All six groups of mice had tumors that were palpable and noticeable by the 3rd day, as shown in Fig. 3A, and as the time passed in each group, the tumors became bigger. The PBS and M11 groups saw greater increases in tumor volume than the other groups, and a significant rise was seen from the 14th to the 24th day after tumor inoculation ($p < 0.05$). The tumor size increased in the MIX group from the 3rd to the 14th day after the CT26 cell line injection, but from the 14th to the 19th day, tumor growth was constant, and from the 19th to the 24th day, tumor size decreased, such that it reached zero on the 22nd day. In the 52b group, the tumor size increased until the 14th day and then decreased from the 17th to the 24th day, such that the tumor size reached zero on the 19th day. Tumor sizes in the AC2 and 19SH groups increased significantly from the 3rd to the 24th day ($p < 0.05$).

Tumor volume and tumor weight were calculated at the same time. As can be seen in Fig. 3B, the highest tumor weight was related to the control or PBS groups, which increased from the 3rd to the 24th day after CT26 cell line inoculation. In the MIX group, the average tumor weight remained constant from the 14th to the 17th day and decreased from the 19th to the 24th day, which was significantly different from the control group ($p < 0.001$). In group 52b, the average weight of mice remained constant from the 14th to the 17th day, decreased on the 19th day, and remained constant until the 24th day. The tumor weight in the M11 group also increased from the 3rd to the 24th day, but its growth rate was lower than that of the control group. Tumor weights in the AC2 and 19SH groups also increased on average from the 3rd to the 24th day.

Survival curve of CT26 tumor–bearing mice fed with probiotic strains

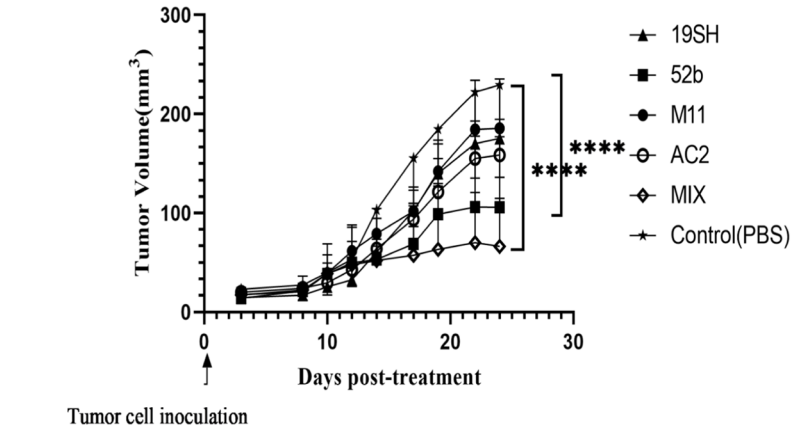
None of the mice in any group died of a tumor. As seen in Fig. 3C, D, all mice in the control group survived 24 days after tumor injection. Only one mouse in the M11 group died on the 10th day, one mouse in the AC2 group on the 9th day, and one mouse on the 6th day before tumor injection in the MIX group. Other mice were ethically sacrificed for tumor antigen preparation.

Cytokine assay in spleen cell culture

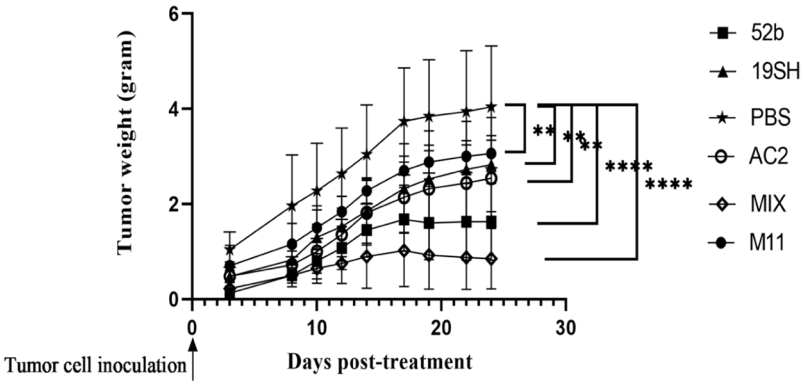
Elevated (IL-12, IFN-γ, TGF-β, GrB, and IL-4) production in response to probiotic bacteria administration

The *L. plantarum* strain M11 (293.33 pg/ml, $p < 0.001$), MIX group (117.73 pg/ml, $p = 0.003$), and group 52b (66.73 pg/ml, $p = 0.024$), respectively, had the highest production levels of IL-12, which was significantly different from the control group (18.05 pg/ml). There were no discernible differences between the fed groups when compared pairwise. The greatest levels of IFN-γ production, which were substantially different from the control group, were found to be associated with groups M11 (282.32 pg/ml, $p < 0.001$), MIX (85.52 pg/ml, $p = 0.004$), and 52b (41.69 pg/ml, $p = 0.047$), respectively. The AC2 and 19SH groups did not substantially vary from the control group, and it should be noted. When the groups were compared to one another, only the M11 group distinguished itself significantly from the AC2 and 19SH groups. According to the findings of this study, mice fed with a mixture of bacteria had a lower level of TGF-β in their spleen cell culture medium (87.02 pg/ml, $p = 0.001$), and *L. gasseri* 52b had a higher level (103.014 pg/ml, $p = 0.005$) than other groups, which was significantly different from the control group. The group fed *L. plantarum* (M11, 174.61 pg/ml) had the highest level of TGF-β production, followed by the control group (188.046 pg/ml). Based on pairwise comparisons between the fed groups, no significant difference was observed between the groups (Fig. 4C).

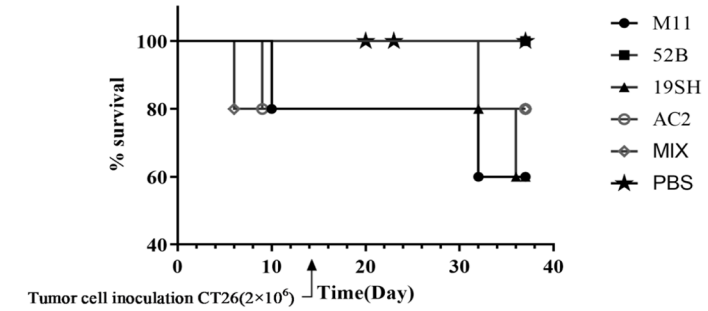
Probiotic bacteria feeding in this research had various impacts on the rate of production (GrB) in mouse spleen cells. The MIX group's peak induction level throughout feeding was 331.993 ng/ml, which differed substantially from the control group ($p = 0.006$). Compared to the control group, groups 52b (264.71 ng/ml, $p = 0.008$) and 19SH (238.715 ng/ml, $p = 0.034$) differed significantly. However, there was no discernible difference between groups M11 and AC2 and the control group. There was no discernible difference between the fed groups based on pairwise comparisons (Fig. 4D). According to the findings shown in Fig. 4E, the control group (33.67 pg/ml) and the M11 group (24.74 pg/ml), which were not statistically different from one another, generated the greatest amounts of IL-4 in spleen cells. The groups that were given the bacteria combination (MIX 5.03 pg/ml) and group 52b (8.40 pg/ml), which differed substantially from the control group, had the lowest amounts of IL-4 generated in spleen cells. There was no discernible difference between the groups AC2 (11.85 pg/ml) and 19SH (12.6 pg/m) and the control



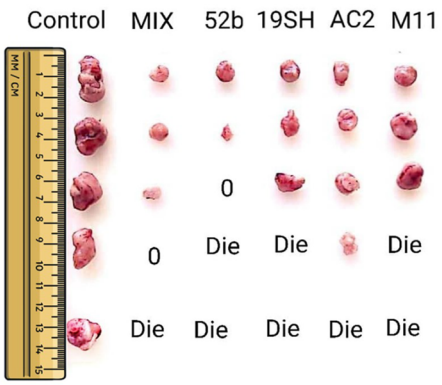
(A)



(B)



(C)



(D)

Fig. 3 **A** Tumor volume. The analysis was performed using the Kruskal–Wallis test to compare the control group (PBS) with 52b (****$p = 0.006$) and MIX (****$p = 0.002$) at $p < 0.05$. **B** Tumor weight. M11 (**$p = 0.0443$), MIX (****$p < 0.001$), 52b (****$p < 0.001$), AC2 (**$p = 0.023$), and 19SH (**$p = 0.042$) compared with the PBS group. **C** Survival curve of CT26 tumor–bearing mice fed with probiotic strains. The survival curves of CT26 tumor–bearing mice fed with probiotic strains were compared using the log-rank statistical test, and statistical significance was set at $p < 0.05$. **D** Tumors were removed from the mice on the 24th day after CT26 cell line inoculation. The quality of the images was improved with CamScanner software (CamSoft, 6.23.0.2208100000)

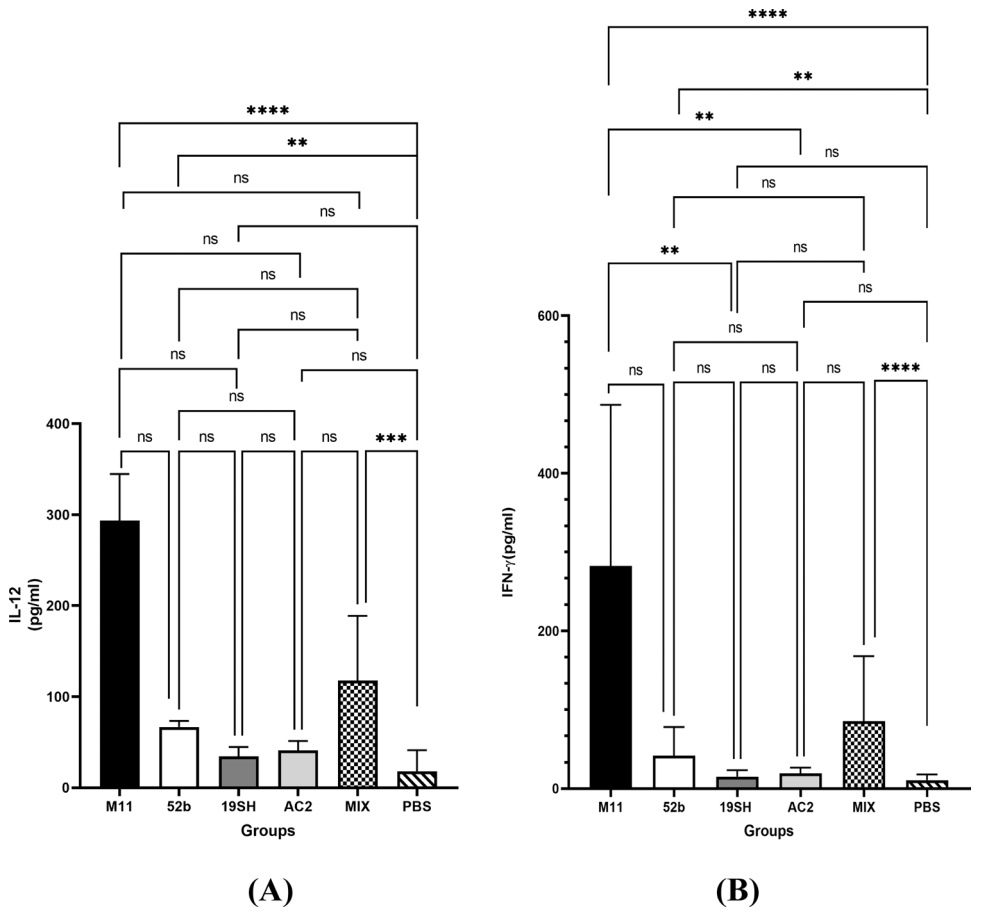


Fig. 4 A Effects of fed probiotic strains on the production of IL-12 by tumor antigen-stimulated spleen cells. The analysis was performed using the Kruskal–Wallis test to compare the control group (PBS) with the other groups as well as pairwise differences between the fed groups at $p<0.05$. M11 (****$p<0.001$), 52b (**$p=0.024$), and MIX (***$p=0.003$) compared with the PBS group. **B** Effects of fed probiotic strains on IFN-γ production in spleen cells stimulated with tumor antigen. Groups M11 (****$p<0.001$), 52b (**$p=0.047$), MIX (****$p=0.004$) compared with the PBS group, M11 compared to AC2 (**$p=0.048$) and 19SH (**$p=0.011$). **C** Effects of fed probiotic strains on TGF-β production in spleen cells stimulated with tumor antigen. 52b (****$p=0.005$) and MIX (****$p=0.001$) compared with the PBS group. **D** Effects of fed probiotic strains on GrB production in spleen cells stimulated with tumor antigen. Groups 52b (****$p=0.008$), MIX (****$p=0.006$), and 19SH (**$p=0.034$) compared with the PBS group. **E** Effects of fed probiotic strains on IL-4 production in spleen cells stimulated with tumor antigen. Groups 52b (****$p=0.007$) and MIX (****$p=0.001$) compared with the PBS group, ns: not significant

group. There was no discernible difference between the groups after pairwise comparisons between the fed groups (Fig. 4E).

Effects of tumor antigen on the proliferation of spleen cells

The findings of an investigation into how feeding bacteria affected spleen cells primed with tumor antigen to proliferate are provided in Fig. 5A. Prior to the experiment, the tumor antigen was produced and employed as a stimulus. Spleen cell proliferation was more rapid in the MIX and 52b groups than in the control, M11, AC2, or 19SH mouse groups ($p<0.05$).

The ratio of interferon gamma to interleukin4 (IFN-γ/IL-4)

Groups MIX, 52b, and M11 showed the highest level of ratio (IFN-γ/IL-4), which had a significant difference compared to the control group (Fig. 5B).

Evaluation of cellular immune response created by DTH assay

After 14 days of tumor injection to the mice, DTH test was performed to evaluate the response of Th1 cells to the specific antigen, tumor antigen was injected into the left footpad, and PBS was injected into the right footpad of the mice. The mice's footpads were assessed for thickness and

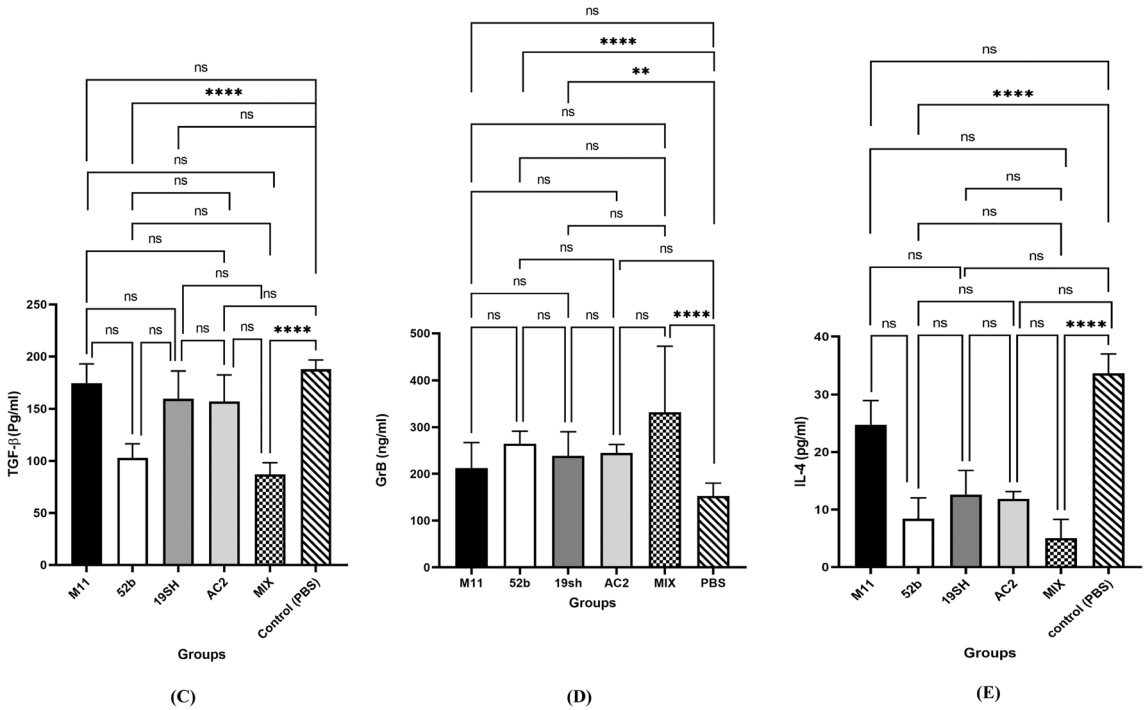


Fig. 4 (continued)

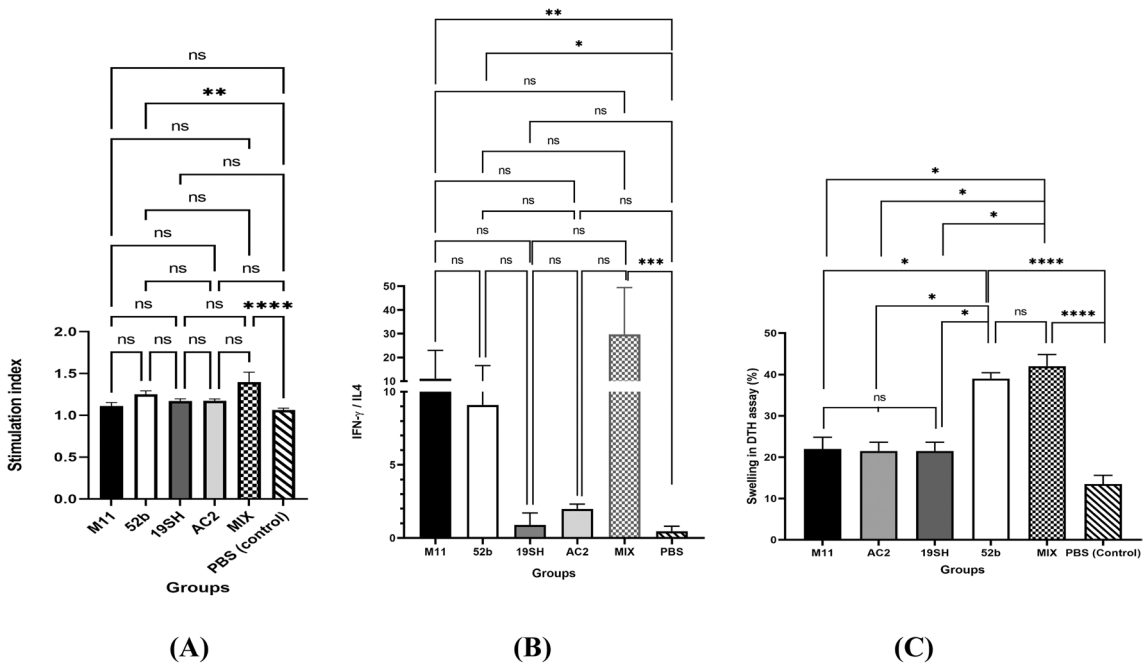


Fig. 5 **A** Comparison of the treated and control groups in terms of lymphocyte proliferation of spleen cells. The analysis was performed using the Kruskal–Wallis test to compare the control group (PBS) with the other groups as well as pairwise differences between the fed groups at $p<0.05$. 52b (**$p=0.031$) and MIX (****$p=0.007$) compared with the PBS group. **B** IFN-γ/IL-4. 52b (*$p=0.010$), MIX (***$p<0.001$), and M11 (**$p=0.008$) compared with the PBS group. **C** The evaluation of DTH response (****p-value <0.001 and *p-value <0.05), ns: not significant

swelling after 48 h. According to Fig. 5C and the obtained data, the largest DTH response was associated with the MIX and 52b groups, which substantially differed from the control and other groups. It indicated that the oral administration of these two probiotic groups enhanced the response of Th1 cells to create effective safety against CT26 tumor.

IL-12 (p70) production by CW components stimulation (in vitro)

Numerous studies have shown that mouse DCs and macrophages generated from bone marrow are capable of releasing inflammatory cytokines when exposed to components of CWs [32]. As illustrated in Fig. 6A, the highest levels of IL-12 production in the splenocyte cells of the experimental groups were related to the CW of *L. plantarum* (M11, 110.66 pg/ml) and *L. gasseri* (52b, 86.53 pg/ml), respectively ($p > 0.05$). It should be noted that the IL-12 production level of the *L. plantarum* M11 CW was significantly different from that of the other groups. The production level of IL-12 by the CW was much higher than the production level of IL-12 by the PGNs of the same bacteria. The lowest level of production of IL-12 was related to the CW of *L. acidophilus* AC2 (54.145 pg/ml), which was not significantly different compared to the level of production by the CW of *L. fermentum* 19SH (44.29 pg/ml). The effects of the CWs and PGNs of the fed bacteria on the production levels of IFN-γ in the splenocyte cells of the mice in the experimental groups were investigated (Fig. 6B). Accordingly, the highest level of IFN-γ production was related to the CW of *L. plantarum* (M11, 304.7 pg/ml), which was a significant difference from the level of IFN-γ production related to the PGN of *L. acidophilus* (AC2, 73.5 pg/ml) and that of *L. fermentum* (19 SH, 69.1 pg/ml). The PGNs of these bacteria had no significant effect on this inflammatory cytokine.

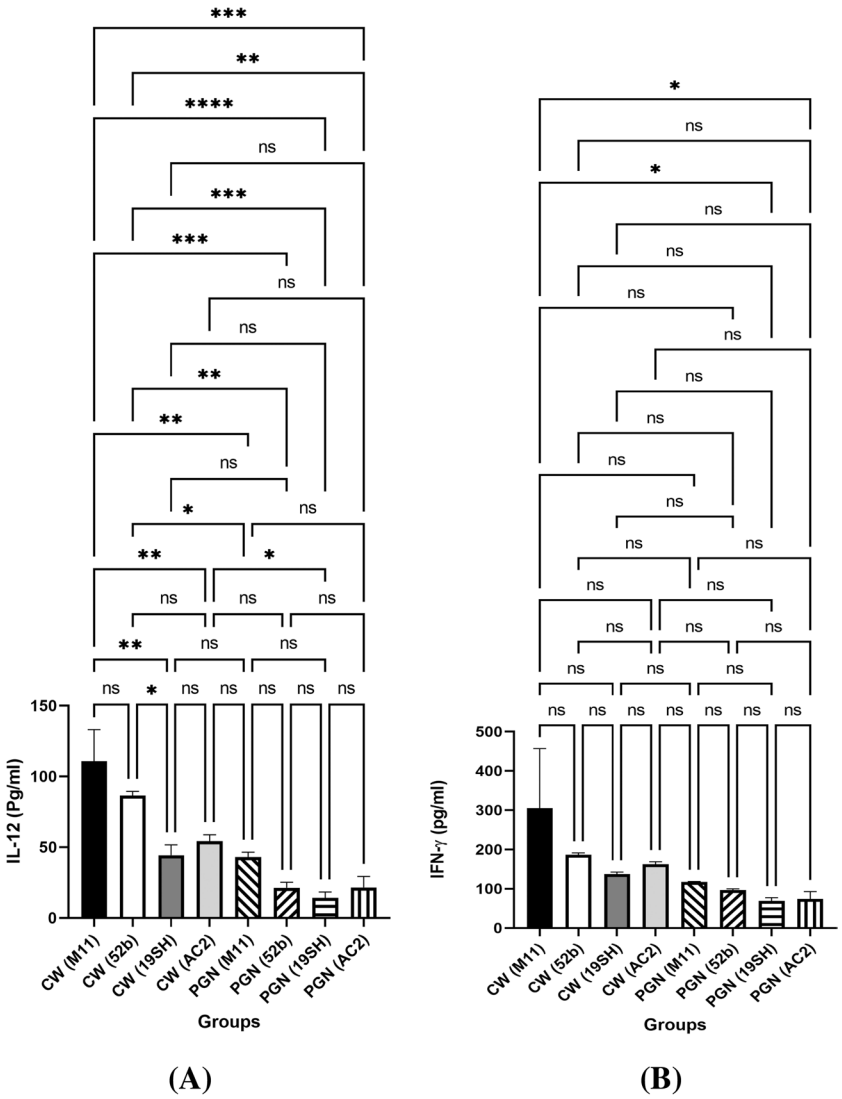


Fig. 6 **A** IL-12 (p70) production by CW components stimulation derived from the mice fed with probiotic strains. Data are presented as mean ± standard deviation, which were analyzed by one-way ANOVA and Tukey's test using Prism 8 software. A p-value of < 0.05 was considered statistically significant (****p-value < 0.0001, ***p-value < 0.0008, **p-value < 0.004, and *p-value < 0.02). **B** IFN-γ production by stimulation of CW components derived from the mice fed with probiotic strains. CW M11 compared to PGN AC2 (*$p = 0.0350$) and PGN 19SH (*$p = 0.0314$), CW: cell wall, PGN: peptidoglycan, ns: not significant

Discussion

According to previous studies, the daily consumption of some probiotic strains promotes an acquired immunity in the body and, specifically, improves Th1-related immune functions. In addition, it improves the quality of the body's health. Findings have indicated that these effects are caused by the production of IL-12 in the body, which leads to the occurrence of a Th1 immune reaction [33]. As a result, many researchers have concentrated on the significance of the function that probiotic bacteria play in causing IL-12 production. A wealth of data has been gathered that demonstrates how probiotics encourage the Th1 type of immune response by causing IL-12 production. They significantly influence the development and functionality of T cells as well as the activation of natural killer cells, which are crucial in triggering immunological responses against solid malignancies [25]. Therefore, based on the results obtained in the current study, among the 25 heat-killed strains, 4 strains of *L. plantarum* M11 were isolated from milk, *L. gasseri* 52b was isolated from human sources, *L. fermentum* 19SH was isolated from Iranian traditional food (Horreh), and *L. acidophilus* AC2 was isolated from sourdough. All were screened for feeding to tumor-bearing mice (colon carcinoma CT26), which had the highest level of IL-12 production in mouse splenocyte cells in vitro. Fourteen days before tumor injection, these strains with a concentration of 1.5×10^8 cfu/ml were fed to mice every day, from the 17th to the 24th day every other day and from the 24th to the 38th day every day with a concentration of 1.2×10^9 cfu/ml by gavage. According to the results obtained in this study, *L. gasseri* and a mixture of the studied bacteria were able to have a positive effect on the treatment process from the 14th day post-injection of tumor cells. On average, the mixed group of these bacteria (MIX) on the 22nd day after tumor injection and the *L. gasseri* 52b group on the 19th day after tumor injection were able to cause apoptosis of tumor cells and reduce tumor size to zero (Fig. 3A, B).

In patients with colorectal cancer, IL-12 production declines, and the serum level of IL-10, an anti-inflammatory Th2 cytokine, rises, according to O'Hara et al.'s [34] research. TGF- and IL-4 are Th2 cytokines with anti-inflammatory properties, just like IL-10. TGF-β stimulates stromal cells, immune cells, endothelial cells, and smooth muscle cells when the environment is favorable for tumor cell growth. TGF-β also suppresses immune response and angiogenesis, which causes cancer to advance and become more aggressive. Additionally, according to other reports, some varieties of lactic acid bacteria can prevent the creation of Th2 cytokines like TGF-β [25]. Th2 cytokines also affect cytotoxic activity, but they also prevent programmed cell death by decreasing apoptosis. For example, IL-4 increases the presence of B-cell lymphoma-extra-large and flice-like inhibitory protein molecules while decreasing CD95 molecules in tumor cells, which prevents programmed cell death [22, 35]. GrB is a crucial component of the Th1/Th2 cytokine pathway in addition to IL-12 and IFN-γ cytokines. GrB is a serine protease that is released by natural killer cells and cytotoxic T lymphocytes (CTLs), and it is vital for the removal of cancer cells and other harmful cells [36]. When the target cells are located, GrB is secreted; it uses perforin to enter the cytoplasm of the cells and trigger cell apoptosis. GrB is among the most promising candidates for advancing the development of cancer therapy because of these inherent qualities [37]. The present study's findings suggest that an increase in immune responses in Th1 cells was justified by a decrease in TGF-β and IL-4 levels in the culture medium of mice that had received *L. gasseri* 52b and a mixture of bacteria. In light of this, it made sense that tumor apoptosis in the MIX and 52b groups compared to the control group (PBS) mice would have decreased tumor growth in experimental mice. Additionally, these two groups increased the immune responses linked to Th1 cells and created tumor treatment by producing a balanced level between inflammatory and regulatory cytokines. Furthermore, the increase in the DTH response in the MIX and 52b groups compared to the control group and the high ratio of IFN-γ/IL-4 in these groups are other proofs of the increased immune reactions in Th1 cells (Fig. 5B, C). The increased proliferation of lymphocytes is another reason for the activation of the immune system by tumor antigen. In this study, the amount of lymphocyte proliferation in the spleen cells of mice in the groups receiving the mixture of bacteria and *L. gasseri* 52b increased in response to the tumor antigen (Fig. 5A).

The noteworthy point here, however, is that the levels of IL-12 and IFN-γ measured in the supernatant of splenocytes of mice fed with *L. plantarum* (M11) were higher than in the MIX and *L. gasseri* 52b groups ($p > 0.05$) (Fig. 4A, B). Therefore, at first glance, it was expected that the greatest effect of tumor treatment and apoptosis would be related to this group; conversely, however, tumor volume and weight of the mice in this group were larger than the other groups (Fig. 3C). The capacity of lactobacilli to stimulate the production of inflammatory and anti-inflammatory cytokines in co-culture with DCs or peripheral blood mononuclear cells indicates that different species of *L. plantarum* stimulate the immune cells in different ways; therefore, they can have a negative effect on the regulation of the immune response [38]. Patients with intestinal inflammation can reduce the inflammation in the affected area by taking probiotics. Moreover, probiotics have the property of stimulating and creating immunity and are also very useful for healthy and

non-diseased consumers. This means that a probiotic substance, in interaction with intestinal cells, causes the release of inflammatory compounds such as cytokines or nitric oxide. Inflammation caused by inflammatory compounds is a very useful mechanism and tool that strengthens the host's immune system against invading bacteria. However, excessive and extreme inflammation can also lead to the occurrence of acute and serious inflammatory diseases, which is probably why the tumor does not heal due to the feeding of *L. plantarum* (M11), despite the excessive induction of the inflammatory cytokine production in this group [39, 40]. In addition, excessive production of IL-12 results in high toxicity and Treg activity; therefore, it is one of the reasons that inhibits and reduces the DTH response and hence reduces the immune system's ability to fight malignancies [41]. The decrease in spleen lymphocyte proliferation in response to tumor antigen and the decrease in DTH test response in the M11 group compared to the MIX and 52b groups are reasons which prove this issue (Fig. 5A, C).

According to studies [42, 43], probiotics' anti-cancer mechanisms primarily involve the enhancement of intestinal flora, binding to DNA and killing cancer cells, immune modulation to reduce chronic inflammation, lowering intestinal pH, and inhibition of enzymes that generate carcinogenic compounds. Unusual gut flora makeup is a risk factor for colorectal cancer. The number of bacteria that induce digestive tract inflammation and may result in toxins and carcinogenic compounds is often greater in the intestinal flora of people with colorectal cancer [44, 45]. According to Chande et al. [46], colorectal cancer patients have much less and a smaller range of lactic acid bacteria in their large intestines than those without the disease [47]. Therefore, we found in this research that probiotic administration increases the quantity and variety of mucosal bacteria and enhances microbial structure.

The literature shows that the structure of the LTA of CW is different and diverse, depending on the source from which the bacterial strain is isolated, mainly due to the amount of D-Ala and the length of the multiple chain (glycerol-phosphate) [30, 41]. Fibronectin is one of the host receptors of LTA. In fact, D-Ala plays a role in the surface adhesion of the cell and protects the cell against cationic antimicrobial peptides. Owing to the presence of a higher percentage of D-Ala in the LTA of the CW, human strains have a greater ability to adhere to intestinal epithelial cells than those of food origin and can cause more stimulation of inflammatory cytokines such as interleukin-12. Indeed, the difference between the food and human strains is associated with the difference in the genes that produce the CW surface proteins and, as a result, the difference in their adhesion to and binding with fibronectin [41].

Darmastuti et al. [48] conducted a study on identifying the genes related to the adhesion and virulence of the *L. plantarum* subsp. *plantarum* Dad-13 and *L. plantarum* subsp. *plantarum* Mut-7 and found out that the two genes of polymerase I and transcript-release factor (PtrF) as well as chaperonin heat shock protein 33 (hsp33) were 100% identified in both strains. The PtrF gene encodes F-protein which is a bacterial superficial protein that binds with fibronectin with high affinity. Another gene that is likely to be involved in the adhesion of the bacteria is hsp33. The expression of this gene is a protein named thermal shock protein which protects unfolded proteins against bacterial accumulation. In addition, this protein can adhere to the host and the microbial cell surface. Therefore, it may be able to facilitate the colonization of the bacteria. hsp 60, hsp 70, hsp 90, and hsp 100 are the other genes playing a role in cell adhesion. Following the phenotype assay of the adhesion genes, it was realized that both strains were highly capable of adhesion and auto-aggregation. Moreover, based on the phenotypical assays of the genes related to virulence, they showed that in both strains, namely *L. plantarum* subsp. *plantarum* Dad-13 and *L. plantarum* subsp. *plantarum* Mut-7, there were no genes related to hemolysin, lipids, and proteins, as one of the pathogenic factors, causing the lysis of red blood cells by disrupting their cell membranes. They stated that consumption of *L. plantarum* subsp. *plantarum* Dad-13 did not negatively affect the general health, body weight, leukocyte profile, glutamic-oxaloacetic transaminase activity, plasma malondialdehyde concentration, or the intestinal morphology of the tested rats. Therefore, based on these findings, it was suggested that both probiotic bacteria are safe for consumption [48].

The previous study demonstrated the adhesion ability and pathogenicity of these strains, namely *L. gasseri* (52b), *L. plantarum* (M11), *L. acidophilus* (AC2), and *L. fermentum* (19SH). It was realized that the adhesion rate was the only probiotic property that differentiated the human strain of *L. gasseri* (52b) from the other feeding groups, which was significantly higher than those of the other bacteria in both in vitro and in vivo conditions. Furthermore, by regularly feeding these bacteria to the BALB/c mouse model, even at high doses, no negative or harmful effects were observed on the mice's general health, organ weight, hematology, and tissue parameters of the organs and blood. On the other hand, they caused a decrease in intestinal pathogens, due to their high capability of auto-aggregation with intestinal pathogens such as *Salmonella*, *Shigella*, and coliforms under in vitro and in vivo conditions. Consequently, their safety was proven [49].

Hirose et al. [30] investigated the CW and PGN of *L. plantarum* L-137 and *L. plantarum* subsp. *plantarum* JCM1149 strains and concluded that with a higher ratio of D-Ala to diaminopimelic acid in the LTA chain, the production level of IL-12 will be more. They found that because the ratio of D-Ala in the CW of *L. plantarum* L-137 is higher

than the D-Ala in the CW of the *L. plantarum* subsp. *plantarum* JCM1149 strain, it can produce more inflammatory cytokines, which can lead to TLRs 2 stimulation. They also stated that purified PGN does not affect TLRs 2 [30]. In the current study, the highest level of production of inflammatory cytokines (IL-12, IFN-γ) by the CW of the groups fed *L. plantarum* M11 and *L. gasseri* 52b was completely in agreement with the results of experiments on feeding these bacteria alive and related experiments to measure the inflammatory and anti-inflammatory cytokine levels produced by them in mouse spleen cells.

Conclusion

According to the current results, regular feeding of live strains of *L. fermentum* and *L. acidophilus* slowed down the disease process, and *L. gasseri* strains and the mixture of these bacteria caused apoptosis and treatment of tumors (colorectal cancer) in the BALB/c mouse model. Adhesion ability to epithelial cells is one of the important probiotic properties of lactobacilli, which applies more to human strains. In terms of having a different structure in their LTA, i.e., having more D-Ala, a longer acyl chain, and more glucose in their phospholipid anchor, these strains cause more stimulation of TLR2 which induces more inflammatory cytokines and programmed death of tumor cells. Using the mixture of bacteria (MIX) and *L. gasseri* native strains isolated from human sources (52b) can moderate the immune response and increase immune reactions in Th1 cells. It is suggested to use *L. gasseri* strains and the mixture of the tested bacteria as an immunoadjuvant in dairy products, especially yogurt, because of their high resistance in acidic conditions. However, before that, clinical and immunological tests must be conducted on patients who are at the beginning of the tumor growth stages. Overall, it needs more investigation to be used in the industry.

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Author contribution Samaneh Hatami: conceptualization, methodology, formal analysis, and investigation, writing (original draft preparation, review, and editing), resources, and software. Masoud Yavarmanesh: conceptualization, methodology, writing (review and editing), supervision, and project administration. Mojtaba Sankian: supervision and project administration. All authors approved the final version submitted.

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Data availability The data generated or analyzed that support the findings of this study are available and included in this published article.

Declarations

Ethics approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study involves animal testing (mice). The ethical criteria with the code of IR.UM.REC.1400.004 (Ferdowsi University of Mashhad, Iran) were observed.

Consent for publication All authors consent for publication.

Competing interests The authors declare no competing interests.

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