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Original Article

Investigation of Lactobacillus Probiotics Derived from Traditional Dairy Products in Eliciting Anti-Tumor Responses in Mouse Colorectal Cancer Model

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What's Known

- While the impact of standard strains on the restoration of the gut microbiota and the overall health of individuals with CRC has been studied, research on the effects of traditional strains has not been evaluated.

What's New

- The simultaneous use of Lactobacillus strains along with 5-FU can potentially enhance the anti-tumor effects of the standard drug 5-FU, indicating that these isolated lactic acid bacteria could be beneficial for colorectal cancer patients in practical applications.

Abstract

Background: Colorectal cancer (CRC) is a serious health problem, and finding new treatments is important. There is growing evidence for the antitumor activity of probiotics. This study investigated the anti-cancer potential of a probiotic mix containing *Lactobacillus plantarum, L. brevis, L. helveticus,* and *L. delbrueckii,* alone or in combination with the chemotherapy drug 5-fluorouracil, against CRC.

Methods: The research was carried out in Mashhad in 2021. The cytotoxic effect of Lactobacillus isolates on CRC cells was investigated in two-dimensional and three-dimensional cell culture models. Histological staining and molecular approaches were used to investigate the regulatory mechanism of Lactobacillus isolates on cell migration, inflammation, fibrosis, cell cycle progression, apoptosis, and tumor necrosis in the CRC mouse model. Statistical analysis was performed using SPSS software version 20 with a significance level of $P<0.05$. The tests employed included the Kolmogorov–Smirnov, ANOVA, Dunnett's *post hoc*, and Kruskal–Wallis.

Results: Lactobacillus strains effectively suppressed tumor growth in CRC by promoting cell death and inhibiting fibrosis and inflammation. These bacteria regulated apoptosis-related genes such as Bcl-2-associated protein x ($P=0.0033$), and *BCL-2* ($P=0.0029$), leading to increased tumor necrosis. Treatment with bacterial supernatants reduced tumor size and fibrosis by downregulating collagen type I, alpha 1 (*Col1a1*) ($P=0.024$), *Col1a2* ($P=0.0231$), and *actin alpha 2* ($P=0.0466$), and transforming growth factor-beta expression. Additionally, they suppressed inflammation by decreasing tumor necrosis factor-alpha ($P=0.0001$), interleukin 6, and IL-1β ($P=0.0198$) levels in tumor tissues. Furthermore, the treatment inhibited CRC cell migration by modulating epithelial cadherin ($P=0.0198$) and matrix metallopeptidase 2 ($P=0.033$) expression.

Conclusion: Findings indicated that co-administration of Lactobacillus isolates with 5-FU could improve the anti-tumor properties of the standard drug, 5-FU, supporting the therapeutic potential of these safe isolated lactic acid bacteria for CRC patients *in vivo*.

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Keywords ● Probiotic ● *Lactobacillus* ● Colorectal cancer ● Inflammation ● Fibrosis

Introduction

Colorectal cancer is a significant global health concern, accounting for the second-highest number of cancer-related deaths worldwide, claiming over 900,000 lives annually.[1] World Health Organization reports indicate that the prevalence of CRC is rapidly increasing worldwide.[2] Despite recent advances in surgical, radiation, and chemotherapy methods for the treatment of CRC, the survival rates are poor, and patients suffer from cancer recurrence and serious side effects, such as gastrointestinal complications, which affect the quality of life.[3]

CRC is a multifactorial disease associated with various risk factors, including genetic predispositions, physical inactivity, alcohol consumption, and unhealthy diet, which may be responsible for compositional alterations in the gut microbiota.[4] It was shown that altered intestinal microbiota induces tumorigenesis by modulating immune responses, metabolism, and uncontrolled molecular activities in colonocytes.[5] Moreover, recent studies on CRC patients have demonstrated that chemotherapy causes microbial imbalance (dysbiosis), which is positively correlated with tumor recurrence and poor prognosis.[6] Therefore, modulation of intestinal microbiota by bio-interventions such as probiotics offers a promising therapeutic approach for CRC prevention and treatment.[7]

Probiotics are non-pathogenic microorganisms that significantly affect the balance and diversity of the gut microbiome.[8] Probiotics are known to be potentially useful in the treatment and inhibition of cancer cell progression. Numerous studies using human cancer cells demonstrated that probiotics have antiproliferative properties and induce apoptosis in several cancers, including colorectal, stomach, breast, and cervical cancers. As a result, probiotic-based regimens are frequently used as adjuvants alongside anti-tumor drugs.[9-11] These regimens aim to enhance the therapeutic outcomes and reduce the adverse effects associated with cancer treatment. The anticancer mechanisms of probiotics have been reported and established in the literature. These mechanisms encompass the alteration of gut microbiota, enhancement of gut barrier functions, breakdown of potential carcinogens, safeguarding against DNA damage in the intestinal epithelium, and reinforcement of the immune and inflammatory systems within the body.[12, 13]

Previous preclinical studies reported that Lactobacillus probiotics had adjuvant properties against various cancers and gastrointestinal disorders, including infectious diarrhea and inflammatory bowel disease.[14, 15] Lactobacillus supplementation was found to help regulate the microbiota, produce antimicrobial, anti-inflammatory, antioxidant, and anticancer metabolites, and improve intestinal permeability in patients with CRC.[16] Probiotic intervention was found to protect CRC patients from chemotherapy-related gastrointestinal complications.[3, 17] However, the molecular mechanism of probiotics' anticancer activity remains to be investigated.

Since probiotic strains' characteristics are species-dependent, we isolated four Lactobacillus strains from traditional dairy sources in Northeast Iran, including *L. plantarum, L. brevis, L. delbrueckii,* and *L. helveticus*. We further investigated the antitumor properties of these bacterial supernatants in CRC. Their anti-proliferative, anti-inflammatory, and antioxidant properties were examined in CRC cells and mouse models.

Materials and Methods

Isolation and Characterization of Lactobacilli

Lactobacillus strains were isolated from samples of traditional yogurt and milk collected in Northeast Iran (Khorasan Razavi Province). The strains were examined and confirmed by genotyping after the initial biochemical evaluation, including Gram staining and assessments for catalase, oxidase, urease, methyl red, citrate utilization, triple sugar iron, gas production, and fermentation to identify the lactic acid bacteria isolates. Then, their probiotic properties were evaluated.

Biochemical Tests and Molecular Identification

Bacteria were cultured on De Man–Rogosa–Sharpe (MRS) agar plates after being inoculated into MRS broth (Merck, Germany). Next, Gram staining and other biochemical tests were performed on purified colonies. Molecular identification of probiotics was previously performed via *16srRNA* gene sequencing as described.[18]

Antibacterial Activity of Probiotic Strains

Lactobacillus species were isolated from traditional dairy products such as milk and yogurt in northeastern Iran. The isolated strains were then tested for their antibacterial properties against key pathogenic microorganisms, including *Staphylococcus aureus* (ATCC 25923), and *Salmonella enterica* subsp. enterica serotype Typhimurium (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (PTCC 1338), and *Enterococcus faecalis* (ATCC 29212). The evaluation was performed using a disk diffusion method.[19]

Screening of Antibiotic Resistance

The antibiotic resistance patterns of the strains were evaluated according to the guidelines provided by the Clinical and Laboratory Standards Institutes (CLSI-2020). To perform this assessment, antibiotic disks (table 1) were positioned on MRS agar plates, and the zones of inhibition were measured using a caliper, following the described procedure.[20-22]

Acid and Bile Salt Resistance Assays

The study investigated the ability of bacteria to survive in acidic environments and resist the effects of bile salts. Following the growth of each strain on MRS agar, it was transferred to the sterile saline solution (0.85%) to create a 1.0 McFarland suspension. A 10 µL aliquot of the suspension was then applied to agar plates containing varying concentrations of Ox-bile (0.3%, and 1.0%, w/v, Sigma-Aldrich). The plates were anaerobically incubated at 37 °C and assessed after 24 hours. Plates without any bacterial growth were classified as negative, while those with colonies were considered positive. Plates lacking Ox-bile served as the control.[22]

Resistance to Digestive Juices

To assess the resistance of the strains to simulated gastric and intestinal juice, the following steps were performed. A 24-hour culture of each strain in 30 mL of MRS broth was centrifuged at 8,000×g for 5 min at 4 °C. The supernatant was carefully removed, and the collected cells were washed twice with 10 mL of 50 mM Phosphate-buffered saline (Sigma-Aldrich, Germany) (pH=6.5). The washed cells were then re-suspended in 3 mL of PBS buffer. One mL of each strain, containing approximately 9 log CFU/mL of bacteria, was added to 9 mL of simulated gastric juice that was prepared by adding pepsin (3 g/L) from Sigma-Aldrich and adjusting the pH to 2.5. The prepared gastric juice with the bacterial suspension was incubated at 37 °C for 3 hours. After incubation, the suspension was subjected to centrifugation at 3,800 rpm for 10 min. The supernatant was carefully extracted, and the bacterial pellets were washed with PBS. The pellet was re-suspended in simulated intestinal juice containing 0.1% w/v pancreatin (Sigma-Aldrich, Germany), 0.15% w/v bile salt, and adjusted to pH 8.0. The suspension was incubated for 3 hours at 37 °C. After incubation, the surviving bacteria were counted, and the results were reported as log CFU/mL.[23]

Hemolysis Assay

The Lactobacillus strains were cultivated in MRS medium for 18–24 hours at 37 °C. To evaluate their ability to cause hemolysis, the streak plate technique was utilized on agar plates containing 5% sheep blood agar.

In-Vitro Experiments

Preparation of Lactobacilli Cell-Free Supernatant: Each strain equally was cultured in MRS medium and placed in anaerobic conditions at 37 °C for 24 hours. The concentration of bacteria ($5*10^{11}$ CFU/mL) in each solution (ranging from 10 to 10,000 bacteria per cell) was determined by measuring the optical density (OD) at 600 nm using a spectrophotometer) Jen Way, England). The samples were then centrifuged to remove bacteria and debris, and the bacterial supernatant was filtered through a 0.2 µm membrane filter. Since the supernatant had an acidic pH, NaOH (Sigma-Aldrich, Germany) was added to adjust the pH for optimal cell growth. Subsequently, the supernatant was mixed with a cell culture medium containing Fetal bovine serum purposes.

Cell Culture: Human colon cancer cell line (SW-480(and colon tumor 26 (CT-26), two colorectal cancer cell lines, along with 3-day transfer, inoculum $3×10^5$ cells (3T3), a normal fibroblast cell line, were cultivated in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Sigma-Aldrich, Germany) or Roswell Park Memorial Institute (RPMI 1640) (Sigma-Aldrich, Germany) medium supplemented with 10% FBS and 1% streptomycin/penicillin (Sigma-Aldrich, Germany). The cells were then maintained at a temperature of 37 °C in a 5% CO_2 atmosphere.

Table 1: The bacterial antibiotic resistance patterns (mm) based on the CSLI guidelines

Antibiotics	Resistant (R)	Intermediate (I)	Sensitive (S)
Ciprofloxacin (5 µg)	≤15	16-20	≥21
Erythromycin (15 µg)	≤15	16-20	≥21
Gentamicin (10 µg)	≤12	13-14	≥15
Vancomycin (30 µg)	≤9	10-11	≥12
Tetracycline (30 µg)	≤18	19-22	≥23
Clindamycin (2 µg)	≤15	16-18	≥19
Ampicillin (10 µg)	≤12	13-15	≥16
Chloramphenicol (30 µg)	≤12	13-17	≥18
sulfamethoxazole/trimethoprim (25 µg)	≤10	11-15	≥16

Cell Viability Assay (MTT): To assess the potential cytotoxic property of the bacterial supernatants on CRC cells, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was carried out as described.[24] Briefly, SW-480 and CT-26 cells were exposed to a variety of concentrations of bacterial cell-free supernatants (100, 1000, 10000, and 100000 bacteria per cell), and the inhibitory concentration (IC_{50}) was measured using a microplate reader (Sunrise, Switzerland). All experiments were performed in triplicates and were repeated and confirmed.

Multicellular Spheroids: To develop a three-dimensional (3D) tumor model, 10^4 cells/mL were seeded in an agarose-coated cell culture plate (Maxwell, China). Tumor spheroids were analyzed using the Leica DMI300B inverted phase contrast microscope (Leica, Wetzlar, Germany) to assess their size and shape with or without bacterial cell-free supernatants.

Migration Assay: For the scratch assay, SW-480 cells were cultured under conditions of 37 °C and 5% CO_2 until they reached 70% confluence. Subsequently, the cells were scratched using a p200 pipette tip (Maxwell, China), and any unattached cells were washed away with PBS. The extent of the scratch area was quantified using a 10× objective on a ZEISS microscopy system (Germany). The cells that were not scratched served as the control group to evaluate the movement of the scratched cells. The rate of wound healing was analyzed using the ImageJ software developed by the National Institutes of Health (NIH) (Bethesda, MD).[25]

Cell Cycle Analysis: Cell cycle analysis was performed to determine the inhibitory impacts of bacterial supernatants on cell cycle progression. In this study, colorectal cancer cells were treated with bacterial cell-free supernatants and subsequently, incubated with RNase enzyme (Sigma-Aldrich, Germany). The treated cells were then stained with propidium iodide (PI) (STEMCELL, China), and the distribution of cells in different phases of the cell cycle was analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA). The cell cycle was then evaluated utilizing the FlowJo V10_CL software (BD Biosciences, USA).[26]

Apoptosis Assay: To investigate the impact of bacterial supernatant on apoptosis in CRC cells, a flow cytometric method using Annexin V (FITC-conjugated) and Propidium iodide (PI) (STEMCELL, China) was employed, as previously described.[27] In summary, CRC cells were exposed to probiotic supernatants for 24 hours. The treated cells were then incubated in Annexin binding buffer and stained with Annexin V-FITC/PI staining solution. Subsequently, a BD FACSCalibur flow cytometer from BD Biosciences was used to determine the percentages of viable, apoptotic, and necrotic cells. The excitation/emission wavelengths of 488/525 nm and 488/675 nm were utilized for Annexin-V and PI, respectively. Then, the results were evaluated by the FlowJo V10_CL software.

In-Vivo Experiments

Animal Experiment: The animal experiments conducted in this study were carried out in compliance with the guidelines for the Care and Use of Laboratory Animals approved by the ethics committee of Mashhad University of Medical Sciences and housed in standard conditions approved by the Animal Ethics Committee Institute (temperature 22±2 °C, humidity of 54±2%, and 12 h light/dark cycle).[27] The study Approval ID was (IR.MUMS.MEDICAL.REC.4001008). Thirty male BALB/c mice (aged 8 weeks, weight 23-25 g) were used. Once the tumors reached a size ranging from 80 to 100 mm^3, the animals were randomly categorized into four unisexual groups: 1- Only treated with PBS (cancer model) (N=6); 2- 5FU-treated (5 mg/Kg every other day, intraperitoneal injection) (N=6); 3- treated with a mix of probiotics, orally administered with 0.2 mL (bacterial supernatant, 5×10^{11} colony-forming units) bacterial cocktail for 3 weeks (N=6); and 4- combination group treated with 5-FU and bacterial cocktail (N=6). The tumors were monitored every other day, and the size of the tumors was measured with the digital caliper. On day 21, the mice were euthanized, and the colon tumors were gathered for histological analysis using Hematoxylin-Eosin (H&E) and Masson's trichrome staining.[28]

Real-Time PCR Analysis: The methodology for real-time PCR was conducted as previously described.[29] In summary, total RNA was extracted from tumor tissues and converted into cDNA using a reverse transcription kit following the manufacturer's instructions (TaKaRa Bio, Shiga). Real-time PCR was carried out using specific primers for the target genes (table 2) on an ABI-PRISM StepOne instrument (Applied Biosystems, Foster City, CA).

Enzyme-Linked Immunosorbent Assay (ELISA): The tumor tissues were processed by homogenization, and the protein concentrations were assessed using a BCA quantification kit (Sigma-Aldrich, Germany). Subsequently, the concentrations of Tumor necrosis factor alpha (TNF-α), Interleukin 6 (IL-6), and Transforming growth factor beta (TGF-β) in the tissue samples were quantified utilizing an ELISA kit (Elabscience, USA).

Table 2: The qPCR primer sequences

Gene	Source	Primer	Sequence
GAPDH	Mouse	Forward Reverse	CAACGACCCCTTCATTGACC CTTCCCATTCTCGGCCTTGA
MMP-2	Mouse	Forward Reverse	AACTGTTGCTTTTGTATGCCCT CGATGTCAGACAACCCGAGT
MMP-9	Mouse	Forward Reverse	GCGTCGTGATCCCCACTTAC CAGGCCGAATAGGAGCGTC
p53	Mouse	Forward Reverse	GGACAGCTTTGAGGTTCGTG TCATTCAGCTCCCGGAACAT
Bax	Mouse	Forward Reverse	AGACAGGGGCCTTTTTGCTAC AATTCGCCGGAGACACTCG
E-cad	Mouse	Forward Reverse	GTCTACCAAAGTGACGCTGA GGGAAACATGAGCAGCTCTG
Col1a1	Mouse	Forward Reverse	GGCAATGCTGAAATGTCCCA CCTTCAACAGTCCAAGAACCC
Col1a2	Mouse	Forward Reverse	GTTCTCAGGGTAGCCAAGGT CCTTCAAAACCAAAGTCATAGCC
IL-1β	Mouse	Forward Reverse	GACTTCACCATGGAATCCGT TGCTCATTCACGAAAAGGGA
TNF-α	Mouse	Forward Reverse	AGGCTGTCGCTACATCACTG CTCTCAATGACCCGTAGGGC
MCP-1	Mouse	Forward Reverse	GTGAAGTTGACCCGTAAATCTGA ACTAGTTCACTGTCACACTGGT
BCL2	Mouse	Forward Reverse	GCTACCGTCGTCGTGACC CCCCACCGAACTCAAAGG
Acta2	Mouse	Forward Reverse	CCCAGACATCAGGGAGTAATGG TCTATCGGATACTTCAGCGTCA

Statistical Analysis

All experiments were conducted in triplicate, and the results are presented as the mean±SD. The Kolmogorov–Smirnov test was carried out to test the normality of continuous data. In the case of normally distributed data, a one-way analysis of variance (ANOVA) was performed, followed by Dunnett's *post hoc* test. If the data were not normally distributed, the Kruskal–Wallis test was performed, and Dunn's test was used for pairwise comparison. Repeated Measure ANOVA was employed to assess the differences in days across various treatment groups. Statistical significance was considered when $P<0.05$.

Results

Biochemical Tests and Molecular Identification

The results obtained from biochemical and molecular tests indicated that all four isolated strains of Lactobacillus bacteria exhibit characteristics of being catalase -ve, citrate -ve, H2S -ve, oxidase -ve, urease -ve, Gram +ve, Methyl Red -ve.

Antibacterial Activity of Probiotic Strains

The antibacterial activity tests conducted against various pathogenic bacteria demonstrated that all four strains exhibit antibacterial activity against *S. aureus* (ATCC 25923) and *S. enterica* subsp. enterica serotype Typhimurium (ATCC 14028). Moreover, *L. brevis, L. helveticus,* and *L. delbrueckii* strains have antibacterial activity against *P. aeruginosa* (ATCC 9027), while *L. plantarum* showed no inhibitory effect on *P. aeruginosa* (ATCC 9027). All four strains exhibited no antimicrobial activity against *E. faecalis* (ATCC 29212). Moreover, among the four strains, only *L. delbrueckii* exerted antimicrobial activity against *E. coli* (PTCC 1338) and showed the highest inhibitory effect on standard pathogens (table 3).

Screening of Antibiotic Resistance

The assessment of antibiotic sensitivity for commercially available antibiotics revealed that the isolates were susceptible to a range of common antibiotics, including erythromycin, ampicillin, chloramphenicol, and tetracycline. However, they showed significant resistance to ciprofloxacin, followed by gentamicin. Detailed results are presented in tables 1 and 4.

Acid and Bile Salt Resistance Assays

The growth of all four selected isolates was influenced differently under acidic and bile salt conditions. As shown in table 5, the results were reported qualitatively. Plates without bacterial growth were considered negative, and those with colonies were considered positive. Two isolates were tolerant to bile salt, and three were tolerant to low pH.

Table 3: Antibacterial activity of Lactobacillus strains against pathogenic strains

Strain	*Salmonella enterica* serotype Typhimurium	*Escherichia coli*	*Pseudomonas aeruginosa*	Enterococcus faecalis	Staphylococcus aureus
L. plantarum	Sensitive	Resistant	Resistant	Resistant	Sensitive
L. brevis	Sensitive	Resistant	Sensitive	Resistant	Sensitive
L. helveticus	Sensitive	Resistant	Sensitive	Resistant	Sensitive
L. delbrueckii	Sensitive	Sensitive	Sensitive	Resistant	Sensitive

The inhibition zone (mm) around the paper disc containing the microbial cell-free supernatant was classified as Sensitive: >12 mm; Intermediate: 10-11 mm; Resistant: >9

Table 4: The bacterial antibiotic resistance patterns based on the diameter (mm) of the inhibition zones

Antibiotic	L. helveticus	L. delbrueckii	L. brevis	L. plantarum
Ciprofloxacin	0 (R)	15 (R)	0 (R)	0 (R)
Erythromycin	17 (I)	31 (S)	24 (S)	23 (S)
Gentamicin	13 (I)	0 (R)	4 (R)	10 (R)
Vancomycin	0 (R)	25 (S)	24 (S)	0 (R)
Tetracycline	21 (I)	30 (S)	28 (S)	20 (I)
Clindamycin	10 (R)	8 (R)	18 (I)	19 (S)
Ampicillin	27 (S)	27 (S)	26 (S)	32 (S)
Chloramphenicol	30 (S)	26 (S)	26 (S)	27 (S)
Sulfamethoxazole/ trimethoprim	30 (S)	0 (R)	0 (R)	16 (S)

S: Sensitive; I: Intermediate; R: Resistant

Table 5: Acid and Bile tolerance of isolates after 4 hours

Species	0.3% bile	1% bile	pH 3.0
L. plantarum	+	-	+
L. brevis	+	+	-
L. helveticus	+	+	+
L. delbrueckii	+	-	+

+: Bacterial growth; -: No growth

Table 6: The resistance of Lactobacillus spp to simulated gastric and intestinal fluid (log/mL)

Strain	Gastric (0 hour)	Gastric (2 hours)	Intestine (4 hours)	Intestine (6 hours)
L. helveticus	7.477 (log/mL)	6.560 (log/mL)	4.356 (log/mL)	3.736 (log/mL)
L. brevis	7.280 (log/mL)	6.571 (log/mL)	3.435 (log/mL)	0
L. delbrueckii	7.338 (log/mL)	4.657 (log/mL)	3.259 (log/mL)	0
L. plantarum	8.134 (log/mL)	3.435 (log/mL)	3.259 (log/mL)	0

Tolerance to the GIT Condition

For a probiotic strain to be effective, it must survive and pass through the gastrointestinal tract without significant reduction in numbers, as shown in table 6. Out of the Lactobacillus isolates tested, all four strains survived after 0, 2, and 4 hours under the conditions simulated by the gastrointestinal tract. After 6 hours, only *L. helveticus* was viable.

Hemolysis Assay

Our findings showed that none of the strains exhibited α or β-hemolysis, indicating that they did not cause any damage to red blood cells (RBCs). As a result, these strains were deemed safe based on this assessment.

Lactobacilli Cell-Free Supernatant Inhibits CRC Cell Growth and Cell Cycle Progression

As shown in figure 1A, administration of Lactobacillus supernatant significantly decreases CRC cell growth with an IC_{50} of 10^3 "the ratio of bacteria to the cancer cell". The MTT result indicated that treatment with the same concentration of bacterial supernatant had no cytotoxic effects on normal fibroblast cells (3T3). To further investigate the anti-proliferative effects of Lactobacillus strains cell-free supernatant, the cell spheroids were treated with bacterial supernatant for 5 days. Consistent with the MTT results, Lactobacillus strains cell-free supernatant significantly decreased the size of spheroids in the 3D cell culture model (figure 1B).

It was found that the administration of these bacterial supernatants, either alone or in combination with 5-FU induced a G1 phase arrest in the CT-26 cells (60.3% and 64.1%, respectively). As presented in figure 1C, administration of probiotics significantly increases

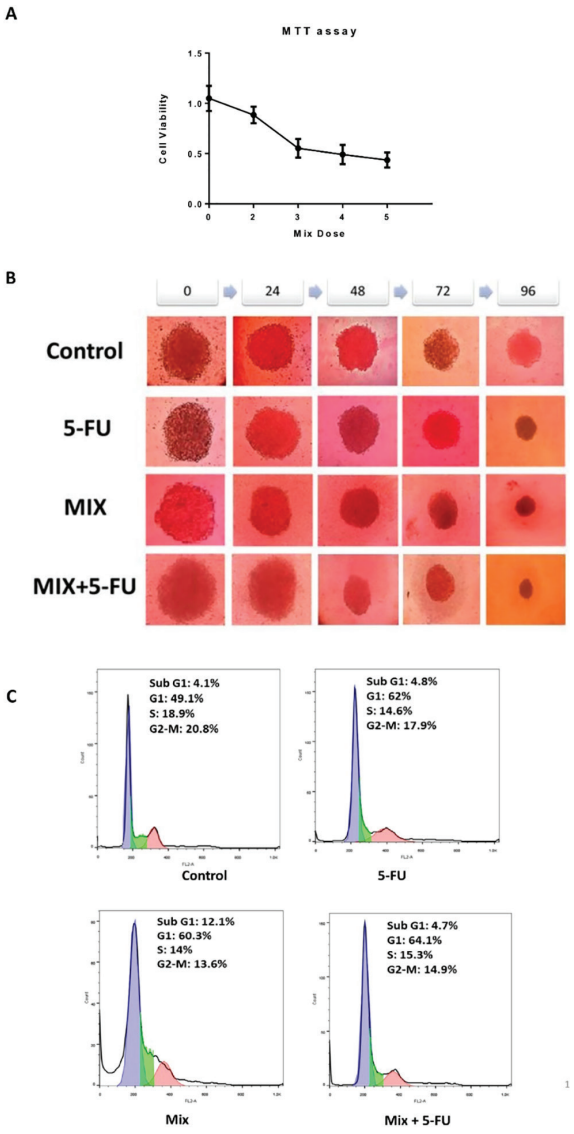

Figure 1: Cell-free supernatant from Lactobacillus strains inhibited cell proliferation and cell cycle progression (n=6 in each group). (A) CT-26 cells were treated with Lactobacillus strains cell-free supernatant for 24 hours, and the cell viability was determined by MTT assay. (B) The effect of Lactobacillus strain cell-free supernatant on CRC spheroids after 0, 24, 48, 72 and 96 hours. (C) The cell cycle analysis by Propidium iodide (PI) staining is shown. CT-26 cells were treated with Lactobacillus strain's cell-free supernatant for 24 hours, and the cells were stained with PI and analyzed by flow cytometry.

CRC cells in the sub-G1 phase (12.1%).

Lactobacillus Strains Cell-Free Supernatant Inhibits Tumor Growth in Mouse Model of CRC

As shown in figure 2 (A-C), gavage administration of bacterial cell-free supernatants decreased the size and weight of tumor tissues. A repeated measure ANOVA test was performed (figure 2A), but the difference in the trend between groups was not statistically significant. However, when specific time points between groups were compared, statistically

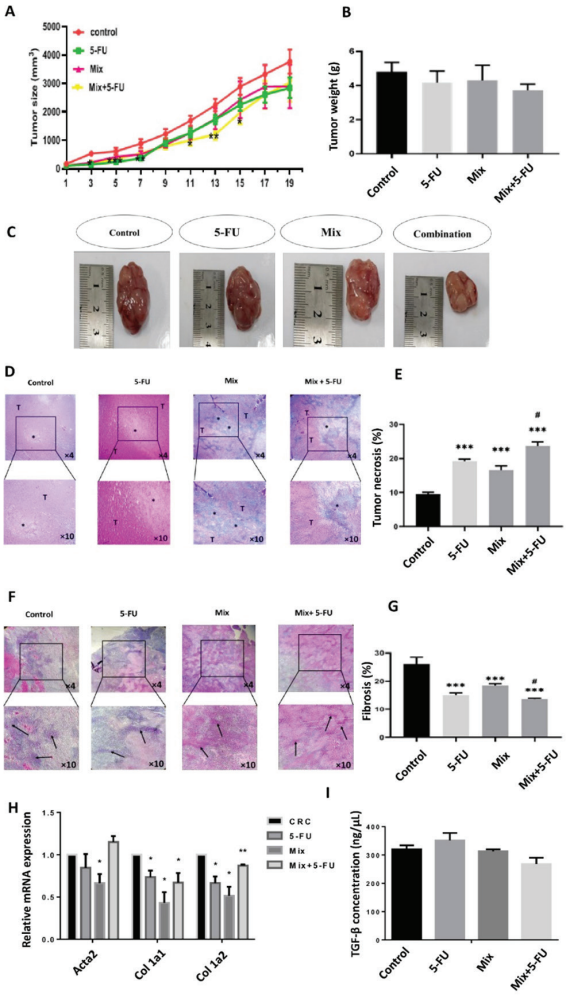

Figure 2: Cell-free supernatant from Lactobacillus strains suppressed tumor growth in a colorectal cancer mouse model (n=6 in each group). (A-C) Tumor size and weight in CRC mice model treated with Lactobacillus strains cell-free supernatant, 5-FU, and their combination, respectively (*: indicates P<0.05, **:P<0.01, ***: P<0.0001); (D) Lactobacillus strains cell-free supernatant induced necrosis in tumor tissue of colorectal cancer (n=5 in each group). H&E staining of tumor sections showed that mix, 5-FU, and mix+5-FU induced necrosis; (E) Percent of tumor necrosis (*: indicates the necrotic area, T: Tumor cells); (F) Lactobacillus strains cell-free supernatant reduced Fibrosis in tumor tissue of colorectal cancer (n=5 in each group). Masson trichrome staining of tumor sections showed that mix, 5-FU, and mix+5-FU reduced fibrosis (black arrows indicate fibrotic area); (G) Percent of tumor fibrosis; (H) Tumor extracts were subjected to q-PCR analysis to evaluate *Col1a1* (P=0.024), *Col1a2* (P=0.0231), and *Acta2* expression (P=0.0466). GAPDH serves as an internal control; (I) CRC tumor tissues were subjected to ELISA to evaluate the protein levels of TGF-β in tumor tissues.

significant differences were seen on some days. Interestingly, compared to the 5-FU treated group, co-administration of bacterial supernatants plus 5-FU, could enhance the anti-cancer properties of 5-FU (figure 2B). The histological staining revealed that Lactobacillus strains cell-free supernatant caused tissue necrosis in the CRC model (figures 2D and E). Fibrosis results from

disturbances in the wound healing process, manifested by the high collagen content of the extracellular matrix (ECM). As illustrated in figure 2 (F-H), our findings indicated that Lactobacillus strains cell-free supernatant considerably reduced collagen deposition and suppressed the expression of Collagen type I, alpha 1 (*Col1a1*), *Col1a2,* and Actin alpha 2 (*Acta2*). To further assess the anti-fibrosis mechanism of Lactobacillus strains cell-free supernatant in CRC, the protein level of TGF-β was measured in mice tissues. Comparing protein expression levels of TGF-β in tissue samples showed that co-administration of probiotics plus 5-FU could downregulate TGF-β expression in the CRC mice model (figure 2I).

Lactobacillus Strains Cell-Free Supernatant Induces Apoptosis in CRC Cells

The pro-apoptotic effect of probiotics significantly increased after co-administration with 5-FU in the cancer cell lines compared to the control group (figure 3A). Additionally, our findings illustrated that the administration of probiotics modulated pro-apoptotic genes such as protein 53 *(p53),* Bcl-2-associated protein x *(Bax),* and *BCL-2* in CRC tissues (figure 3B).

Lactobacilli cell-free supernatant prevents CRC cell migration

Figure 4A shows that migration ability was decreased in probiotics-treated cells. Moreover, co-administration of probiotics with 5-FU increased its anti-migratory effects on cancer cells (figure 4A). Consistent with these results, treatment with probiotics decreased the mRNA expression of Matrix metallopeptidase 2 *(MMP2)* and *MMP9*, whereas upregulated the expression of E-cadherin in CRC tissue samples (figure 4B). These results suggested that the administration of probiotic supernatants suppressed the migratory behaviors of CRC cells.

Lactobacillus Strains Cell-Free Supernatant Exerts Anti-inflammatory Effect on Tumor Tissues

Our findings demonstrated that administration of Lactobacillus strains cell-free supernatant significantly reduced the expression of *IL-1β*, *TNF-α*, and Monocyte Chemoattractant Protein-1 *(MCP-1)* compared with the control group (figure 5A). To further confirm the anti-inflammatory effects of Lactobacillus strains cell-free supernatant, we determined the protein level of inflammatory factors in the CRC model. Co-administration of probiotics with 5-FU significantly reduced the protein level of TNF-α in CRC tissues (figures 5B and C).

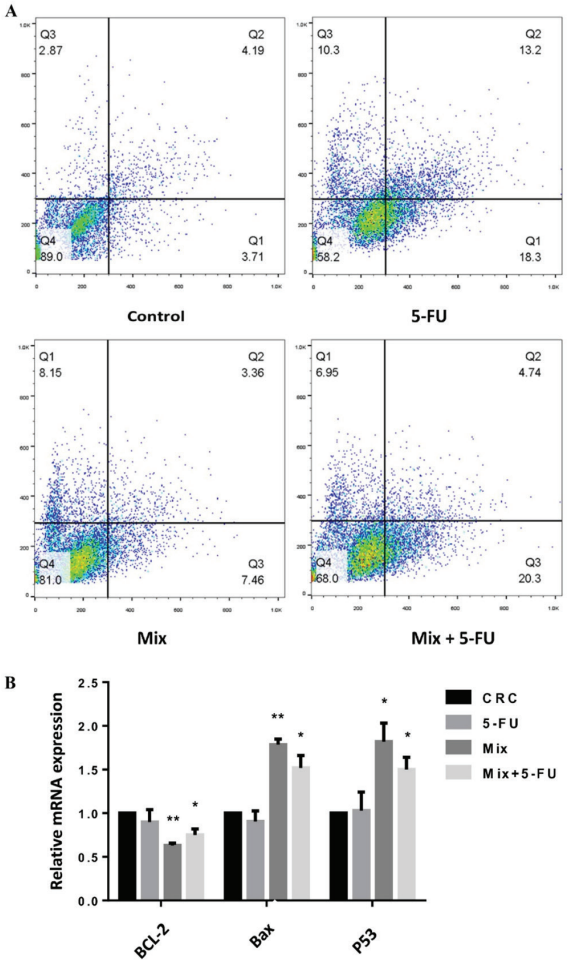

Figure 3: Cell-free supernatant from Lactobacillus strains induced cell apoptosis in colon cancer cells (n=6 in each group). (A) CT-26 was treated with Lactobacillus strains cell-free supernatant for 24 hours and apoptosis was explored by flow cytometry using annexin V/PI staining. The values of the lower right and the upper right area indicate the percentage of the cells in early and late apoptosis, respectively. (B) CRC tumor models were subjected to q-PCR analysis to evaluate pro-apoptotic and tumor suppressor mRNA expression of BCL-2 (P=0.0029), Bax (P=0.0033), and p53. GAPDH serves as an internal control.

Bacterial Supernatant Showed No Histopathological Alterations in Mice Organs

Our results showed no toxicity-associated morphological changes or damage to these tissues. No infiltration of inflammatory cells to the liver or kidney, nor re-arrangement of myofiber in the heart was observed in probiotic-treated groups, supporting the safety of the mix of probiotics supernatant on non-cancerous organs (figure 5D).

Discussion

In this study, we evaluated the anti-tumor properties of four different strains of *L. plantarum, L. brevis, L. helveticus,* and *L. delbrueckii* with or without 5-FU in CRC cells. Our results showed

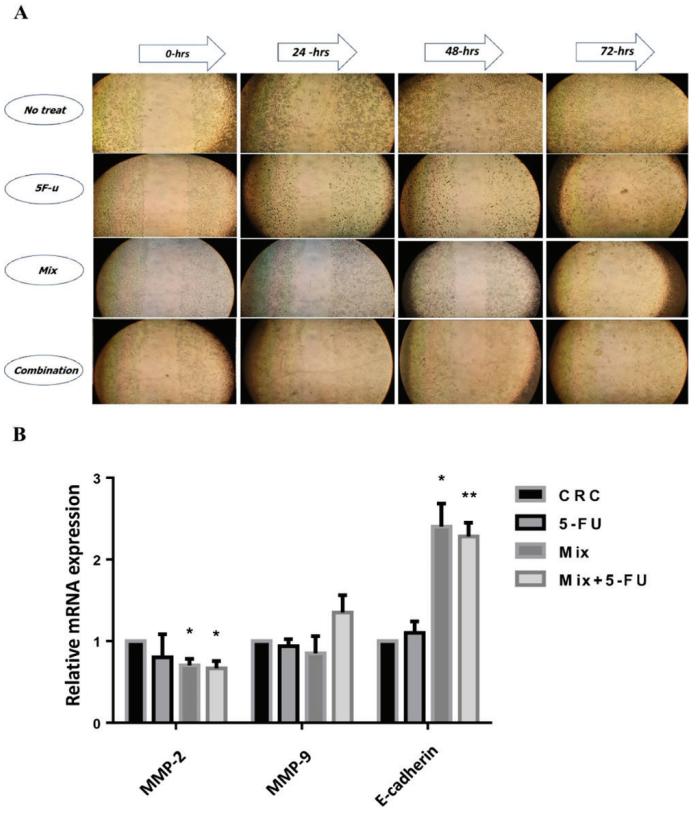

Figure 4: Cell-free supernatant from Lactobacillus strains regulated CRC cell migration (n=6 in each group (A). CT-26 cells were treated with Lactobacillus strains cell-free supernatant and cellular migration was measured. (B) CRC tumor tissues were subjected to q-PCR analysis to evaluate E cadherin (P=0.0198), *MMP-2* (P=0.033), and *MMP-9* gene expressions.

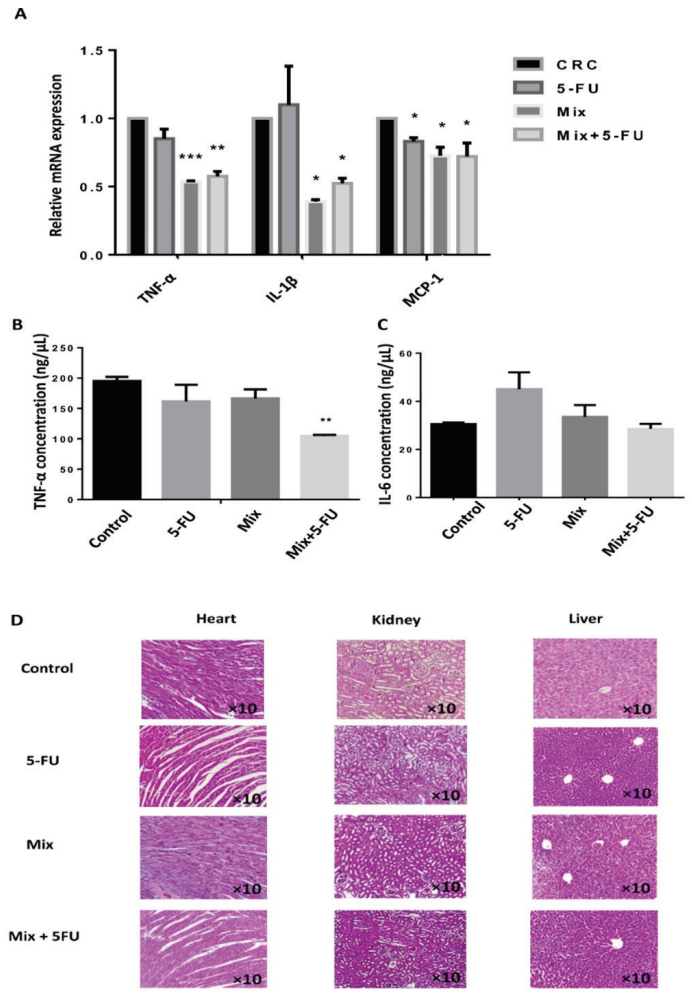

Figure 5: Cell-free supernatant from Lactobacillus strains showed no cytotoxic effects on non-cancerous organs and suppressed inflammatory responses (n=6 in each group). (A, B) CRC tumor tissues were subjected to qPCR and ELISA analysis to evaluate proinflammatory cytokine expression TNF-α (P=0.0001), IL-1β (P=0.0198), mcp-1, TNF-α, and IL-6. (C) Morphological details of indicated organs were investigated using hematoxylin-eosin staining. No significant pathological changes in the heart, liver, and kidney were observed in mice following treatment with Lactobacillus strains cell-free supernatant.

that Lactobacillus cell-free supernatant exerted anti-cancer effects by decreasing tumor size, inducing cell cycle arrest and apoptosis, and regulating inflammatory and fibrotic responses in *in-vitro* and *in-vivo* CRC models. Moreover, it was shown that the administration of Lactobacillus probiotics could improve the therapeutic potential of 5-FU in cellular and animal experiments. Dairy and dairy-based products have long been recognized as a valuable source of probiotics. Various microorganisms, such as lactic acid bacteria (LAB) and bifidobacteria, are derived from fermented milk and have been used for centuries. Different regions, such as Mongolia and Africa, have a rich history of spontaneously fermenting milk, and the use of beneficial microorganisms in fermented dairy products has been passed down through generations. Traditional fermented milk contains diverse LAB species, making it an excellent source of probiotic strains.[30, 31] In a study, researchers isolated 148 LAB strains from Kurut, traditional naturally fermented yak milk from China. The predominant microbial populations in Kurut were found to be *L. delbrueckii* subsp. bulgaricus and *Streptococcus thermophilus*.[32] Furthermore, yeasts and Lactobacillus strains with probiotic properties have been isolated from kefir grains, Masai milk, and Koumiss, which are all fermented milk products. These microorganisms and their secondary metabolites such as organic acids, fatty acids, and proteinaceous compounds, can modulate immune responses, making them beneficial for human health.[33-35.]

Increasing evidence suggests that the altered composition of gut microbiota is directly correlated with CRC tumorigenesis.[14, 15] Recent studies have shown that probiotics can decrease CRC growth and development by regulating gut microbiota. Therefore, probiotic bacteria have been widely studied as a preventive or adjunctive treatment for CRC.[16, 17] Probiotics elicit anti-cancer effects by modulating cell cycle arrest, apoptosis, and immune responses. Moreover, we evaluated the role of the Lactobacillus cell-free supernatant on CRC growth and cell apoptosis. Our findings demonstrated that administration of Lactobacillus probiotics decreases CRC tumor size and induces tumor cell apoptosis and necrosis by up-regulation of apoptosis-related genes such as *Bax* and *p-53*.

The acquisition of immune cells and upregulation of proinflammatory cytokines were shown to have critical effects on tissue repair and host defense. However, prolonged exposure to inflammatory agents causes chronic inflammation, which is associated with uncontrolled cell proliferation and cancer progression.[18, 19] Regarding the relationship between inflammation and tumor initiation, inhibition of inflammatory responses is considered an efficient approach in cancer prevention and treatment.[36, 37] Consistent with these data, Yue and others evaluated the anti-cancer mechanism of *L. plantarum* YYC-3 in a mouse model of CRC. Their results indicated that *L. plantarum* YYC-3 inhibits CRC initiation and progression by reducing immune cell infiltration and downregulating several proinflammatory cytokines including *IL-6, IL-17,* and *IL-22*. Further studies on the anti-tumor mechanism of Lactobacillus probiotics against CRC revealed that administration of *L. Paracasei,*[20] *L. casei,*[21] and *L. plantarum,*[22] exerted anti-inflammatory effects by downregulating the expression of pro-inflammatory markers including IL-6, IL-17, IL-23, TNF-α, and INF-*γ.* In agreement with these results, we demonstrated that administration of Lactobacillus cell-free supernatants suppressed inflammatory responses by decreasing inflammatory cells in tumor tissues and suppressing IL-1β, TNF-α, and MCP-1.

Chronic fibrosis is regarded as a significant risk factor for CRC carcinogenesis. Tumor fibrosis is characterized by chronic inflammation, increased secretion of extracellular matrix proteins, growth factors, and inflammatory cytokines that promote cancer cell growth and proliferation.[38] Anti-fibrotic properties of probiotic bacteria have been investigated in recent studies. For instance, Park and colleagues reported that administration of *Lactobacillus acidophilus* in colitis mice inhibits inflammatory responses and intestinal fibrosis by suppressing *Acta2* and *type-I collagen*.

Further investigations on the therapeutic potential of probiotics revealed that treatment with bacterial probiotics inhibits myocardial,[24] hepatic,[25] and renal[26] fibrosis. We performed Masson trichrome staining to investigate the regulatory effects of bacterial supernatants on the fibrosis process and collagen accumulation in tumor tissues. In agreement with recent studies, our results demonstrated that treatment with Lactobacillus cell-free supernatants decreased collagen deposition and fibrosis by suppressing fibrosis-related genes including *Col 1a1, Col 1a2, Acta2,* and *TGF-β* in CRC tissues.

To achieve more accurate and realistic results in future studies, the use of xenograft animal models using human tumor samples is suggested. Additionally, considering the promising results of using probiotic bacteria in the treatment of colon cancer, investigating the effectiveness of these bacteria in the treatment of other common cancers, such as breast, lung, and stomach cancers, in future studies is suggested.

Conclusion

In conclusion, our findings indicate that treatment with supernatants of newly isolated lactic acid bacteria improves the anti-tumor properties of the standard drug, 5-FU, by inducing apoptosis and suppressing pro-inflammatory and pro-fibrotic cytokines in CRC cells and tumors. Further clinical investigations are required to evaluate the exact molecular mechanisms and examine the therapeutic efficiency of probiotics in CRC treatment.

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Authors' Contribution

Sh. R: Cellular and animal experiments, Data gathering, and drafting; E.G: Cellular and animal experiments, Data gathering, and drafting; SE.N: Animal experiments, Real-time PCR, and ELISA test, data gathering, and reviewing the manuscript; F.R: Animal experiments, Real-time PCR, and ELISA test, data gathering, and reviewing the manuscript; SM.H: Designing the Cellular part of the project and reviewing the manuscriptMB.HN: Data analysis, data interpretation, and reviewing the manuscript; As.A: Data analysis, data interpretation, and reviewing the manuscript; A.A: Study concept, drafting, and reviewing; M.R: Study concept, drafting, and reviewing; S.S: Study design and reviewing the manuscript; M.K: Study design and reviewing the manuscript; M.K: All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Conflict of Interest: None declared.

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