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Original Article

Cytotoxic Effects of Cytoplasmic Extracts from Traditional Yogurt-derived *Lactobacillus* Strains on HT-29 Human Colorectal Cancer Cells

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Abstract

Introduction: Traditional yogurt is a rich source of probiotic *Lactobacillus* species with potential anticancer properties. This study aimed to isolate *Lactobacillus* strains from traditional yogurt and evaluate the cytotoxic effects of their cytoplasmic extracts on human colorectal cancer cells (HT-29).

Methods: This in vitro study was conducted between 2023 and 2024. A total of 21 yogurt samples from various regions near Isfahan were processed via serial dilution and cultured on MRS agar anaerobically. Based on the results, 8 *Lactobacillus* isolates were identified through biochemical characterization and 16S rRNA sequencing. Three representative strains, including *L. casei* (MGB0470), *L. casei* (LcA), and *L. fermentum* (AGR1487), were selected for cytotoxicity evaluation. Cytoplasmic extracts were obtained following cell lysis via freeze–thaw cycles and sonication. HT-29 cells were treated with extracts (0.5-2 mg/mL) for 24 hours, and cell viability was measured using the MTT assay. Data were analyzed using one-way ANOVA and Tukey's post hoc test.

Results: All extracts exhibited a dose-dependent reduction in HT-29 cell viability ($P<0.001$). At 2 mg/mL, viability decreased by over 70%. Additionally, *L. fermentum* (AGR1487) demonstrated the most potent inhibitory effect, with IC_{50} values ranging from 1.2 to 1.4 mg/mL. Phylogenetic analysis confirmed the genetic identity of the isolates.

Conclusion: Cytoplasmic extracts from *Lactobacillus* strains isolated from traditional yogurt significantly inhibited colorectal cancer cell viability in a dose-dependent manner. These results suggest that native probiotic strains, especially *L. fermentum* (AGR1487), hold promise as potential adjunctive agents in anticancer therapy. Further molecular and preclinical investigations are warranted to elucidate their mechanisms and therapeutic potential.

Keywords: Lactobacillus, Yogurt, Probiotics, Colorectal neoplasms, Cytotoxicity, Tumor cell line

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Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality worldwide, with incidence steadily rising, particularly in developing regions (1). By 2030, over 2.2 million new CRC cases are expected annually, largely due to dietary shifts, sedentary lifestyles, and alterations in gut microbiota (2). Conventional treatments, including surgery, radiotherapy, and chemotherapy, can be effective but often cause off-target effects such as tissue damage, immune suppression, and long-term metabolic disturbances (3). These limitations have prompted the search for safer adjunctive therapies.

Probiotics, live microorganisms that confer health benefits when consumed adequately, have attracted special attention for their antitumor potential (4). Among them, lactic acid bacteria (LAB), particularly *Lactobacillus* spp., can modulate gut microbiota, strengthen the intestinal barrier, and produce bioactive metabolites including organic acids, hydrogen peroxide, bacteriocins, short-chain fatty acids, and conjugated linoleic acid (5,6). These compounds contribute to antimicrobial, anti-inflammatory, and anticancer effects, and LAB are generally recognized as safe (GRAS), making them suitable for clinical and dietary applications (7).

Recent studies have shown that both whole LAB cells and derivatives such as cell-free supernatants and cytoplasmic extracts can induce cytotoxicity in colorectal cancer cell lines, including HT-29 (8,9). These effects are mediated via apoptosis, modulation of Bcl-2 and caspase proteins, regulation of oxidative stress, and suppression of tumor-promoting inflammatory mediators (10). Some LAB strains can also enhance the efficacy of conventional chemotherapy while reducing gastrointestinal side effects (11).

Yogurt is a rich source of probiotic LAB (12). *Lactobacillus* strains isolated from yogurt have demonstrated the ability to produce cytoplasmic metabolites that selectively target malignant cells without affecting healthy tissues (13). Given this diversity and therapeutic potential, further investigation into yogurt-derived LAB cytoplasmic extracts is warranted (14).

In this study, the cytotoxic activity of cytoplasmic extracts from *Lactobacillus* strains isolated from

traditional yogurt was evaluated against HT-29 human colorectal adenocarcinoma cells. By examining their effects on cancer cell viability, this research aims to support the potential of probiotic-derived postbiotics as adjunctive therapeutic agents in the management of colorectal cancer.

Materials and Methods

This in vitro experimental study was conducted to evaluate the cytotoxic effects of 62 cytoplasmic extracts from traditional yogurt-derived *Lactobacillus* strains on the HT-29 human colorectal cancer cell line.

Isolation of Bacteria from Yogurt Samples

A total of 21 traditional yogurt samples were collected from various cities and villages around Isfahan from households and local dairy markets using a convenience sampling method. Approximately 100-150 g of each sample was aseptically collected in sterile containers and immediately transferred to the Microbiology Laboratory at Islamic Azad University, Falavarjan Branch, and stored at 4°C until processing. Traditional non-industrial yogurt samples without added preservatives or starter cultures were included in the study. Industrial products and samples with visible spoilage or contamination were excluded from the study. Each sample was serially diluted in Ringer's solution up to 10^{-7}, and 100 µL of each dilution was spread onto MRS agar plates (15). The plates were incubated under microaerophilic conditions at 37°C for 48 hours using anaerobic jars connected to an Anoxomat system (16).

Biochemical Identification of Lactobacillus

Presumptive *Lactobacillus* isolates were identified using standard biochemical tests, based on previous studies (17). The isolates were rod-shaped, Gram-positive, and catalase-negative. They exhibited negative fermentation of sucrose and lactose and positive fermentation of maltose, trehalose, galactose, arabinose, mannitol, and fructose. Additional characteristics included negative indole production, mostly non-motile cells, negative oxidase activity, and the ability to grow at 15, 37, and 45°C. The isolates were facultative anaerobes or microaerophilic bacteria, consistent with standard *Lactobacillus* identification methods reported in the literature.

Acid and Bile Tolerance Assays

To assess the tolerance of *Lactobacillus* strains to acidic conditions, overnight cultures grown in MRS broth at 37°C were used. Briefly, 0.1-mL aliquots of each active culture were adjusted to pH 3.0 and 2.0 using 5 N HCl and incubated at 37°C for 3 hours. Samples were collected every hour, and viable bacterial counts were determined by the pour plate method with 10-fold serial dilutions prepared in 0.1% peptone water. Bacterial growth was also monitored by measuring optical density at 600 nm using a spectrophotometer (18).

Bile tolerance was evaluated by adding a sterile-filtered saturated bile solution (0.3%) to overnight MRS cultures, while cultures without bile served as controls. The cultures were incubated at 37°C for 3 hours, with samples taken every hour. Viable bacterial counts were determined using pour plate counts on serially diluted samples in 0.1% peptone water. Bacterial growth was simultaneously monitored by measuring optical density at 600 nm (18).

Molecular Identification and 16S rRNA Sequencing

Genomic DNA was extracted from the *Lactobacillus* isolates using a commercial DNA extraction kit (Karmania Pars Gene, Iran) according to the manufacturer's instructions. Briefly, bacterial cells from liquid cultures were collected by centrifugation and resuspended in 400 µL lysis buffer. The suspension was vortexed for 15 seconds and incubated at 65°C for 20 minutes to achieve complete cell lysis. After adding 300 µL precipitation buffer and mixing briefly, 40 µL elution buffer was added and incubated at room temperature for 10 minutes. The lysate was applied to a spin column, centrifuged at 8000 rpm for 1 minute, washed sequentially with 300 µL Wash Buffer I and 400 µL Wash Buffer II, and DNA was finally eluted in 50 µL DNase-free water.

PCR amplification of the 16S rRNA gene was carried out in a 20 µL reaction containing 10 µL Amplicon Master Mix (with Taq DNA polymerase, dNTPs, and $MgCl_2$), 2 µL universal 16S rRNA primers (Table 1, sequences adopted from reference (19), 3 µL extracted DNA, and 5 µL nuclease-free water. The thermal cycling program was as follows: initial denaturation at 94°C for 5 minutes (1 cycle), denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute (32 cycles), followed by a final extension at 72°C for 1 minute (1 cycle). Positive and negative controls were included in each PCR run.

PCR products were analyzed on 1% agarose gel in 1X TAE buffer using standard electrophoresis equipment. DNA bands were visualized under UV light and compared with a DNA ladder to confirm the expected product size.

Sequencing and Phylogenetic Analysis

PCR products along with the 16S rRNA primers were submitted to Pishgam Company (Iran) for Sanger sequencing. The obtained sequences were analyzed using BLAST against the NCBI database to determine species identity. For phylogenetic analysis, sequences were aligned and a phylogenetic tree was constructed using MEGA 10 software. Based on sequence similarity and phylogenetic relationships, 3 isolates showing the closest match to known *Lactobacillus* strains were selected for subsequent cytotoxicity experiments.

Table 1. Universal 16S rRNA Primers Used for PCR Amplification

Primer	Sequence (5′→3′)	Temperature (°C)
27F	AGAGTTTGATCCTGGCTCAG	56
1492R	CTACGGCTACCTTGTTACGA	56

Preparation of Cytoplasmic Extracts

Overnight *Lactobacillus* cultures grown in MRS broth at 37°C were used as inocula for larger cultures (1 mL into 50 mL fresh medium). Growth continued at 120 rpm until mid-logarithmic phase ($OD_{600} = 1$). Cells were pelleted at 5000 rpm for 10 minutes, rinsed with phosphate buffer, and disrupted by 7 freeze–thaw cycles combined with sonication. Cytoplasmic fractions were separated by centrifugation, quantified via the Bradford method, sterilized using 0.22 µm filters, and adjusted to final concentrations ranging from 0.5 to 2 mg/mL for cytotoxicity analyses (18).

Cell Culture

HT-29 cells, derived from human colorectal adenocarcinoma, were supplied by the Pasteur Institute Cell Bank (Iran). Cultures were grown in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% FBS and 1% penicillin–streptomycin and incubated at 37°C under 5% CO_2 with controlled humidity. Cells were seeded and incubated for 24 hours to ensure proper adhesion before cytotoxicity testing.

MTT Assay for Cell Viability

Cell viability was assessed using the MTT assay (20). HT-29 cells were seeded at a density of 1×10^5 cells/well in 96-well plates and incubated for 24 hours to allow adherence. Then, cytoplasmic extracts at various concentrations (0.5-2 mg/mL) were added, and the plates were incubated for an additional 24 hours. All treatments and controls were performed in triplicate.

After treatment, cells were washed with phosphate-buffered saline (PBS), and 20 µL of MTT solution (5 mg/mL) was added to each well. Plates were incubated at 37°C for 4 hours to allow formazan formation. The formazan crystals were dissolved in 20 µL dimethyl sulfoxide (DMSO) for 5 minutes, and absorbance was measured at 490 nm using a microplate reader. Cell viability was calculated using the following formula:

Cell Viability (%) = 100 × (Absorbance of treated cells / Absorbance of control cells)

Statistical Analysis

Each experiment was independently repeated 3 times, and data were shown as mean values with their corresponding standard deviations. Statistical evaluation was performed using one-way ANOVA together with Tukey's post hoc test to assess differences between the groups. *P*-values < 0.01 and < 0.001 were considered statistically significant.

Results

Isolation and Molecular Identification of Lactobacillus

A total of 21 traditional yogurt samples were screened, and 8 *Lactobacillus* isolates with typical round off-white colonies were obtained. Biochemical and physiological characterization, including Gram staining, catalase reaction, carbohydrate fermentation profiles, motility, growth at various temperatures, and oxygen tolerance, confirmed that all 8 isolates belonged to the genus *Lactobacillus*. Among these 8 confirmed isolates, 3 representative strains (L1, L2, and L3) were selected for molecular analysis. PCR amplification using universal 16S rRNA primers produced a single band of approximately 1540 bp in 1% agarose gel, confirming successful amplification. BLAST analysis of the sequences revealed the closest known Lactobacillus strains, as summarized in Table 2.

Cytotoxicity Assessment

The cytotoxic effects of cytoplasmic extracts from all three *Lactobacillus* strains were evaluated on HT-29 colorectal cancer cells at concentrations ranging from 0.5 to 2 mg/mL. After 24 hours of incubation, all extracts caused a significant dose-dependent reduction in cell viability.

All tested concentrations of *L. casei* (MGB0470) reduced cell viability compared to the control group, with the minimum effective concentration observed at 0.5 mg/mL. The cytotoxic effect increased gradually with higher concentrations, indicating a clear dose-dependent trend (Figure 1).

Data are presented as Mean ± SD. Asterisks indicate statistical significance versus control ($P < 0.001$). With increasing extract concentration from 0.5 to 2 mg/mL, HT-29 cell viability significantly decreased ($P < 0.001$). All concentrations showed significant reductions compared to each other ($P < 0.001$), except for 1 and 1.5 mg/mL, which differed at $P < 0.01$. The lowest effective concentration was 0.5 mg/mL.

L. casei (LcA) also decreased cell viability in a dose-dependent manner, although its overall inhibitory effect was slightly lower than MGB0470. The minimum effective concentration for this strain was 0.75 mg/mL,

Table 2. 16S rRNA Sequence Similarity of Selected *Lactobacillus* Isolates with Reference Strains

Selected isolate	Closest reference strain	Similarity (%)
L1	*Lactobacillus* casei (MGB0470)	99
L2	*Lactobacillus* casei (LcA)	99
L3	*Lactobacillus fermentum* (AGR1487)	99

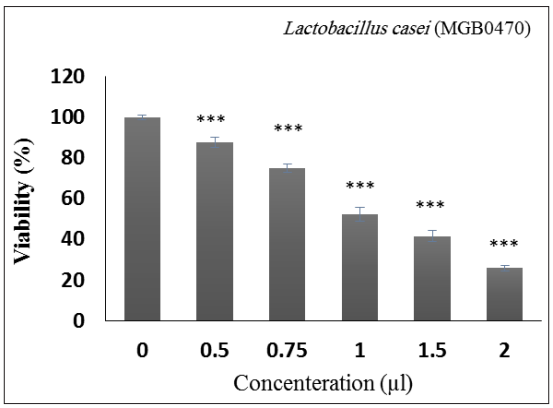

Figure 1. Viability of HT-29 Cells Treated with *Lactobacillus Casei* (MGB0470) Extracts

and a progressive reduction in viability was observed at higher doses (Figure 2).

Except for 0.5 mg/mL, all concentrations significantly reduced cell viability ($P<0.001$). All pairwise comparisons were significant at $P<0.001$. The minimum effective concentration was 0.75 mg/mL.

L. fermentum (AGR1487) exhibited the strongest cytotoxic effect among the 3 strains. Significant reduction in cell viability was observed even at the lowest concentration tested (0.5 mg/mL), and the highest concentration (2 mg/mL) caused the most pronounced decrease, highlighting its potent anticancer potential (Figure 3).

Cell viability decreased significantly from 0.5 to 2 mg/mL ($P<0.001$). Except for 0.5 and 0.75 mg/mL, all concentrations significantly differed from each other ($P<0.001$). The lowest effective concentration was 0.5 mg/mL.

Comparative analysis at 0.75 mg/mL revealed that *L. fermentum* (AGR1487) had the highest inhibitory effect on HT-29 cell viability, followed by *L. casei* (MGB0470), while *L. casei* (LcA) exhibited the lowest cytotoxic activity among the three strains (Figure 4). These results demonstrate that all 3 *Lactobacillus* cytoplasmic extracts possess significant anticancer activity against HT-29 cells, with *L. fermentum* (AGR1487) showing the highest potency.

Lactobacillus fermentum (AGR1487) exhibited the highest inhibitory effect, whereas *Lactobacillus casei* (LcA) showed the lowest at this concentration. Data are presented as Mean ± SD. Asterisks indicate statistical significance ($P<0.001$***, $P<0.01$**).

L1: *Lactobacillus casei* (MGB0470), L2: *Lactobacillus casei* (LcA), L3: *L. fermentum* (AGR1487)

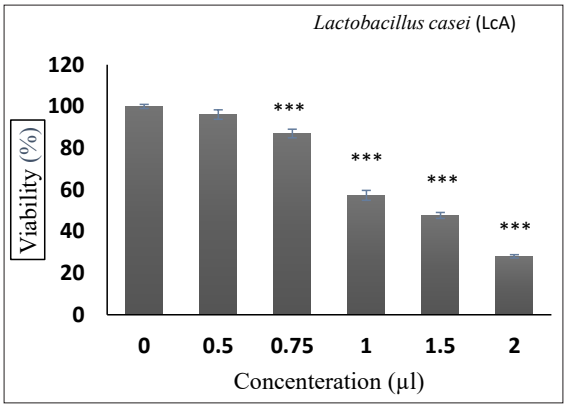

Figure 2. Viability of HT-29 Cells Treated with *Lactobacillus Casei* (LcA) Extracts

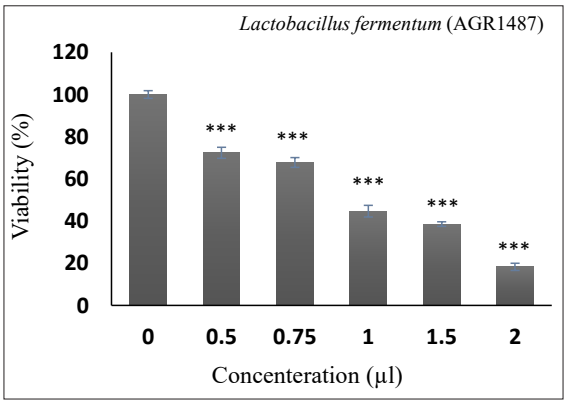

Figure 3. Viability of HT-29 Cells Treated with *Lactobacillus Fermentum* (AGR1487) Extracts

Discussion

In the present study, lactic acid bacteria were isolated from traditional yogurt, which has long been considered a valuable reservoir of probiotic microorganisms. Unlike industrially produced yogurt that usually contains a limited number of standardized starter cultures, traditional yogurt often harbors a wide range of indigenous *Lactobacillus* species adapted to local environments and production methods. This microbial diversity increases the likelihood of finding strains with unique biological functions, including anticancer potential (21).

Of the initial 8 isolates obtained, three representative strains were selected and identified as *L. casei* (MGB0470), *L. casei* (LcA), and *L. fermentum* (AGR1487). The selection criteria were based on their phenotypic and genotypic characteristics, ensuring inclusion of distinct species and avoiding redundancy. Similar findings have been reported in previous studies. For example, Gharekhani et al (2019) isolated several *Lactobacillus* species, including *L. casei* and *L. fermentum*, from Iranian traditional yogurt and confirmed their probiotic potential through acid and bile tolerance assays (22). In another study, Tokatlı et al (2015) demonstrated that Turkish artisanal yogurts contained diverse *Lactobacillus* strains, some of which exhibited significant antimicrobial and health-promoting properties (23). These reports are consistent with our findings and highlight the importance of traditional dairy products as reservoirs of functionally promising probiotic bacteria.

The cytotoxic effects of the cytoplasmic extracts from these isolates were subsequently evaluated against HT-29 colorectal cancer cells. Our results demonstrated that all three strains significantly reduced cell viability in a dose-dependent manner. At the highest tested concentration (2 mg/mL), more than 70% of HT-29 cells lost viability, with *L. fermentum* (AGR1487) exerting the most potent effect. This observation underscores the therapeutic promise of

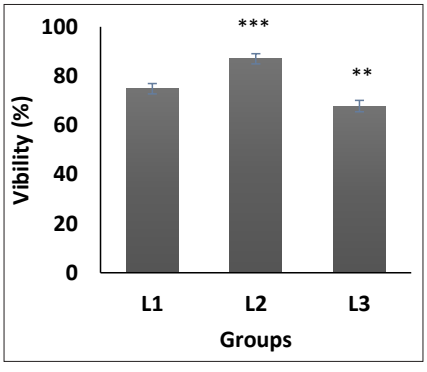

Figure 4. Comparison of HT-29 Cell Viability among the Three *Lactobacillus* Strains at 0.75 mg/mL

specific yogurt-derived strains as anticancer probiotics. Similar findings have been reported by Corpetti et al, who observed that *L. casei* extracts effectively reduced colorectal cancer cell growth, and by Jacotet et al (2017), who demonstrated that *L. casei* BL23 prevented colorectal tumor development by modulating the gut microbiota and promoting apoptosis (24, 25). Likewise, Escamilla et al reported that both live cells and cell-free supernatants of *L. casei* significantly inhibited CaCo2 cell proliferation, achieving up to 91% growth suppression, while Tsai et al identified antimicrobial peptides from *L. casei* as potent inducers of apoptosis in SW480 cells (26, 27).

The dose-dependent pattern observed in our study is also in line with previous investigations (28, 29). Higher concentrations of cytoplasmic extracts generally exert stronger cytotoxicity, suggesting that bioactive metabolites within the extracts act in a concentration-dependent manner. Importantly, *L. fermentum* (AGR1487) exhibited superior cytotoxic effects compared to both *L. casei* strains, which may be attributed to strain-specific differences in metabolite production, such as higher levels of short-chain fatty acids (SCFAs), conjugated linoleic acid (CLA), and bacteriocins (30). These strain-specific differences highlight the need to screen and characterize native isolates for their anticancer potential.

The mechanisms underlying these effects are likely multifactorial. Probiotic-derived cytoplasmic extracts can disrupt the metabolic activity of tumor cells through acidification of the microenvironment, membrane destabilization via bacteriocins and antimicrobial peptides, and suppression of tumor-promoting inflammatory mediators such as TNF-α, IL-6, and IL-1β (31). Additionally, they may modulate oxidative stress, leading to mitochondrial dysfunction and caspase-mediated apoptosis (32). Previous reports have also shown that *Lactobacillus* metabolites can regulate the expression of apoptotic genes, including upregulation of Bax and downregulation of Bcl-2, further promoting programmed cell death (33, 34).

Collectively, our findings are consistent with the growing body of evidence supporting probiotics and their derivatives as promising adjuncts in the management of cancer. The potent cytotoxic effect of *L. fermentum* (AGR1487) emphasizes the importance of exploring local and traditional sources of probiotic strains, which may have unique functional properties not found in widely studied commercial strains. Future studies should expand on these findings by identifying the precise bioactive components responsible for the observed cytotoxicity and by validating these effects in vivo, where interactions with the host immune system and gut microbiota may further enhance their anticancer potential.

While the current study provides valuable insights into the cytotoxic and pro-apoptotic effects of *Lactobacillus* cytoplasmic extracts on HT-29 cells, several limitations should be acknowledged. First, this research was conducted entirely in vitro, and the findings may not fully reflect the complex interactions within the human body. Therefore, further in vivo studies and animal models are required to confirm these findings. Second, we evaluated only one human colorectal cancer cell line (HT-29), and future studies involving a broader range of cell lines would be beneficial. Lastly, the specific bioactive molecules in the cytoplasmic extracts responsible for the observed anticancer effects have not been fully characterized, and this remains an area for further investigation.

Conclusion

The cytoplasmic extracts from three *Lactobacillus* strains, *L. casei* (MGB0470), *L. casei* (LcA), and *L. fermentum* (AGR1487) isolated from traditional yogurt, exhibited significant dose-dependent cytotoxicity against HT-29 colorectal cancer cells. Higher extract concentrations produced stronger inhibitory effects, and the estimated IC50 values ranged from 1.2 to 1.4 mg/mL. These results highlight the therapeutic potential of native probiotic strains derived from traditional dairy products as effective anticancer agents. Further studies are warranted to identify the bioactive components responsible for the observed cytotoxicity and to validate their efficacy in preclinical and in vivo models.

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Authors' Contribution

Conceptualization: Mozhgan Ghiasian, Fereshte Ghandehari.
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Competing Interests

The authors declare that they have no conflict of interests.

Ethical Approval

All procedures were carried out according to ethical guidelines for working with laboratory animals. This study was also approved by the Ethics Committee of Islamic Azad University, Falavarjan Branch (IR.IAU.FALA.REC.1403.035).

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