Original Article

J Adv Med Biomed Res. 2024; 32(155): 449-457

Inhibition of Proliferation and Invasion of Human Colon Carcinoma Cell Line (Caco-2 cells) by Cell-Free Supernatants from *Lactobacillus Rhamnosus* and *Lactobacillus Acidophilus*

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Article Info

10.30699/jambr.32.155.449

Received: 2024/01/28;
Accepted: 2025/01/15;
Published Online: 15 Feb 2025 ;

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ABSTRACT

Background & Objective: Colorectal cancer (CRC) is one of the most common types of cancer in the world and is considered a leading cause of cancer-related death. The present study aimed to investigate the inhibitory effect of *lactobacillus acidophilus* (PTCC 1643) supernatant (LAS) and *lactobacillus rhamnosus* (PTCC 1657) supernatant (LRS) on the growth and invasiveness of the human colon carcinoma cell line (Caco2) in-vitro.

Materials & Methods: In this study, the antiproliferative activity and anti-invasion potential of LAS and LRS were determined by MTT and transwell chamber assays, respectively. Furthermore, the expression of mitochondrial membrane potential-9 (MMP-9) and matrix metalloproteinase-12 (MMP12) genes were analyzed by real-time PCR.

Results: The results of the MTT assay indicated that LAS and LRS had cytotoxic effects on Caco-2 cell proliferation at a concentration of 25% and higher after 72 hours (p<0.0001). Thus, the minimum concentrations (25%) of supernatants were chosen for further experiments. LRS and LAS could significantly suppress the invasiveness of cells, p= 0.028 and p=0.002, respectively. Also, the expression of MMP12 was significantly increased in cells when treated with LAS (p<0.001), whereas LRS had no significant effect on MMP-12 expression. Furthermore, the expression of MMP-9 was statistically decreased in cells treated with both supernatants (p<0.00001).

Conclusion: In general, it was shown that LAS and LRS exert anti-cancer activity against the growth, invasion, and metastasis of Caco2 cells. These two bacteria could be used as prophylactic and therapeutic agents for the prevention and treatment of CRC.

Keywords: *Lactobacillus*, Colon cancer, Probiotic, Invasion assay, Metastasis

Introduction

CRC is ranked as the fourth leading cause of mortality in the world higher rates in the West and rising in developed Asian countries. Risk factors include genetics, diet, and gut microbiota imbalance, which has garnered recent attention (1, 2). Recently, numerous studies have indicated that gut microbiota homeostasis is closely related to the risk factors mentioned earlier (3, 4). It has been reported that abnormal gut microbiota leads to the emergence of different pathophysiological events associated with diseases such as CRC (5). However, the precise mechanism of microbiota in the development of CRC is still opaque. Among the factors mentioned above, diet plays an essential role in the emergence of CRC (6).

Probiotics are vital microorganisms in a healthy human microbiota environment. As members of the gut

microbiota, Lactic acid bacteria (LAB), especially Lactobacillus, exert health-promoting activity closely associated with the suppression of allergic responses and anti-inflammatory and anti-tumor effects (7). Several lines of evidence demonstrated that the supplementation of LAB could act as a prophylactic strategy for the prevention and cure of CRC due to their probiotic properties (8, 9).

Nevertheless, the beneficial role of LAB in inhibiting CRC progression and its effects on tumor microenvironments remain largely unknown. Several investigations suggested that LAB exerts anti-neoplastic activity by promoting the immunity or modulation of immune responses. They also enhance the DNA repair process, stimulate programmed cell death, and inhibit the proliferation of colon cancer cells (10, 11). Although accumulative evidence supports the role of probiotic LAB in the prevention of the early stages of the development of colon cancer, little is known about the effect of LAB's role in later stages of CRC, especially metastasis.

Matrix metallopeptidases (MMPs) are ECM-remodeling enzymes linked to cancer progression and poor prognosis (12). MMPs can digest ECM proteins, such as gelatin, elastin, and collagen. These types of proteins can eradicate the structural barriers and facilitate the migration of cells. Besides, by hydrolyzing the extracellular proteins released by MMPs, they can change the activity of numerous signal peptides, including cytokines, growth factors, and chemokines. Increased activity or expression of MMPs is markedly associated with higher invasiveness and the ability to metastasize in almost all types of human cancer and poor prognosis (13, 14).

The MMP-9 protein digests all ECM proteins. The levels of MMP-9 have been shown for poor prognosis in CRC, along with other types of cancer, such as cervical and breast cancer, while MMP-12 has protective roles in CRC prevention (15).

To the best of our knowledge, research on the role of cell-free supernatants of L. acidophilus (LA) and L. rhamnose (LR) in the prevention of Caco-2 cells and measuring cell death, invasion rate, and metastasis markers like MMP-9 and MMP-12 is lacking.

Materials and Methods

We purchased DMEM (Dulbecco's Modified Eagle's medium), extracellular matrix (ECM), Lactic acid, Trypan blue, penicillin, ampicillin, streptomycin, and MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) from Sigma-Aldrich (Merk, Germany), Fetal Bovine Serum (FBS) from Gibco (Thermo Fisher Scientific, USA), and the real-time PCR assay kit from Amplicon, USA.

Preparation of bacterial strains, cell-free supernatant (CSF)

The following lactobacillus strains, namely LA, and LR, were stored in the De Man, Rogosa, and Sharpe (MRS) broth medium (MRS broth, Scharlau, Spain) (pH=6.5, Merck, Germany) supplemented with 20% (v/v) glycerol at −80 °C. Before the experiments, each strain was cultured in MRS broth and incubated under the anaerobic condition at 37 °C.

For preparing CSF, bacterial cells (109 CFU/ml) at the logarithmic phase of growth (after 24 and 48 h) were centrifuged at 5000 g for 15 min, and the supernatants were filtered using a 0.22 µm filter. CSF was adjusted to pH 7.4 using a bicarbonate buffer. Afterward, various concentrations (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50% from supernatant) were prepared for the cell viability assay.

Cell proliferation, migration, and invasion assays

This experiment focused on analyzing the effects of *lactobacillus acidophilus* (PTCC 1643) supernatant (LAS) and *lactobacillus rhamnosus* (PTCC 1657) supernatant (LRS) on the growth inhibition of Caco2 cells (NCBI C139, Pasteur Institute of Tehran, Iran). Briefly, 4 ×104 cells/well were cultured overnight in an incubator (5% CO2, 37 0C). Then, the cells were treated with different concentrations of lactobacillus culture supernatants (0-50%) collected after 24h and 48h of the incubation period. Cell growth inhibitory effects were determined by the MTT assay and the results were subsequently analyzed after 24, 48, and 72 h. The MTT solution (0.5 mg/ml) was added to the wells and incubated at 37°C for 4 h. The precipitated formazan crystals were solubilized by adding 100 µl of DMSO, and the wells' optical absorbance was measured using an ELISA reader (BIO-RAD, Hercules, USA). The inhibition rate (IR) was evaluated using the following equation:

Inhibition ratio (%) = 1- ODexp / ODcon × 100

Where ODexp and ODcon are the optical absorbance values of treated and untreated cells, respectively.

Cell invasion was assessed using the Transwell method (16). To this aim, 2 ×104 cells were cultured in the DMEM medium at the top of the transwell membrane chamber (Costar; Corning, 8-µm pore size). The cell culture medium supplemented with 10% FBS was added to the bottom of the chamber. The migration assay was carried out after 36 hours at 37°C in 5% CO2 humidified incubator. The cells grown at the upper surface of the membrane were carefully scraped off after the incubation period. Cells migrated to the bottom surface were fixed in 100% methanol for 5 min. Then, the cells were stained with a crystal violet staining solution for 2 min. afterward, cells were counted under a light microscope at different random fields at ×300 magnifications. The number of Caco-2 cells was expressed as the mean number per group.

RNA extraction, cDNA synthesis, and Real-time quantitative PCR (qRT-PCR)

To analyze the effect of LAS and LRS on the expression of MMP-9 and MMP-12 genes, Caco2 cells were cultured in the presence of LAS and LRS at a concentration of 25% for 36 h, and then total RNA was isolated from treated and untreated cells using the RNeasy Mini Kit (Hilden, Germany). The quality and quantity of the extracted RNA were spectrophotometrically determined using a Nanodrop instrument (Thermo Scientific, USA). The SYBR Premix Ex Taq 11 reagent kit (Takara Bio, Japan) was used to reverse transcription of RNA, and then the mRNA expression of target genes was analyzed using qRT-PCR.

The gene expression analysis of purified mRNA genes, including MMP12 and MMP9, was performed using Applied Biosystems Step One Real-Time PCR (Thermo Fisher). The master mix reaction solution used for real-time PCR comprised 250 ng cDNA, 2x master mix (10 µl), 10 pmol of each primer pair adjusted by ddH2O up to the final reaction of 10 µl. The sequences of primers were designed by the NCBI PRIMER BLAST tool. The sequences of primers for qRT-PCR analysis are presented in (Table 1).

The thermal cycling program was initiated by cDNA denaturation at 95˚C for 30s, followed by 40 cycles of 95˚C for 5 seconds and 61˚C for 34 seconds. The experiments were carried out in triplicate for each target gene. To examine the specificity of primers used and the absence of primer dimmer, the melting curve analysis was conducted after each amplification run.

Statistical Analysis

Data analysis was performed by Graph Pad Prism version 8.4.1. The data were expressed as mean values ± standard deviation from three independent experiments. One-way ANOVA was performed for comparisons involving more than two groups, followed by the Tukey post hoc tests for multiple comparisons. The level of statistical significance was set at $p<0.05$.

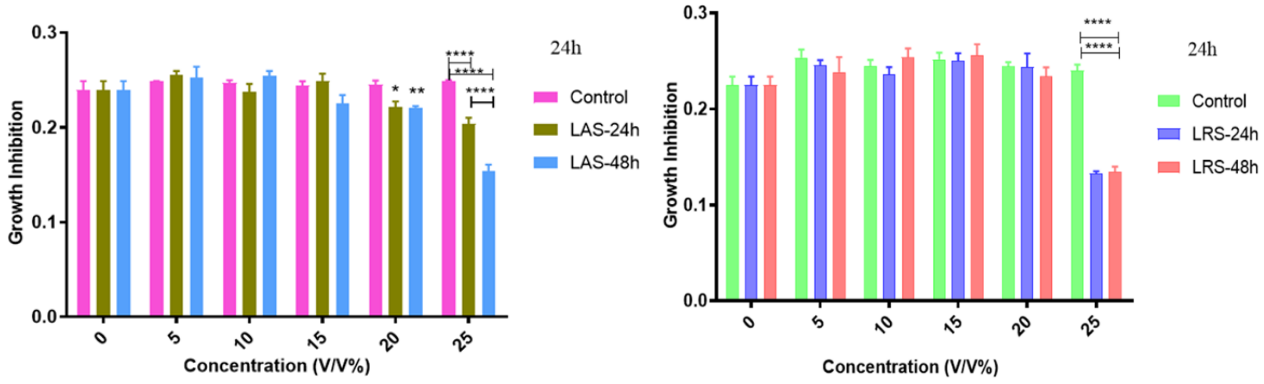
Table 1. The sequence of primers

Primer	Forward	Reverse
MMP9	5′- AAGGATGGGAAGTACTGGCG -3′	5′- GCTCCTCAAAGACCGAGTCC -3′
MMP12	5′- TTTGGTGGTTTTTGCCCGTG -3′	5′- GGAACAAGTTTGTGCCTCCTG -3′
β-Actin	5′- TGAAGATCAAGATCATTGCTCCC -3′	5′- AGTCATAGTCCGCCTAGAAGC -3′

Results

Cytotoxic effect of LA and LR strain culture supernatants on Caco2 cell growth

The cells were treated with different concentrations of lactobacillus culture supernatants (0-50%) collected after 24h and 48h of the incubation period. The results were subsequently analyzed after 24, 48, and 72 h. Cell growth inhibitory effects were determined by the MTT assay (Figure 1). The results demonstrated that LAS and LRS (collected supernatants after 24 and 48h of incubation) had a significant inhibitory effect on Caco2 cell proliferation compared with cells treated with the MRS solutions or those left untreated. LAS and LRS inhibited 50% of the cell proliferation at a concentration of 25% V/V (IC50) after 24h. Also, the cell viability was significantly reduced at 20% and 25% concentrations of LAS and LRS after 72h. The results indicated that LRS collected after 48 h exhibited greater cytotoxic activity against the Caco-2 cell line than those collected after 24 h. However, there was no significant difference in cell viability when Caco-2 cells were treated with LAS after 24 h and 48 h.



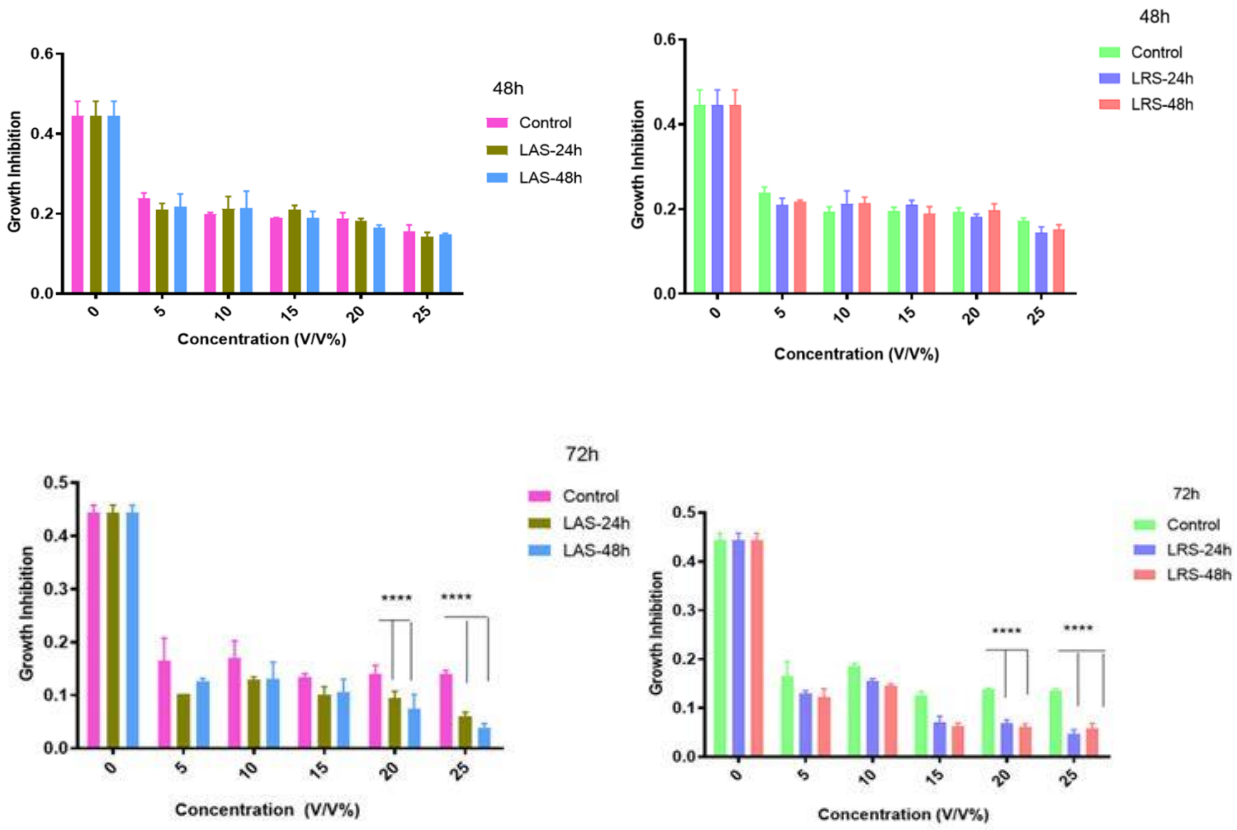


Figure 1. The MTT assay represents cytotoxicity effects of LRS, LAS, and control or MRS with different concentrations on Caco2 cells; A1&A2)) after 24h, B1&B2) after 48h treatment, C1&C2) after 72h treatment. *; *p*<0.05, **; *p*<0.01, *; *p*<0.001, and ****; *p*<0.0001.**

Inhibition of invasiveness by LA supernatant

In this study, we evaluated the effect of LAS and LRS on cell invasion through the transwell chamber (Figure 2). In this method, cells that migrated into the lower surface of the membrane were fixed and then stained.The percentages of migration and invasion of cells were significantly lower when the cells were incubated with LAS (*p*=0.002) and LRS (*p*=0.028) after 36h compared with cells treated with the MRS solution.

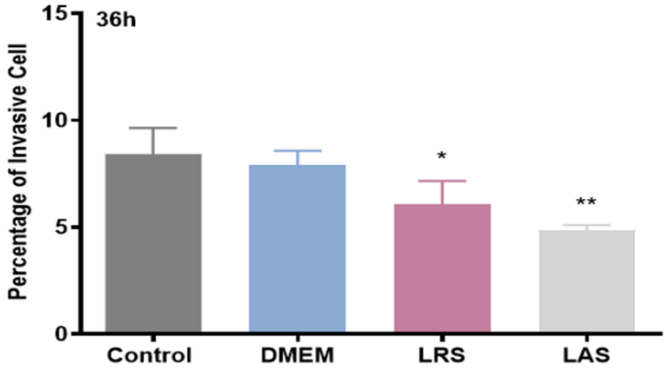


Figure 2. Cell migration/invasion assay in LRS- and LAS-treated Caco2 cells. Cells (2 × 104 cells/ml) were treated with 25% concentrations of LAS and LRS for 36 h. The mean number of cells from 6 random fields was expressed, and the values are represented as the mean ± SD of three independent experiments. The asterisks indicate a statistically significant difference compared with control. Control: 25% Mrs. * *p* = 0.028 and ***p*=0.002.

Effects of LAS and LRS on the expression of MMP-9 and MMP-12 genes in Caco2 cells

To analyze the effect of LAS and LRS on the expression of MMP-9 and MMP-12 genes, Caco2 cells were cultured in the presence of LAS and LRS at a concentration of 25% for 36 h, and then the total RNA of cells was isolated, and RT-PCR was performed as described earlier. As depicted in (Figure 3B), the expression of MMP-9 was significantly decreased in Caco2 cells treated with LRS and LAS compared with the MRS-treated Caco-2 cells. As shown, the expression of MMP-12 was significantly increased in cells treated with LAS, while LRS had no significant effect on the expression of the MMP12 gene when compared with cells treated with the MRS solution (Figure 3A).

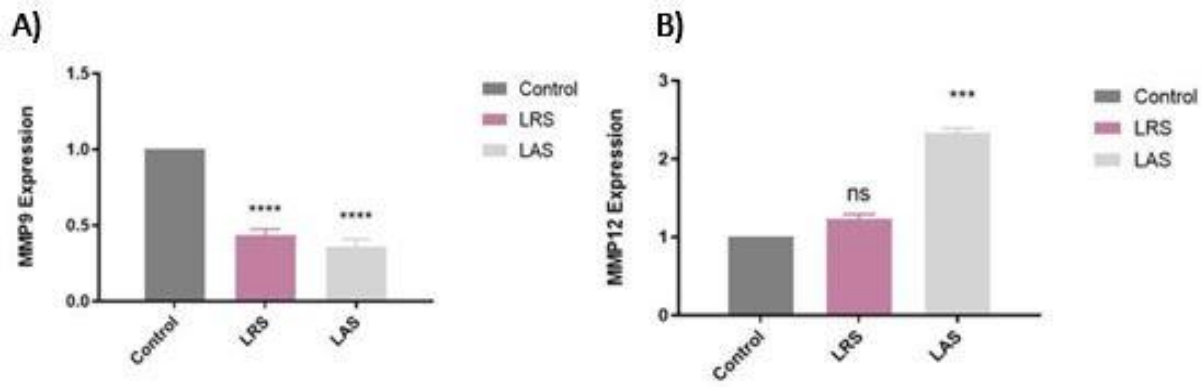


Figure 3. Cell migration/invasion assay in LRS- and LAS-treated Caco2 cells. Cells (2 × 104 cells/ml) were treated with 25% concentrations of LAS and LRS for 36 h. The mean number of cells from 6 random fields was expressed, and the values are represented as the mean ± SD of three independent experiments. The asterisks indicate a statistically significant difference compared with control. Control: 25% Mrs. * *p* = 0.028 and ***p*=0.002.

Discussion

The invasion of primary tumors into distant organs, such as the lungs and liver, is considered the principal cause of mortality in patients afflicted with CRC (17). The epidemiological studies suggested that colorectal cancer is a lifestyle disorder, and various genetic components and environmental factors are involved in the pathogenesis of the disease (18). Unfortunately, a vast majority of patients with CRC are diagnosed at the advanced stages of the disease, especially when the initial tumors invade other organs (survival rate of 10%). Alternatively, therapeutic options, including radiotherapy and chemotherapy, have serious adverse effects on human and mainly emerge as gastrointestinal toxicity, diarrhea, nausea, and vomiting (19). Therefore, prophylactic strategies and alternative treatments are needed to prevent the emergence of CRC.

In the present study, the impact of two probiotic Lactobacillus sp., namely LA and LR, on the inhibition of the growth and invasion of Caco2 cells was determined by analyzing the cell invasion assay and the expression of MMP-9 and MMP-12 genes. Studies indicated that the proteolytic activity of the MMP-9 enzyme contributes to the digestion of the ECM in the colon, facilitating the cell invasion process during metastasis (20, 21). Conversely, evidence showed that the expression of MMP-12, a metalloelastase enzyme, has inhibitory effects on the growth and proliferation of CRC, and it is associated with increased survival of CRC patients (18).

We showed that LAS and LRS significantly affected the viability of Caco2 cells compared with control after 72 h (p<0.0001). Also, our results revealed that LRS and LAS containing secreted bioactive compounds significantly reduced the invasion of metastatic Caco2 cells (Fig 3, p = 0.028 and p=0.002, respectively) as shown by decreased expression of MMP-9 (Fig 4A, p<0.0001). Furthermore, LAS increased the expression levels of MMP-12 (Fig 4B, p<0.001), while LRS did not affect MMP-12 expression. To the best of our knowledge, the current research is the first study reporting that released bioactive compounds from LA and LR can regulate the expression levels of MMP-9 and MMP-12 genes in Caco-2 cells, suggesting their potential role in inhibiting colon cancer cell invasion.

It is now known that MMPs have detrimental roles in the metastasis of colon cancer, promoting the invasion of primary tumors through the digestion of collagen in the ECM (20). It has been shown that the MMP-9 enzyme has proteolytic activity, participating in the reconstruction and breakdown of the ECM, a phenomenon observed in the invasion and metastasis of CRC. The MMP-9 protein is capable of regulating the tumor microenvironment and increasing the levels of vascular endothelial growth factor (VEGF), which is

involved in angiogenesis. Also, MMP-9 effectively contributes to the formation of early metastatic niches process (22). Several preclinical analyses demonstrated that the selective inhibition of MMP-9 can decrease tumor proliferation and metastasis rates in CRC. It can also induce programmed cell death in pancreatic cancer cells (23, 24). Escamilla and colleagues showed that cell-free supernatants extracted from probiotic *Lactobacillus rhamnosus* GG led to a marked reduction in the growth and invasion of HCT-116 cells, thereby diminishing the expression and activity of MMP-9 (10).

On the other hand, a large body of evidence indicates that the inhibition of MMP-12 has deleterious effects on the treatment course of cancer (25, 26). While elevated expression of MMP-12 has been reported in patients with CRC, its expression level has been higher in patients with no liver metastasis than those with liver metastasis (26). Besides, the expression of MMP-12 can lower the expression rate of VEGF and increase the expression of angiostatin, which is an endogenous inhibitor of the angiogenesis process (27). Consistent with these statements, several investigations demonstrated that the expression of MMP-12 is associated with increased overall survival of patients and reduced tumor growth. The degree of MMP-12 expression has been conversely attributed to the metastasis process of primary colon cells [40]. Our findings showed that the effect of LAS on cell invasion and proliferation was more pronounced than that of LRS. This may be due to the amplifying role of LAS in the expression of MMP-12. In addition to the anti-proliferative, pro-apoptotic, and anti-metastatic effects of lactobacilli (28, 29), our results showed that LA and LR also have remarkable anti-proliferative and anti-metastatic effects on CRC cells.

Several studies demonstrated the relationship between a diets enriched with Lactobacillus and reduced risk of CRC (28, 30). Several studies revealed that probiotics, like the Caco2 cell line, modulate cancer cells, proliferation, and apoptosis (30-33). Furthermore, it is now known that probiotics have various properties, including radio-protective, antioxidant, biocompatibility, immunomodulatory effects, and toxin neutralization. They can improve the intestinal microbial environment and immune system response (33-37) .Thus, they could be an alternative therapy instead of invasive treatments, such as radiotherapy and chemotherapy.

Conclusion

In conclusion, our results revealed that the CFS extracted from LA and LR significantly reduced the invasion of metastatic Caco2 cells, as shown by decreased expression of MMP-9. Furthermore, LAS increased the expression levels of MMP-12, while LRS did not affect MMP-12 expression.

Several lines of evidence have shown the significance of probiotic balance in maintaining homeostasis, which could contribute to optimizing cancer therapy. Our findings suggest that these two bacteria could be used as complementary therapy or prophylactic agents for treating and preventing CRC.

Overall, further research is required to characterize bioactive factors' precise mechanism of action in probiotic-containing functional foods. These insights could lead to new preventive strategies against CRC cell proliferation and invasion.

Acknowledgements

The authors have special thanks to Department of Hematology Lab Director.

Conflict of Interest

The authors declare no conflict of interest.

Funding

This research received no external funding.

Ethics approval and consent to participate

The research registration code in Payamnur University is 2471026.

Authors' contribution

AS, MS, and SA contributed to the concept of the manuscript. AS, MS, and SA were responsible for the reference selection and writing of the manuscript. AS prepared figures 1-3. All authors read and approved the final manuscript.

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How to Cite This Article:

Shokati A, Soleimani M, Abroun S. Inhibition of Proliferation and Invasion of Human Colon Carcinoma Cell Line (Caco-2 cells) by Cell-Free Supernatants from *Lactobacillus Rhamnosus* and *Lactobacillus Acidophilus*, J Adv Med Biomed Res. 2024; 32(155): 449-457.