The 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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Research Article

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

Posted Date: March 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-148685/v2

Abstract

Background: The epidemiological studies indicated that colorectal cancer 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.

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

Results: The results indicated that supernatants of these two lactobacilli had cytotoxic effects on Caco-2 cells at a concentration of 25% v/v and higher. Thus, the minimum concentrations (25% V/V) of supernatants were chosen for further experiments. LAS and LRS could significantly suppress the invasiveness of Caco-2 cells. Also, the expression of MMP12 was significantly increased in Caco-2 cells when treated with LAS, whereas LRS had no significant effect on the invasive capacity and the gene expression levels of MMP12. The expression of MMP-9 was statistically decreased in Caco2 cells treated with LAS and LRS ($P<0.00001$).

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

1. Introduction

According to epidemiological studies, colorectal cancer is ranked as the fourth leading cause of mortality in the world [1]. The incidence of colorectal cancer is higher in western countries and rapidly increasing in developed Asian countries [2]. There are various risk factors associated with colorectal cancer, including external factors, such as poor diet, alcohol consumption, tobacco use, and sedentary work, as well as internal factors, such as genetic predisposition, hormone, and immunological imbalances [3]. Recently, the role of gut microbiota in the development of colorectal cancer has attracted much attention, and numerous studies have indicated that the homeostasis of gut microbiota is closely related to the risk factors mentioned earlier [4–6]. It has been reported that abnormal gut microbiota leads to the emergence of different pathophysiological events associated with numerous diseases, such as colorectal cancer [5, 7]. However, the precise mechanism of microbiota in the development of colorectal cancer is still opaque. Among the factors mentioned above, diet plays an essential role in the emergence of colorectal cancer [3, 8].

Probiotics are vital microorganisms in a healthy human microbiota environment [9]. *Lactobacillus* species is one of the most commonly used probiotics. Lactic acid bacteria (LAB) exert health-promoting activity

closely associated with the suppression of allergic responses as well as anti-inflammatory and anti-tumor effects [10–12]. As members of the gut microbiota, lactic acid bacteria (LAB), especially *Lactobacillus*, exert health benefits for the host when administered in adequate amounts [13, 14]. Several lines of evidence demonstrated that the supplementation of LAS could act as a prophylactic strategy for the prevention and cure of colorectal cancer as a result of their probiotic properties [15–18].

Nevertheless, the beneficial role of LAB in the inhibition of colorectal cancer progression and their effects on tumor microenvironments remain largely unknown. A number of investigations suggested that LAB exert anti-neoplastic activity by promoting the immunity or modulation of immune responses [19]. They also enhance the DNA repair process [20], stimulate programmed cell death, and inhibit the proliferation of colon cancer cells [21]. 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 role in later stages of colorectal cancer, especially metastasis.

Matrix metallopeptidases belong to the matrix metalloproteinase (MMP) family, whose activity is dependent on the zinc ion and they are categorized as protolithic enzymes that are responsible for the remodeling and the degradation of the components of the extracellular matrix (ECM) [22, 23]. Proteins incorporated into the ECM contribute to the regulation of cancer cell functions and tumor microenvironments. Also, it has been shown that abnormal ECM deposition leads to tissue remodeling and progression of tumorigenesis [24]. MMPs are able to digest proteins present in the ECM, such as gelatin, elastin, and collagen I, IV, and V.

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 are capable of changing 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 [25].

The MMP-9 protein belongs to the matrix metalloproteinase family, and it is able to digest all extracellular matrix proteins. The levels of MMP-9 level have been shown as a biological index for poor prognosis in colorectal cancer, along with other types of cancer, such as cervical and breast cancer [26]. MMP-12, also named metalloelastase, does not belong to the MMP family; however, it has been demonstrated that this protein can degrade a broad range of substrates. A group of studies has shown that MMP-12 is mainly expressed in macrophages and its protective roles in the prevention of colorectal cancer.

To the best of our knowledge, no study was conducted on the role of cell-free supernatants of probiotics in the prevention and elimination of human colon carcinoma cell line (Caco-2). Thus, cell death, invasion rate, and the indices of metastasis, such as MMP-9 and MMP-12, were measured in Caco-2 cells.

2. Materials And Methods

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) were purchased from Sigma-Aldrich (Merk). Fetal Bovine Serum (FBS) was procured from Gibco (Thermo Fisher Scientific- US). The real-time PCR assay kit was procured from Amplicon.

2.1. Bacterial strains and culture medium

The following lactobacillus strains, namely L. *acidophilus*, L. *rhamnose*, 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.

2.2. Preparation of cell-free supernatant (CSF)

For preparing cell-free supernatant (CSF), bacterial cells (10^9 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 bacterial filter. Cell-free supernatant was adjusted to pH 7.4 using 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.

2.3. Cell proliferation, migration and invasion assays

This experiment focused on analyzing the effects of *L. acidophilus*, *L. rhamnose* supernatants on the growth inhibition of Caco2 cells (NCBI C139, Pasteur Institute of Tehran, Iran). The yellow tetrazolium salt, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is enzymatically reduced by viable cells, producing insoluble formazan crystals with purple color. So, such a change in the color of the product could be quantifiable by spectrophotometry. Briefly, 4×10^4 cells/well were seeded onto a 96-well plate, and the cells were cultured overnight at 37 ^{0}C in a CO2 incubator (5% CO2) to adhere to the bottom of the wells. Then, cells were treated with different concentrations of lactobacillus culture supernatants (24h and 48h) (0–50% V/V). The results were subsequently analyzed after 24, 48, and 72 h. The MTT solution (0.5 mg/ml) was added to the wells, followed by incubation at 37°C for 4 h. The precipitated formazan crystals were solubilized by adding 100 µl of DMSO, and the optical absorbance of the wells was measured at 570 nm, using an ELISA reader (Model 680, BIO-RAD, Hercules, CA, USA). The inhibition rate (IR) was evaluated using the following equation:

Inhibition ratio (%) = 1- OD_{exp} / OD_{con} × 100

Where OD_{exp} and OD_{con} are the optical absorbance values of treated and untreated cells, respectively.

Cell invasion was assessed using the Transwell method. To this aim, a total of 2×10^4 cells were cultured in the DMEM medium at the top of the transwell membrane chamber (Costar; Corning, 8-µm pore size). In the bottom of the chamber, the cell culture medium supplemented with 10% FBS was added (Fig. 1). The migration assay was carried out after 36 hours at 37°C in 5% CO_2 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 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 of cells per group.

2.4. RNA extraction, cDNA synthesis, and real-time quantitative reverse transcription-polymerase chain reaction

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 for the 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 of 250 ng cDNA, 2x master mix (10 µl), 10 pmol of each primer pairs adjusted by ddH_2O up to final reaction of 10 µl. The sequences of primers were designed by the NCBI PRIMER BLAST tool. The primers used in real-time PCR were as follows: MMP9 forward primer: AAGGATGGGAAGTACTGGCG, reverse primer: GCTCCTCAAAGACCGAGTCC, MMP12 forward primer: TTTGGTGGTTTTTGCCCGTG, reverse primer: GGAACAAGTTTGTGCCTCCTG, and β-Actin (reference gene) forward primer: TGAAGATCAAGATCATTGCTCCC, reverse primer: AGTCATAGTCCGCCTAGAAGC. 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. In order to examine the specificity of primers used, as well as the absence of primer dimmer, the melting curve analysis was conducted after each run of amplification.

2.5. Statistical Analysis

The obtained values are expressed as the means and standard deviation (means ± SD). The difference between the experimental groups and the untreated group were analyzed by two-tailed Student T-test. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1 Cytotoxic effect of L. *acidophilus* and L. *rhamnosus* strain culture supernatants on Caco2 cell growth

The cells were treated with different concentrations of lactobacillus culture supernatants (0–50% V/V) 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 (Fig. 2). The results demonstrated that LAS and LRS (collected supernatants after 24 and 48h of the incubation periods) had a

significant inhibitory effect on Caco2 cell proliferation in comparison 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 obtained results indicated that LRS collected after 48h had more cytotoxic activity against the Caco2 cell line compared with those collected after 24 h. There was no significant difference in cell viability when Caco-2 cells were treated with LAS after 24h and 48h.

3.2. Inhibition of invasiveness by *L. acidophilus* supernatant

The cell invasion analysis was performed in the matrigel basement membrane using a transwell system, as described in the section of “Materials and methods.” In this method, cells that migrated into the lower surface of the membrane were fixed and then stained (Fig. 3). The percentages of migration and invasion of cells were significantly lower when the cells were incubated with LAS ($P < 0.01$) and LRS ($P < 0.05$) after 36h compared with cells treated with the MRS solution.

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

In order 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 shown in Fig. 4A, 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. As depicted in Fig. 4B, the expression of MMP-9 was significantly decreased in Caco2 cells treated with LRS and LAS compared with the MRS-treated Caco-2 cells.

4. 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 colorectal cancer [27]. Unfortunately, a vast majority of patients with colorectal cancer are diagnosed at the advanced stages of the disease, especially when the initial tumors invaded other organs. This phenomenon reduces the survival rate of patients by 10%. Alternatively, all therapeutic options, including chemotherapy, surgery, and radiotherapy, can remarkably affect the quality of life of patients. It has been shown that both radiotherapy and chemotherapy have serious adverse effects on the human and mainly emerged as gastrointestinal toxicity, namely mucositis, enteritis, diarrhea, nausea, and vomiting [28]. Therefore, it seems prophylactic strategies and alternative treatments are needed to prevent the emergence of colorectal cancer.

In the present study, the impact of two probiotic *Lactobacillus* sp., namely L. *acidophilus* and L. *rhamnosus*, on the inhibition of the growth and metastasis of Caco2 cancer cells was determined through analyzing the cell invasion assay *in-vitro* 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 extracellular matrix in the colon, facilitating the cell invasion process during metastasis [29–32]. Conversely, evidence showed that the expression of MMP-12, which is a metalloelastase enzyme, has inhibitory effects on the growth and proliferation of colorectal cancer cells, and it is associated with increased survival of patients with colorectal cancer [33].

We showed that LAS and LRS had a significant effect on the viability of Caco2 cells compared with those treated with the MRS medium (control). Also, our results revealed that LAS and LRS containing secreted bioactive compounds significantly reduced the invasion of metastatic colon cancer cells in-vitro (Fig. 3), as shown by decreased expression of MMP-9 (Fig. 4A). Furthermore, LAS increased the expression levels of MMP-12 (Fig. 4B), while LRS had no effect on MMP-12 expression. To the best of our knowledge, the current research is the first study reporting that released bioactive compounds from *L. acidophilus* and L. *rhamnosus* can regulate the expression levels of MMP-9 and MMP-12 genes in Caco-2 cells, suggesting their potential role in the inhibition of colon cancer cell invasion.

It is now known that MMPs have detrimental roles in metastasis of colon cancer, promoting the invasion of primary tumors through the digestion of collagen in the ECM [29, 30]. It has been shown that the MMP-9 enzyme has proteolytic activity, participating in reconstruction and breakdown of the ECM, a phenomenon observed in the invasion and metastasis of colorectal cancer. 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 the angiogenesis process [34]. Also, MMP-9 effectively contributes to the formation of early metastatic niches [35]. A number of preclinical analyses demonstrated that the selective inhibition of MMP-9 is able to decrease tumor proliferation and metastasis rates in colorectal cancer. It can also induce programmed cell death in pancreatic cancer cells [36, 37]. 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 [21].

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 [38, 39]. While elevated expression of MMP-12 has been reported in patients diagnosed with CRC, its expression level has been higher in patients with no liver metastasis compared with those with liver metastasis [40]. Besides, the expression of MMP-12 is able to lower the expression rate of VEGF and increase the expression of angiostatin, which is an endogenous inhibitor of the angiogenesis process [41]. Consistent with these statements, a number of investigations demonstrated that the expression of MMP-12 is associated with increased overall survival of patients and reduced tumor growth [42, 43]. 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 the 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 [44–46], our results showed that *L. acidophilus* and *L. rhamnosus* also have remarkable anti-proliferative and anti-metastatic effects on colorectal cancer cells.

A number of studies demonstrated the relationship between a diet enriched with *Lactobacillus* and reduced risk of colon cancer [47]. Several studies, in vitro and in vivo, revealed that probiotics modulate cancer cells, like Caco2 cell line, proliferation and apoptosis [48–50]. Furthermore, it is now known that probiotics have various properties, including radio-protective, antioxidant, and antagonistic activity, as well as toxin neutralization. They are able to improve the intestinal microbial environment and immune system response [51, 52]. Thus, they could be used as an alternative therapy instead of using invasive treatments, such as radiotherapy and chemotherapy.

Conclusion

In conclusion, this study revealed that the expression level of *MMP-9* gene, as indices of metastasis, are influenced by the cell-free supernatants extracted from *L. acidophilus* and L. *rhamnosus*. Moreover, the modulation of the MMP-12 gene by *L. acidophilus* resulted in the anti-invasion activity of Caco-2 cells.

Several lines of evidence showed the significance of probiotic balance in the maintenance of homeostasis, which paves the way to achieve optimal cancer therapy. Recent studies suggest the application of probiotics in cutting-edge cancer therapies. Clinical studies and animal models performed on *L. Acidophilus* and *L. Rhamnosus* suggest the possibility of utilizing such probiotics as alternative approaches to prevent metastasis and cure cancer.

Altogether, regarding the above findings, further research is required to characterize the precise mechanism of action of bioactive factors in probiotic-containing functional foods. This provides insight into seeking preventive strategies to combat cancer cell proliferation and invasion.

Abbreviations

Colon carcinoma cell line Caco-2

DMEM Dulbecco's Modified Eagle's medium

ECM Extracellular matrix

FBS Fetal bovine serum

Lactic acid bacteria LAB

lactobacillus acidophilus supernatant LAS

lactobacillus rhamnosus supernatant LRS

Matrix metalloproteinase MMP

Mitochondrial membrane potential-9 MMP-9

Vascular endothelial growth factor VEGF

Declarations

• Ethics approval and consent to participate

At the time of this study had been done, study on cell line did not need to ethic Committee permission at my university.

• Consent for publication

Not applicable.

• Availability of data and materials

The original data are available upon reasonable request to the

corresponding author.

• Competing interests

The authors declare any competing interests.

• Funding

No any funding supported this study.

• Authors' contributions

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 1-4. All authors read and approved the final manuscript.

• Acknowledgements

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

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Figures

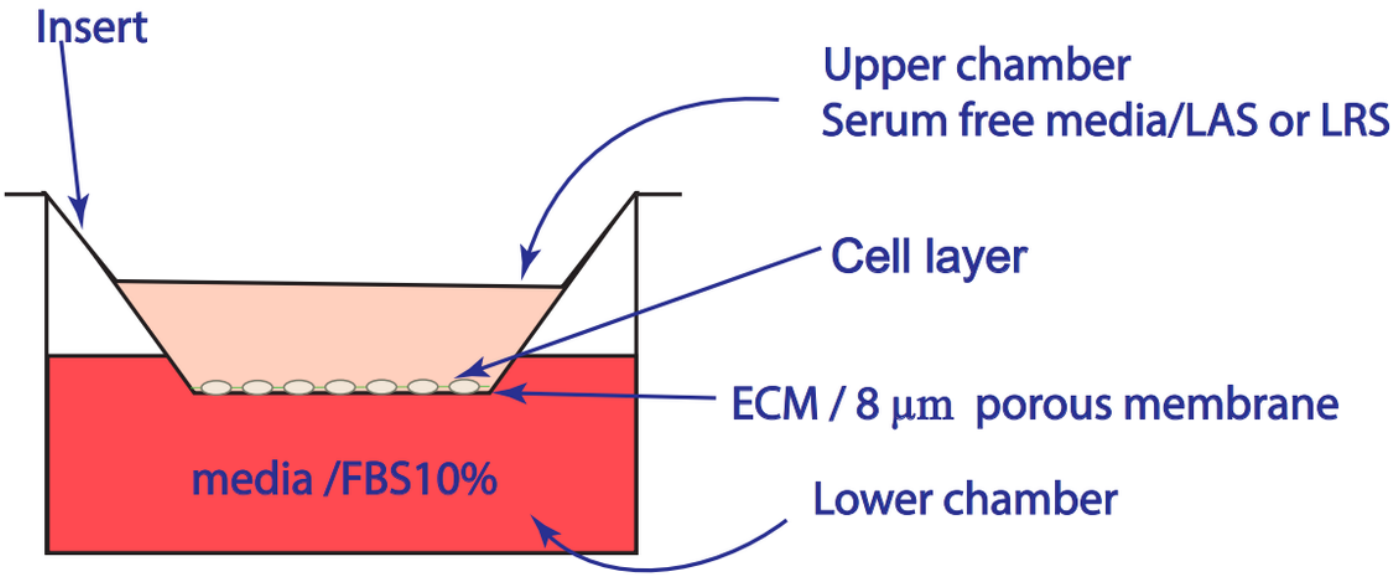

Figure 1

Transwell assay. In this method, cells that migrated into the lower surface of the membrane were fixed, stained and then counted.

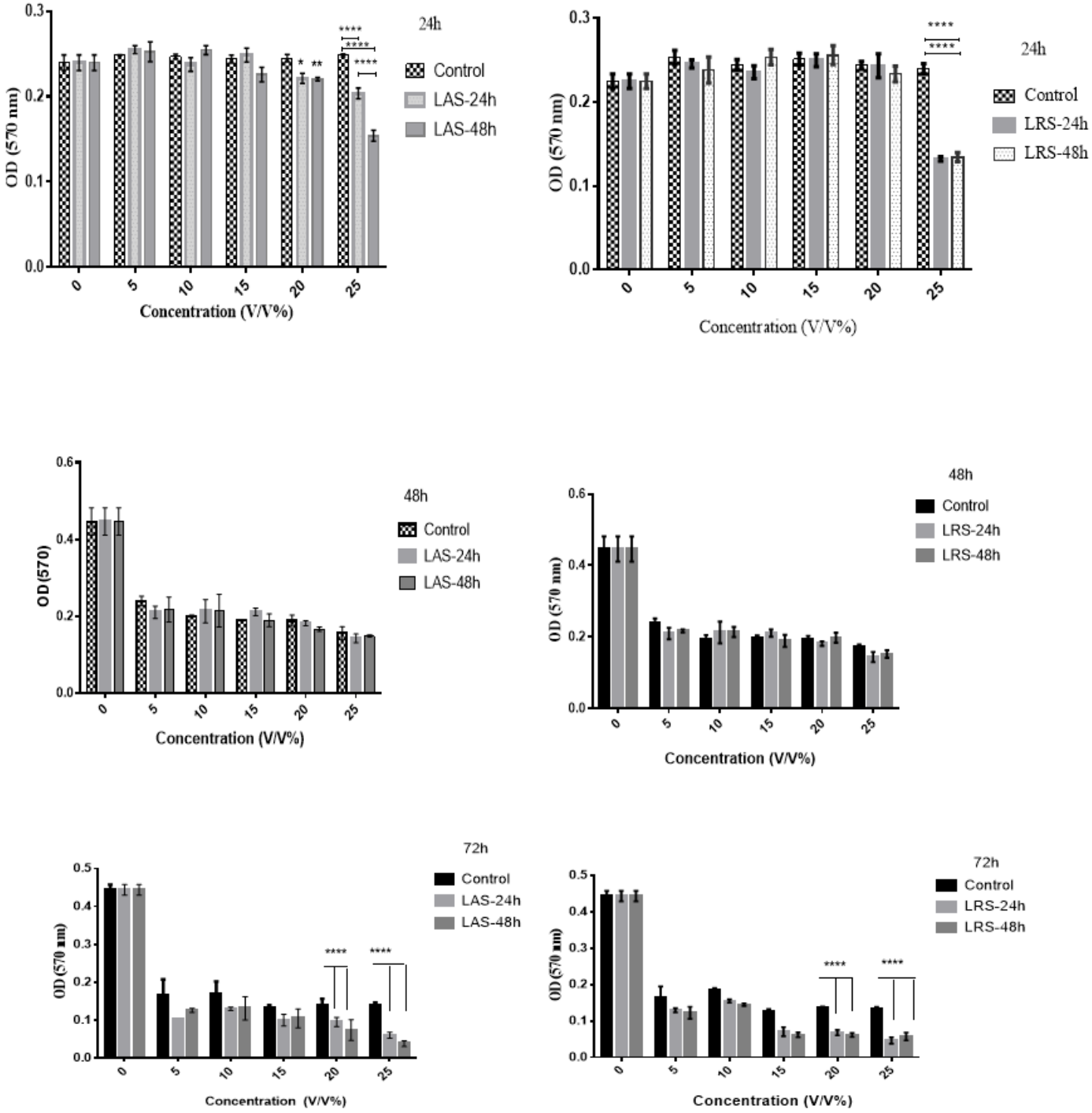

Figure 2

The MTT assay represents cytotoxicity effects of LRS, LAS, and MRS (collected after 24h and 48h) with different concentrations on Caco2 cells; A_1&A_2)) after 24h, B_1&B_2) after 48h treatment, C_1&C_2) after 72h treatment. LRS; *Lactobacillus rhamnosus* supernatant, LAS: *Lactobacillus acidophilus* supernatant. MRS; De Man Rogosa Sharpe. The mean value is represented with three separate experiments for each point. *; P<0.05, **; P<0.01, ***; P<0.001, ****; P<0.0001.

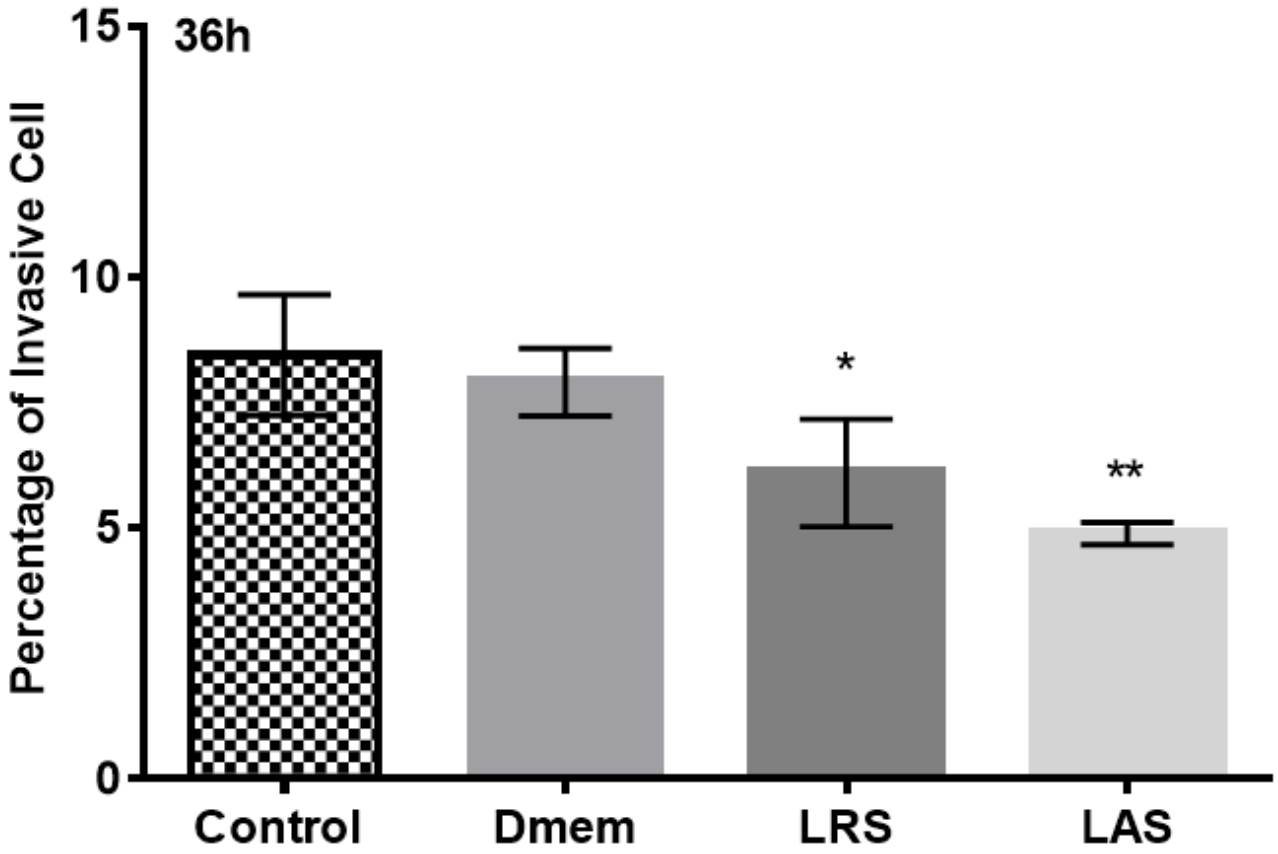

Figure 3

Cell migration/invasion assay in LRS- and LAS-treated Caco2 cells. Cells (2×10^4 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. * $P < 0.05$ and **P<0.01.

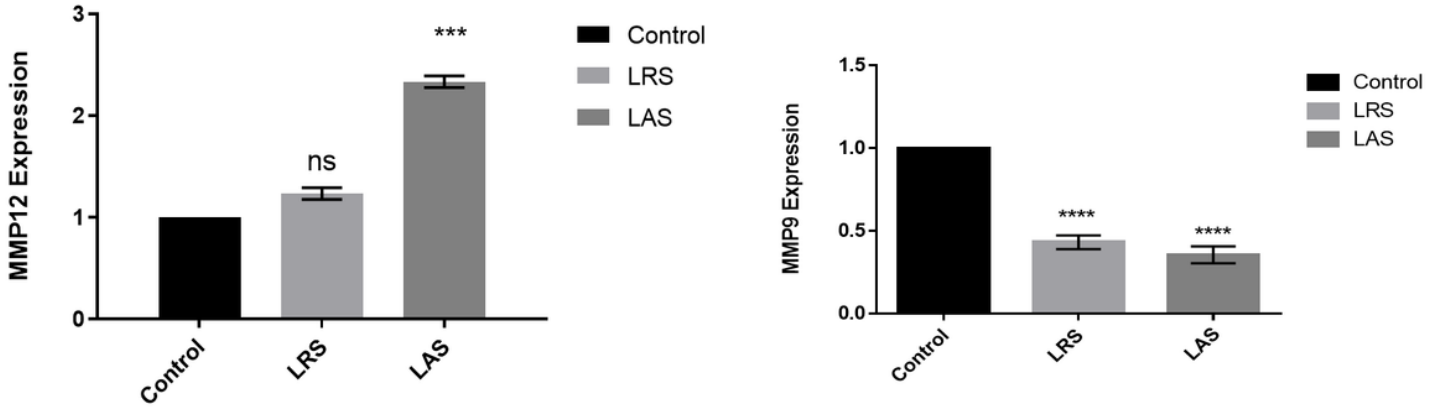

Figure 4

The impact of LAS and LRS on the expression of *MMP-12* and *MMP-9* genes. **A.** The effects of MRS, LAS, and LRS on the expression of the *MMP-12* gene. **B.** The effects of MRS, LAS, and LRS on the expression of the *MMP-9* gene in Caco2 cells. The relative expression analysis of target genes was performed by the 2−ΔΔCt method. The values are expressed as the means ± SD from three independent experiments.

* P<0.05, **; P<0.01, ***; P<0.001, ****; P<0.0001.

Supplementary Files

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