Metastasis of cervical cancer cell line affected by probiotics in real-time PCR assay

Azher Adnan and Zainab M Farhan

DOI: https://doi.org/10.22271/27069567.2024.v6.i3a.570

Abstract

Objective: The term "probiotic" refers to live microorganisms that have been linked to advantageous outcomes for the host. Nonetheless, a large body of research suggests that probiotic lactic acid bacteria (LAB) may be helpful in preventing colon cancer in its early stages. However, nothing is known about how probiotic LAB may affect colorectal and cervical cancer in their later phases, particularly with regard to metastasis. The present research sought to examine the impact of Lactobacillus crispatus supernatant (LCS) on cervical cell lines derived from human tissue, specifically focusing on the HeLa cell line. Throughout our inquiry, this was going to be executed.

Materials and Methods: Using the MTT test, the experiment's activities impact of the LCS was ascertained. Through the utilization of a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), an investigation into the expression of the MMP2 and MMP9 genes was carried out after the cells had been synchronized.

Results: Probiotics called Lactobacillus crispatus supernatant cytotoxicity affected HeLa cells. Furthermore, LCS did not affect normal cells adversely. Additionally, it was demonstrated that treatment with the lactobacilli under study resulted in a decrease in the expression of the MMP2 and MMP9 genes. Our research indicates that LCS effectively inhibits metastatic potency in HeLa cells by down regulating MMP2 and MMP9 expression while up regulating the expression of their inhibitors.

Conclusion: We have shown that a probiotic can delay the progression of late-stage cancer illness and has anti-metastasis effects on cell lines. LCS exhibited both cytotoxic and anti-metastatic effects on HeLa cell lines. HeLa cervical cancer cells are resistant to proliferation when exposed to L. crispatus. It is required to conduct additional evaluations in order to assess our findings on the other cancer cell lines before considering the use of these probiotics in larger trial studies.

Keywords: Probiotic, HeLa, metastasis, gene expression, cervical cancer

1. Introduction

Probiotics are described as living bacteria, or germs, that, when ingested, boost human health [1]. Friendly bacteria, sometimes referred to as probiotic living bacteria, have the ability to lessen potentially hazardous microorganisms in the intestines. The two most popular kinds of microorganisms used as probiotics are lactobacilli and bifidobacteria, whose positive effects are being thoroughly studied [2]. Pregnant or nursing mothers may take probiotics to support their microbial ecosystem that may also benefit the health of the mother and the child. Researchers examined the characteristics and (Health) behaviors of pregnant women who took probiotics and the effects on the fetuses’ health [3]. These are vital in the digestive, respiratory, and immune functions of the body among other systems. In addition, they have the capability of reducing the risk of acquiring communicable diseases in children and other vulnerable persons tremendously [4]. The synthesis of anti-mutagenic compounds and also the degradation of carcinogetic material by the action of the probiotic bacteria prove the anti-tumor activities of the bacteria that lowers the risk factor for cancer [5]. In countries that are considered to be developing, cervical cancer is the most prevalent disease that is diagnosed in females. Additionally, it is the second most prevalent cancer in females worldwide [6]. The majority of probiotics are members of the Lactobacillus genus, which is naturally occurring in the human vagina and has a significant role in defending the host against urogenital infections [7]. Additionally, a large body of research suggests that probiotic lactic acid bacteria (LAB) may be helpful in preventing colon cancer in its early stages. However, little is known about the impact of LAB in the later stages of colorectal and cervical cancer, particularly with regard to metastasis.
Extracellular matrix (ECM) penetration by cells is necessary for tissue invasion and metastasis; during the metastatic process, matrix metalloproteinases (MMPs) break down the ECM [8]. The most widely recognized risk factor for this disease is infection with the Human Papillomavirus (HPV). Even though HPV infection is a primary contributing element to the pathogenesis of cervical cancer, other environmental and host variables, such as the pre-existing infections and microbial flora in the cervical region, are likely also linked to the development of the illness. Lactobacilli, including Lactobacillus crispatus and Lactobacillus gasseri, are the predominant microorganisms in the healthy human vaginal and cervical ecosystems [9]. Vaginally acquired infections are killed or have their proliferation inhibited by substances produced by some lactobacillus species [10]. Additional lactobacilli inhibit pathogen adhesion to urogenital epithelial cells in vitro [11].

2. Material and methods

2.1. Cell culture

A cell line derived from human cervical carcinoma, commonly referred to as HeLa was obtained from the Pasteur Institute, which is a component of the National Cell Bank of Iran. HeLa is a human cell line. In order to carry out this experiment, the HeLa cell line must be utilized. Following a period of twenty-four hours during which the cells were allowed to incubate in Roswell Park Memorial Institute (RPMI) media that had been supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (both of which were supplied by Invitrogen, USA), the cells were then permitted to continue incubating. With a temperature of 37 degrees Celsius and a carbon dioxide concentration of 5%, the cells were maintained in a humidified environment.

Fig 1: Human cervical cancer cell line (HeLa)

2.2. Lactobacillus supernatant preparation

The L. crispatus strain SJ-3C-US was cultivated in De Man Rogosa Sharpe (MRS) broth (pH=6.5, Merck, Germany) at 37 °C for 24 hours, using microaerophilic conditions. When the bacterial cultures had been cultured for a period of twenty-four hours, they were centrifuged at a speed of seven thousand revolutions per minute for a period of seven whole minutes. This process was repeated seven times. The concentration of the bacterial cultures was 2×10^8 colony-forming units per milliliter. A membrane filter with a thickness of 0.2 millimeters was used to filter the lactobacilli supernatants (LS) in order to remove any bacteria and debris that may have been left behind. To distinguish the effects of lactate generated by L. crispatus supernatant (LCS) from the influence of pH change, the pH of MRS (6.5) the liquid was modified to correspond with the pH of LS (4.2±0.1). This was done in order to find out the difference between the two results. This was done in order to achieve such a separation. This was done by utilizing lactate. This was done while maintaining the pH of MRS broth. MRS is the abbreviation for this specifically adapted control. The experiment involved testing the following conditions: The LCS solution has a pH of 4.2, whereas the MRS solution has a pH of 6.5. These solutions were used in HeLa cells.

Fig 2: The L. crispatus strain was cultivated using De Man Rogosa Sharpe (MRS) broth (pH=6.5, Merck, Germany).

2.3. MTT Assays

There are particular conditions under which NAD (P) H-dependent cellular oxidoreductase enzymes are able to perform the role of an indication of the quantity of live cells that are present in the sample. In order to determine the level of metabolic activity that is present in cells, a colorimetric method known as the MTT test is utilized. Through the action of these enzymes, the tetrazolium dye MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) can be converted into formazan, a chemical that is insoluble and contains a purple color. In order to examine the cytotoxicity (The killing of cells) and cytostatic activity (The change from cell proliferation to quiescence) of possible medicinal medications and hazardous substances, tetrazolium dye assays can be performed. MTT tests are typically conducted under conditions of total darkness due to the light sensitivity of the MTT reagent. The MTT substrate is produced in a solution that is balanced to
simulate the circumstances that are found in the body. After that, it is introduced to cells that have been grown at a concentration that ranges from 0.2 to 0.5 mg/ml. A length of time ranging from one to four hours is subsequently spent incubating the cells. A plate reading spectrophotometer is used to detect variations in absorbance at 570 nm in order to determine the amount of formazan present. This is done because it is thought that the amount of viable cells is directly connected to the amount of formazan present. This purple formazan product has a maximum absorbance close to 570 nm, and it is produced when MTT is converted into it by cells that are alive and have an active metabolism. Due to the fact that dying cells are unable to convert MTT into formazan, color formation is a reliable and useful signal that can only be used for living cells.

2.4 Cell synchronization for RNA extraction
The HeLa cells were cultivated in RPMI media that was treated with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) for a period of twenty-four hours. The growth process was carried out in a culture medium. In the following step, the cell lines were counted, and then an equal number of cells were moved to four flasks with a capacity of 25 cm³ for the purpose of sub-culturing. After that, the cells that were contained within three of these flasks were utilized for a period of four hours to undergo treatment with LS, MRS, and MRS+HCL. In order to serve as a control, the final flask was tested without being subjected to any kind of treatment.

2.5 RNA isolation, cDNA synthesis and quantitative reverse transcriptase-polymerase chain reaction
Through the utilization of the TriPure Reagent kit (Roshe Applied Science, Germany), RNA was extracted from each and every cell, regardless of whether or not it had been treated. The assessment of RNA quality and quantity was performed using a spectrophotometer known as Nanodrop, produced by Thermo Scientific in the United States. The reverse transcription of RNA was performed using the PrimeScript RT reagent kit from Takara Bio, Japan. After that, the quantitative real-time polymerase chain reaction, also known as qRT-PCR, was applied in order to assess the levels of mRNA expression in the genes that were the primary focus of the inquiry. It is the job of the Australian business Corbet to do real-time measurement of particular mRNA genes. These genes include MMP2, MMP9, and gene 6000, amongst others. In order to carry out the real-time master mix reaction, two master mixes were utilized. One of these master mixes was acquired from Takara Bio in Japan. Additionally, two hundred and fifty nanograms of cDNA and ten micromoles of each primer pair were utilized. After adjusting the volume of the reaction using ddH2O, the total volume of the reaction was brought down to ten microliters. With the use of the Primer 3 software and the NCBI-BLAST database, the primer sequences for each of the genes that are the subject of the current investigation were chosen from previous publications. These sequences were then verified before being used in the investigation. The primer sets are assembled in Table 1, which is a thorough compilation. A denaturation of the cDNA was performed at 95 degrees Celsius for ten seconds as part of the thermal cycling technique. This was then followed by fifty cycles of two-step amplification. The procedure involved applying denaturation at a temperature of 95 degrees Celsius for a period of ten seconds, then annealing and extension at a temperature of sixty-five degrees Celsius for a period of thirty seconds. The denaturation process was repeated three times. During the course of the investigations, each data point was examined twice, to ensure accuracy. After each cycle of amplification, a melting curve analysis was carried out in order to verify that there was no synthesis of primer dimers and to determine the specificity of the products obtained from the qRT-PCR. To verify that the primer dimer formation was not present, this was carried out in order to affirm with certainty.

Table 1: Sequences used in this study

<table>
<thead>
<tr>
<th>Primer sequences</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2 F: GGCAGTIACAATACCTGAAACAC R: GTCTGGGGGAGTCCAAAGAACT</td>
<td>111</td>
<td>(27)</td>
</tr>
<tr>
<td>MMP9 F: GCACGACGTCTTCCAGTACC R: CAGGATTCATAGGTCACGTAGC</td>
<td>124</td>
<td>(28)</td>
</tr>
</tbody>
</table>

Fig 3: The process of converting RNA into complementary DNA (cDNA) and subsequently measuring the expression of certain genes using quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed
3. Results
3.1. The cytotoxic effect of *L. crispatus* strain SJ-3C-US culture supernatant on HeLa cell growth
The inhibitory effects on cell growth were assessed using the MTT test. During this trial, it was demonstrated through the findings that LCS was able to successfully inhibit the development of HeLa cells compared to the cells subjected to MRS solutions. Based on the data, it was determined that the acidity did not play a primary role in the inhibition of the proliferation of HeLa cells. Furthermore, the IC50 value of LCS against HeLa cells was shown to be 11% (v/v), which suggests that a component present in LCS, in addition to lactate, selectively affects the cervical cancer cells (HeLa) while having no effect on normal cells. This was demonstrated further by the fact that the IC50 value was 11%.

![Chart 1: The effect of *Lactobacillus crispatus* strain SJ-3C-US culture supernatant on the proliferation of HeLa cells](image1)

![Fig 4: The photographs illustrate the cytotoxic impacts of LCS, MRS+HCL, and MRS on the HeLa cell line at different doses, as evaluated using the MTT assay. Each point is denoted by the average value derived from three distinct experiments. The acronyms MRS (De Man Rogosa Sharpe), MRL (MRS with lactic acid), and LCS (*Lactobacillus crispatus* supernatant) are employed](image2)

3-3. MMP2 and MMP9 genes expression in HeLa cells treated with LCS
The levels of mRNA expression of the *MMP2* and *MMP9* genes were measured using quantitative real-time PCR after a treatment with LCS, MRL, or MRS for a period of four hours. The treatment was carried out during the course of the experiment. Upon comparing HeLa cells injected with LCS to those handled with MRS+HCL or MRS, we observed a decrease in mRNA expression of the MMP2 and MMP9 genes in the LCS-treated cells. There are significant discrepancies between the LCS, MRS+HCL, and MRS for MMP2 all of which compare. The LCS, on the other hand, represents a major departure from the other two groups in the instance of MMP9. The conclusion that can be drawn from this is that there is no discernible difference of statistical significance between MRS+HCL and MRS in MMP9.
4. Discussion
Scientific evidence has demonstrated that probiotics can exert anti-cancer effects through many pathways, including as stimulating immune responses and exhibiting anti-proliferative, anti-apoptotic, and anti-microbial activity. There is evidence indicating that cervical cancer cells have the ability to spread to other organs, including the liver, lungs, and brain [11, 12] by stimulating the activation of MMP2 and MMP9, the extracellular matrix (ECM) is subsequently broken down [13]. A recent research was conducted to examine the reasoning for the practice of restocking the vagina with nonpathogenic organisms, such as lactobacilli that provide probiotic functions [14]. It has been demonstrated that Matrigel™ is capable of promoting a natural cell morphology and behavior, and it has been successfully exploited for a range of applications, including cell growth and differentiation, angiogenesis, and invasion experiments [15]. Matrigel™ has been widely utilized to promote acinus development in human breast MCF-10A cells and MDCK cells [16, 17]. However, problems connected with the limited availability, the passaging of infections, immunogenicity, and the fact that Matrigel™ originated from animal tumors suggests that it will not be used as a clinical material. Therefore, the research for a practical, tolerable, and adjustable artificial ECM for 3D cell culture and tissue engineering has attracted many laboratories, including ours.

Since the *L. acidophilus* 36YL strain may have antagonistic and anticancer activity in the vaginal ecosystem, this research applied several *in vitro* methods. What was found out was that this position slows the growth of bacteria that can be harmful to mankind and other dangerous bacteria. According to the observations, it emerged that this specific stress has the characteristics of probiotics that are highly effective in combating cancer and in delaying the formation of cancerous agents [18]. For this reason, we thought it expedient to assess the anticancer properties of the *L. crispatus* SJ-3C-US strain on cervical cancer cells. This was because the *L. acidophilus* 36YL strain showed comparatively lower efficacy than the *L. crispatus* SJ-3C-US strain, when applied to the same cell line. When the produced metabolites of the *L. crispatus* SJ-3C-US strain were compared with human cervical cancer cells called HeLa cells, it was observed that the former was most toxic. In the earlier studies, we proved that the culture supernatants of lactobacilli possessed a high cytotoxic effect on the cells which were cervical cancer cells [19]. This study aims to establish the consequences of using *L. crispatus* culture supernatant on HeLa and MRC-5 cells. An assay with the MTT test was conducted on cells in order to assess the effects. Furthermore, the effects of the impacts were analyzed by determining the metastatic activity of genes, including MMP-2 and MMP-9, as well as their inhibitors on the transcriptional level. That we were able to also observe a decrease in the genes MMP2 and MMP9 in HeLa cell lines after exposure to LCS provide evidence of this probiotic as an inhibitor of cervical cell lines. As mentioned in this paper, through the experiments it has been confirmed that *L. crispatus* possesses the efficacy of down regulating the enzymatic action of MMP2 and MMP9 [20]. Based on the conclusion of this study, LCS exhibited an inhibitory impact on the viability of HeLa cells. Outcomes that were stated in the previous sections [21]. *L. acidophilus, L. casei, L. rhamnosus, B. longum,* and *B. lactis* supernatants have been identified to possess the ability to suppress the development of colorectal carcinoma based on past experimental studies [22, 23].
The induction of Autophagy pathway is another possible way that how probiotics may suppress cell proliferation. The present work clearly showed that, following the treatment of HeLa cells with LCS or LRS, the expression of a vast amount of genes involved in autophagy was significantly decreased [22]. It was reported in a recent study carried out on various malignant cell lines on the metabolites secreted by the L. acidophilus 36YL strain. These cell lines included HeLa, MCF-7, AGS, and HT-29. The study compared the secretion of metabolites from the L. acidophilus 36YL strain to that of the normal cells (HUVEC). In addition to reducing the viability of all malignant cell types, the metabolites produced by these bacteria did not have any detrimental effects on normal cells during the experiment [23]. A study revealed that the primary cause of HeLa cell death induced by LS was not the acidity level. Thus, it is possible that the cause of cervical tumor cell death in LS may be attributed to a chemical other than lactate. Furthermore, they elucidated through the use of qRT-PCR that LS has the ability to decrease the expression of two genes that play crucial roles in autophagy, as well as CASP3. Previous reports have indicated that LS causes down-regulation of CASP3 [24]. Our findings demonstrate a substantial decrease in the transcript level of just E6 following treatment with lactobacilli supernatants. As far as we know, this is the initial report demonstrating the reduction of the HPV E6 oncogene by lactobacilli. Suppressing the naturally occurring HPV oncogenes can effectively reverse the development of cervical cancer [25]. Lactate generation and pH levels in cultures are responsible for some of the probiotic activities that occur [26]. We conducted a comparison between the effects of the LS and those of MRS and MRS+HCL. Remarkably, MRS+HCL had a stronger inhibitory Impact on cellular cultures compared to MRS, despite both having equal pH levels. Hence, in the current investigation, a distinction exists between lactate production and pH due to lactate production above the acidic pH threshold. Furthermore, the inhibitory impact of lactobacilli was mostly attributed to the formation of lactate rather than only relying on pH levels.
5. Conclusion
It is important to note that probiotics contain the primary natural bacteria of the cervix. There is a possibility that it could offer a fresh method for the prevention or possibly the inhibition of cell invasions that are associated with cervical cancer. It is required to do additional research in order to analyze the impact that the supernatant fraction has on the different types of cancer cells. L. crispatus has been shown to have the ability to limit the proliferation of HeLa cervical cancer cells, according to the available evidence. The precise mechanism that is responsible for this influence is not yet fully understood. The down-regulation of HPV oncogenes, on the other hand, has the potential to partially induce this cytotoxicity of the virus. The therapeutic effects of lactobacilli in malignancies are currently being studied, and the outcomes of this study provide additional data in favor of those effects—which are currently being evaluated.

6. Reference


