Effects of Probiotic Lactobacillus acidophilus and Lactobacillus casei on Colorectal Tumor Cells Activity (CaCo-2)

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Abstract
Background: The probiotic microorganisms are live normal flora that provide nutritional benefits. When probiotic administered in adequate amounts, they also confer a health benefit on the host. Different mechanisms of probiotic effects include the following: stimulating the immune system, modifying the composition of normal intestinal flora and preventing the carcinogenic activity of fecal enzymes. In this study, direct effects of probiotic lactobacilli on tumor cells were investigated.

Methods: Supernatants and bacterial extracts of two standard Lactobacillus species (L. acidophilus and L. casei) were prepared and CaCo-2 cells were treated with them. Probiotic effects on cell proliferation, necrosis, apoptosis, migration and invasion were assessed.

Results: The supernatants of Lactobacilli decreased cell proliferation and increased cell apoptosis, however, no significant effect on cell necrosis was reported. In contrast, Lactobacilli extract, reduced cell proliferation and increased cell apoptosis. Lactobacilli extract also led to cell necrosis. Furthermore, both supernatants and cell extracts of the probiotic agents resulted in decreased cells’ migration and invasion.

Conclusion: In this study, it was shown that Lactobacilli probiotics useful effects are not confined to the enhancement of the immune system; however, they effectively suppress the malignant phenotypes of colorectal cancer cells.

Keywords: Colorectal cancer, lactobacillus acidophilus, lactobacillus casei, probiotic

Introduction

Probiotics refer to harmless microorganisms that could have nutritional advantages. They also provide health benefits, when administered in adequate amounts.1 Since 1953, numerous positive effects of probiotics on ulcerative colitis, diarrhea and ectopic eczema have been reported.2-5 A number of clinical studies have been performed on the ability of probiotic prevention, control and treatment of various cancers, especially the gastrointestinal tract.6,7 Due to the large quantities of probiotic bacteria in the gut (1011 CFU/g of the intestinal content), probiotics seems to be one of the most interesting candidates for the treatment of colorectal cancer (CRC).8 Lactic acid bacteria (LAB) or the Lactobacilli bacteria are commonly used in the dairy industry. Some LAB strains, known as probiotics, theoretically stimulate the immune system, leading to the prevention of colorectal cancer.9 Considering these points, several studies were performed on the health benefits of milk fermented with Lactobacillus casei and L. acidophilus.10-12 The results of these studies indicate the positive effects of these probiotics on prevention of diarrhea caused by antibiotic treatment in hospitalized patients. In addition, the use of probiotics could reduce the Clostridium difficile-associated diarrhea outbreak.13 More studies on these two strains, demonstrated positive effects of these probiotics on increase of tumor cell apoptosis in response to 5-fluouracil treatment.14 Furthermore, oral uptake of L. acidophilus led to enhance the host immunity by increasing the level of IgG, IgM and gastrointestinal IgA.15,16 As reported previously, only 20% of germ-free animals develop chemically induced colon tumors, compared with 93% of those with a normal flora.17 Reddy, et al. showed that a stimulated growth of Bifidobacteria in the colon can give rise to the inhibition of colon carcinogenesis.18 Considering these findings, the present study aimed to evaluate the effects of standard L. acidophilus ATCC 4356 and L. casei ATCC 39392 on the inhibition of malignant phenotype of colorectal cancer CaCo-2 cells.

Materials and Methods

Probiotic materials
Standard strains of L. acidophilus (ATCC 4356) and L. casei (ATCC 39392) were cultured on de Man, Rogosa and Sharpe (MRS) agar. Bacterial colonies were introduced into liquid MRS and cultured overnight. Then, 1 ml of the culture was sub-cultured in 50 ml of fresh MRS medium. Absorbance of the medium was measured periodically at 600 nm until reached 1. To separate supernatant and bacterial pellets, the media were centrifuged at 3000 rpm for 5 min. Supernatant samples were sterilized using 0.22 μm filter. The solution was mixed with RPMI1640 medium, containing 10% FBS in different percentages of 5, 10 and 20%. MRS containing RPMI1640 medium was used as negative controls.
The bacterial plate was resuspended in 3 ml of 1 × phosphate buffered saline (PBS) and bacterial lysis was performed using an ultrasonic bath. Then, samples were sterilized using 0.22 μm filter. The suspension in concentrations of 1% and 5% were prepared by adding bacterial lysates to RPMI 1640 medium containing 10% of fetal bovine serum (FBS).

Cell culture
Colorectal cancer cell line (CaCo-2) from the Pasteur Institute (National Cell Bank of Iran), was cultured in RPMI 1640 medium (Invitrogen, USA) containing 20 μg/ml of gentamicin supplemented with 10% of FBS (Invitrogen, USA). Cells were grown at 37 °C, 5% CO₂, and 95% humidity.

Microculture tetrazolium test (MTT assay)
The inhibitory effect of probiotics on the growth and proliferation of CaCo-2 cells was assessed by MTT assay, as previously described. The inhibition rate (IR) of probiotics was evaluated using the following equation:

\[ IR(\%) = 1 - \frac{OD_{exp}}{OD_{con}} \times 100 \]

Where OD_{exp} and OD_{con} are the optical densitometries of treated and control cells, respectively.

Lactate dehydrogenase release assay
Probiotic necrosis inducing ability was measured based on the measurement of lactate dehydrogenase released from necrotic cells. Cells were treated as described in the previous step (except the use of RPMI medium containing 5% of FBS) and LDH activity was evaluated using a LDH assay calorimetric kit (Sigma, Germany), according to the manufacturer’s instructions with minor modifications. After the plate was centrifuged at 250 g for 4 min, 50 μl of the supernatant was transferred into the new plate. After adding 100 μl of the enzyme activity measuring solution (consisting of equal proportions of the LDH substrate solution, enzyme cofactor solution and dye solution), the plate was wrapped in an aluminum foil and incubated at room temperature for 30 min. Enzymatic reaction was stopped, by adding 15 μl of 1N HCL and 85 μl of 1N NaOH. The optical density of the samples was measured at 550 nm. The inhibition rate (IR) of probiotics was calculated.

Measurement of cell apoptosis
The apoptosis inducing effect of probiotics on CaCo-2 cells was assessed by Caspase 3 activity measurement. Cells were treated in the MTT assay and total protein of the samples was extracted. Briefly, 5 × 10⁴ cells in each well of Caco-2 cell culture plates were cultured overnight. After synchronization, using overnight serum deprivation, cells were treated with different concentrations of cell lysates or supernatant. Treated cells were collected using cell scraper and washed with cold 1 × PBS solution. The resulting cell pellet was dissolved in 1 ml of cell lysis buffer per each million cells. After the complete cell lysis, using successive periods of freezing and melting, the solution was incubated on ice for 15 min and then centrifuged at 14000 rpm for 20 min at 4 °C. Supernatant was removed and protein concentrations were determined using nanodrop 2000. Samples were normalized. Following concentration normalization of the samples, 5 μl of the protein solution were mixed with 85 μl of measurement buffer. Then, 10 μl of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) were added to the mixture. The resulting solution was added into a 96-well plate and incubated at 37 °C for 2 h. The solution absorbance was measured at 405 nm.

Cell migration and invasion assay
To evaluate the probiotic effects on motility and the aggressiveness of colorectal cancer cells, in vitro migration and invasion assay was performed using colorimetric migration and invasion kit (Millipore, USA), according to the manufacturer’s instructions. The optical density of the samples was measured at 560 nm. Inhibition of cell migration and invasion capacity by probiotic treatment was calculated by MTT assay.

Statistical analysis
Data analysis was carried out using the software package SPSS v.19. Statistical significance between two groups was analyzed by Student’s t test. One-way ANOVA was used to compare multiple groups.

Results
Probiotic treatment of cells leads to decreased cell proliferation The antiproliferative effect of Lactobacillus strains on cancer cells is shown in Figure 1. It has been shown that treatment of cells with two strains of Lactobacillus cell, suppressed cell proliferation in a dose-dependent manner. However, the effect of L. acidophilus strain is greater than that of L. casei at all doses.

Since the bacterial secreted substance is one of the effective factors of probiotics on the host cell, the possible effect of probiotic bacterial extracts on the inhibition of host cell proliferation was assessed in the current study. As shown in Figure 1, the extract of both strains of Lactobacillus can suppress cell proliferation. As a result of supernatant treatment, the effect of L. acidophilus strain was found to be more powerful than that of L. casei at both doses.

LABs induce cell necrosis by direct effect but not via secreted substances
As MTT results may show proliferation and death of the host cells, we assessed-necrosis induction potency of the probiotic bacteria. Supernatant and cell extract effects on the cell death by induction of necrosis are shown in Figure 2. According to this figure, none of the supernatant concentrations caused a significant increase in cell necrosis. The increased cell supernatant resulted in the increased cell necrosis; however, this increase can also be seen in increasing MRS concentration. Furthermore, treatment of cells with the bacterial extract enhanced the cell necrosis with more powerful effect than that of the supernatants.

Lactobacillus supernatant and cell extract effectively induce cell apoptosis
Since one of the most desired strategies in cancer therapy is inducing of apoptosis in tumor cells, in this study the effect of...
probiotics on tumor cell apoptosis was assessed by the measurement of caspase-3 activity. According to Figure 3, supernatants and bacterial extracts induce apoptosis in cells and this effect is dose-dependent.

Treatment of cells with probiotic materials decrease migratory ability of tumor cells

To investigate the effect of probiotics on cancer cell motility, cell migration through the nitrocellulose membranes was measured. As it has been shown in Figure 4, cells affected by probiotics (both supernatants and bacterial extracts) had less migratory ability than control cells. Interestingly, it seems that probiotics exert their inhibitory effects on the cell migration indirectly and through their secreted materials.

Treatments of cells by probiotic materials decrease invasion ability of tumor cells

For development and spread of tumor in vivo, cells must invade into neighbor tissue and degrade extracellular matrix. To assess...
Figure 3. Effects of bacterial supernatants and extracts on cell apoptosis. Probiotic materials induced apoptosis in a dose-dependent manner. However, bacterial extracts showed a more powerful effect than that of the supernatants. As revealed in cell necrosis assay, the probiotic species had no significantly different apoptosis inducing effect on CaCo-2 cells. L.A.L: L. acidophilus. lysate, L.C.L: L. casei. lysate, L.A.S: L. acidophilus. supernatant, L.C.S: L. casei. supernatant.


Figure 5. Effects of bacterial supernatants and extracts on invasive phenotype of Caco-2 cells. As stated in the text, L. acidophilus showed a more powerful effect than that of L. casei. MRS: de Man, Rogosa and Sharpe agar, L.A.S: L. acidophilus. supernatant, L.C.S: L. casei. supernatant, L.A.L: L. acidophilus. lysate, L.C.L: L. casei. lysate.
tumor invasion suppressing ability of probiotics, the ability of Caco-2 to degrade collagen matrix and passing from membrane was evaluated using an invasion assay kit (Millipore, USA), in the presence of different concentrations of probiotic materials. According to Figure 5, treatment of cells with probiotics leads to decreased cell invasion capacity. Furthermore, invasion inhibition effect of L. acidophilus is higher than that of L. casei.

Discussion

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women worldwide. This cancer is generally considered as a benign type, therefore 5-year survival rate of early-stage CRC patients is 63%. However, it is the second leading cause of cancer deaths in human populations. Most of CRC patients are diagnosed in advanced stages of the disease, particularly metastatic stage, which reduces the patient survival rate to 10%. On the other hand, all of CRC common treatments including surgery, chemotherapy and radiotherapy, considerably reduce the patient’s quality of life. Considering these points, more effective prevention (prophylaxis) strategies is required to deal with this cancer.

During the past few decades, different studies showed some of gastrointestinal tract normal flora in addition to the production of nutrient component, could have some positive health effects on their corresponding host. These microorganisms, produce different kinds of bacteriocins which regulate combination of the microorganism population of bowel and as a result decrease bacterial infections of the gut. Furthermore, these probiotics prevent toxic materials adhesion to the intestinal wall. Probiotics protect the gut against the formation of precancerous lesions by suppressing the activity of carcinogen enzymes such as azoreductase.

Lactobacillus family members like L. acidophilus, L. casei and L. delbrucki are among the most important parts of human gastrointestinal normal flora. These bacteria are commonly used in dairy products. They are considered as effective factors in enhancing the immune system of consumers. Immunomodulatory effects of Lactobacillus strains are not limited to the digestive system and affect the whole immune system. Recent studies show that Lactobacilli probiotics facilitate the treatment of colorectal cancer using 5-FlouroUracile. These findings suggest that the use of probiotics seems to be a good option in prophylaxis against gastrointestinal tract malignancies, especially colorectal cancers. The anticancer action of probiotics may be due to various mechanisms, including its anticarcinogenic and/or antiprocarcinogenic effects, immunomodulatory properties, modification of differentiation processes in tumor cells, production of short chain fatty acids, alteration of tumor gene-expression, activation of the host’s immune system, inhibition of the bacteria that convert procarcinogens to carcinogens, alteration of colonic motility and transit time, as well as reduction of intestinal pH to reduce microbial activity. Probiotic bacteria with oligosaccharides could enhance bacterial growth in the colon leading to greater quantities of short chain fatty acids such as butyrate, which has been shown to have anti-tumor effects. Animal studies have confirmed that probiotic bacteria inhibit tumor formation and proliferation. As reported by Morotomi, L. casei shirotia strain, a lactic acid bacterium, has a great cancer preven-

tion potential. Similarly, Reddy, et al. found that feeding yoghurt to Swiss mice led to 28% – 35% reduction in Ehrlich ascites tumor cells, compared to control groups fed milk. Studies on the effects of probiotics have had conflicting results on the behavior of different tumor cells. In different cells, different pathways in the regulation of cell proliferation may play a major role, therefore the effects of probiotics on various cells will be different. Varying probiotic species and genera may also have different immunological and physiological effects in different cancer states. Combination probiotics may interact and have an impact on host cell differently than single probiotic preparations. The composition of colonic bacterial micro flora appears to change with aging. It is unknown whether elderly patients should be treated with different probiotics than younger patients. In this study, it was shown that lactobacilli probiotics useful effects are not limited to the enhancement of the immune system but also, they will be effective to suppress malignant phenotypes of colorectal cancer cells.

In conclusion, regarding results achieved in this study and the low-grade nature of the Caco-2 cells, we suggest that the use of lactobacilli probiotics can serve as a promising tool to prevent the incidence of colorectal cancer. Due to positive results from in vivo and molecular studies, use of probiotics for the prevention of colon cancers has attracted much attention. Various mechanisms have been proposed. Despite all the positive findings, other researchers have also reported insignificant protective effects against the colon cancers. Because of increasing interests in this area, further research must be carried out to investigate the involved mechanisms, and to generate uncontroversial experimental evidence on the protective effects of probiotics on colon cancers.

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References

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