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Evaluation of the effect of isolated Lactobacillus from Sistani Yellow Kashk on the U87MG glioblastoma tumor cell line

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Abstract

Consuming nutritious and high-quality foods is increasingly important for health and well-being. Sistani Yellow Kashk, a traditional fermented dairy product from the Sistan region, is notable for its high content of lactobacilli and essential nutrients. These beneficial bacteria are associated with various health benefits, including preventing diarrhea, eczema, and cancer. This study investigates the effects of Lactobacillus supernatants isolated from Sistani Yellow Kashk on the growth of the U87MG Glioblastoma brain tumor cell line and determines the IC50 of the lactobacilli. Using De Man–Rogosa–Sharpe (MRS) Lactobacillus culture medium, Lactobacillus from Sistani Yellow Kashk were isolated and U87MG Glioblastoma cancer cells treated with Lactobacillus supernatants. The MTT (3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide) assay was employed to assess cell metabolic activity, and the IC50 was calculated. Additionally, cells were treated with the IC50 concentration for 48 hours, and apoptosis was analyzed using flow cytometry. A nearly two-year study demonstrated that the supernatant of *lactobacilli* derived from Sistani Yellow Kashk significantly inhibited the growth of U87MG Glioblastoma cancer cells in a dose- and time-dependent manner and induced apoptosis ($p < 0.05$).

Introduction

Historically, the process of fermentation has been utilized to preserve and increase the sensory attributes, functional features, and shelf-life of food products (1), In general, fermented foods are predominantly rich in Lactic Acid Bacteria (LAB), including *Lactobacillus*, *Streptococcus*, *Pediococcus*, and *Leuconostoc* (2). Additionally,

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Lactic Acid Bacteria possess the potential to enhance the nutritional value of food and improve lactose digestion, as well as to prevent certain cancers and regulate serum cholesterol levels. Additionally, LAB plays a role in modulating the body's immune system (3, 4).

Sistani yellow kashk is a traditional fermented dairy and cereal product that is widely consumed in the Sistan and Baluchestan province. Due to its high-calorie content, it is recommended for individuals who are overweight and can help prevent recurrent weakness. It is also beneficial for those with colds, diabetes, hyperglycemia, and constipation. Additionally, Sistani yellow kashk is rich in beneficial bacteria, such as Lactobacillus (5).

However, Lactobacillus species are primarily advantageous due to their use as probiotics in foods and supplements for the prevention and treatment of various diseases (6). In addition, *Lactobacillus* are Gram-positive, non-spore-forming, microaerophilic or anaerobic rods. Their cells frequently exhibit a chain-like arrangement and are seldom motile. These bacteria are regarded as essential components of the human gut microbiome (6, 7). Thereby maintaining and enhancing immune function, reducing bacterial translocation through the intestinal mucosa, and decreasing the incidence of inflammatory bowel diseases and irritable bowel syndrome (8, 9). Additionally, these microorganisms diminish the activity of carcinogenic fecal enzymes, such as azoreductase, thereby preventing the formation of precancerous lesions (10). Therapeutic diets are increasingly being considered to eliminate cancer tumors and improve treatment outcomes (11). Research has demonstrated the potential benefits of a probiotic diet or supplement in combating cancer in the composition of the intestinal microbiota, inhibition of cell proliferation, and stimulation of apoptosis, without presenting any harmful consequences (12). Probiotics play a crucial role in restoring gut flora balance and may potentially aid in cancer prevention. Moreover, probiotics inhibit the invasion and spread of infections that pose a cancer risk (13).

Cancer, with a noticeable increase in cases over the past few decades, has become one of the most prevalent diseases globally (14). In 2020, approximately 10 million people succumbed to cancer, and it is expected to rise to a mortality rate of 16.3 million by the year 2040 (15). Cancer, a genetically heterogeneous disease, is currently the second leading cause of death worldwide, following cardiovascular diseases (16). This disease arises from mutations in genes that regulate crucial cellular pathways, including those involved in growth, development, and apoptosis (17). One of the most common and deadly malignant brain tumors in adults is glioblastoma multiforme (GBM), which falls under the category of gliomas (18). Furthermore, malignant glioma is the cause of 2.5% of cancer deaths (19). In Iran, brain cancer ranks ninth among men and tenth among women (18, 20).

In this study, we investigated the effects of *Lactobacillus* extracted from Sistani yellow kashk on the glioblastoma tumor cell line U87MG. The cytotoxicity of this *Lactobacillus* was assessed using the 3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay across various concentrations and time intervals. Subsequently, apoptosis in these cells was evaluated using flow cytometry.

Materials and methods

Sampling

During this study, 45 samples of Sistani Yellow Kashk were randomly collected from Zabol, Sistan and Baluchestan province, Iran. Sampling was conducted using sterile containers and under strictly sterile conditions. The samples were transferred to the microbiology laboratory of Zabol University under cold conditions.

Sample Preparation and Microbial Culture

10 g of each selected Sistani Yellow Kashk was suspended in a 90 mL bacteriological peptone diluent. After 60 minutes, 5 mL of this solution was

added to 100 mL of de Man Rogosa & Sharpe (MRS) culture medium to cultivate and isolate all species of Lactobacillus. Bacterial colonies were incubated in MRS broth (Merck, Germany) in an anaerobic jar for 24 and 48 hours at 42-45 °C. The growth of bacteria was identified by measuring the optical density at 600 nm. After that, to obtain a single colony from the samples, the streak plate method was performed using a sterile inoculation needle on MRS agar and incubated for 48 hours at 37°C. Then, bacterial colonies were subjected to Gram staining, Catalase test, Oxidase test, motility assessment, and other biochemical tests. Then, 10 mL of each bacterial culture was centrifuged at 4000×g for 5 min then the supernatant was discarded, and cell pellets were gently washed with PBS buffer. Subsequently, the growth rate of the isolates was measured in a spectrophotometer (Eppendorf, Germany) at an optical density of 600 nm (21). Isolated probiotic Lactobacillus species were confirmed by Polymerase chain reaction (PCR) using species-specific primers described by Kwon et al. previously (22).

The viable cell count was calculated by pour plating of serially diluted culture on MRS agar, with three replicates. After incubation time, the number of bacteria was measured by the following equation:

$$N = \frac{\sum c}{(n1 - 0.1n2)d}$$

N: represents the total viable cell count per mL;

∑C: represents the sum of colonies counted on all the dishes retained;

n1: represents the number of dishes retained in the first dilution;

n2: represents the number of dishes retained in the second dilution;

d: represents the dilution factor corresponding to the first dilution.

Cell-free supernatant (CFS) was prepared by centrifugation of the incubated culture at 15,000 rpm for 15 minutes. Different concentrations of cell-free supernatant were prepared in this study at 25, 50, 100, 150, 200, and 250 micrograms per milliliter. The dilution process was conducted using Roswell Park Memorial Institute medium (RPMI 1640 + Glutamax). CFS was filtered through a pre-sterilized 0.22 µm nitrocellulose membrane. Small aliquots of supernatants were stored in a freezer at -80°C until use.

Cell line

The cell line used in this study was the U87MG glioblastoma brain tumor cell line, which was obtained from the Pasteur Institute (Tehran, Iran) in the form of a flask.

Cell Culture

U87MG cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640 + Glutamax) (Bio-idea, Iran) (Cat No. BI 1031), supplemented with 10% fetal bovine serum (FBS) (Gibco) (Cat No. 10270-106) and 1% Penicillin-Streptomycin (100x) (10,000 Units/ml Penicillin, 10,000 µg/mL Streptomycin) (Bio-idea, Iran) (Cat No. BI 1036). The cells were maintained in T25 flasks and incubated at 37°C in an incubator with 5% CO2 and 90% humidity (24).

Microculture Tetrazoliumtest (MTT) assay

This method is based on the reduction of MTT dye (23). The MTT Assay Kit (Cat No. BI1017), purchased from Idea Zist Company, was used for this purpose. For each treatment, 11 concentrations and one untreated sample were analyzed, totaling 12 columns. Three wells were used for each treatment condition, and all experiments were performed in triplicate. Three 96-well plates were utilized for assessments at 24 hours, 48 hours, and 72 hours. The procedure commenced when a T25 flask reached over 80% confluency, and all stages of cell passage were carried out to obtain the cell pellet. Next, the number of cells is counted using a Neubauer chamber (hemocytometer). After incubating the cancer cells with Lactobacillus extract for 24, 48, and 72 hours at 37°C with 5% CO2, the plates were stained with a 0.5 mg/mL MTT solution. Following 4 hours of incubation at 37°C, the supernatant was removed, and 50

microliters of DMSO solution was added to each well. After 10 minutes, the absorbance was measured at 570 nm using an ELISA reader (Organon-Teknika, Netherlands) (24).

Apoptosis Assessment by Flow Cytometry

To determine the percentage of apoptotic cells in a drug-treated cell population and compare it with the negative control, cells were stained with two dyes: Annexin V-FITC and Propidium Iodide (PI). In this study, we used the Annexin-V-FLOUS Staining Kit (Roche) (Cat No: 11858777001). Subsequently, the samples were analyzed using a flow cytometer (25).

Statistical Analysis

Statistical analysis was conducted using SPSS software version 18, while Microsoft Excel 2013 was used for generating some charts. One-way analysis of variance (ANOVA) or independent sample t-test was used to determine the statistical significance and P value <0.05 was considered as significant.

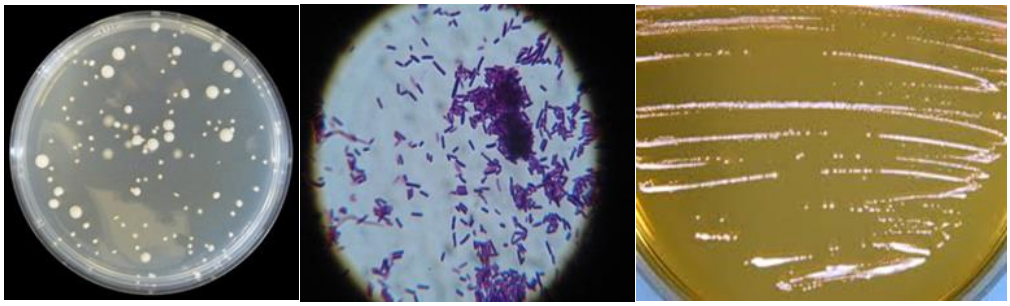

Fig. 1. The growth of *Lactobacillus* in MRS. All rod-shaped isolates were able to grow at pH levels between 4 and 6 but were unable to grow at a pH below 4.

Fig. 2. *Lactobacillus* Microscopic image

Results

Microbial culture results

The growth of Lactobacillus in MRS medium, a specific medium for Lactobacillus, was satisfactory. One of the conditions confirming that the colonies grown where exclusively Lactobacillus was the increase in acidity, achieved by adjusting the culture medium to a pH of 5.7. The genus Lactobacillus, due to its wide distribution in nature, exhibited varied results across different tests. (Figures 1 and 2).

Isolating Lactobacillus results

Identification of the Isolating Lactobacillus was done with the mentioned tests and the results are as described in the following (Table 1).

Table 1. Biochemical Identification Results of *Lactobacillus*
(A, B, C, D, and E represent distinct colonies identified and isolated from Sistani Yellow Kashk).

Biochemical Test Results	Results				
	A	B	C	D	E
Oxidase	Negative	Negative	Negative	Negative	Negative
Gram Test	Positive	Positive	Positive	Positive	Positive
Catalase	Negative	Negative	Negative	Negative	Negative

Cell culture results

The investigations conducted on the cell cultures showed that the growth rate was normal parameters and there were no problems in the process of cancerous cell culture. Furthermore, the contamination, cell size, color of the MRS culture, and growth rate were meticulously assessed.

MTT assay results

Treatment of the U87MG glioblastoma cancer cell line with various concentrations (25, 50, 100, 150, 200, and 250 µg/mL) of supernatants derived from Lactobacillus, using the MTT assay over 24 hours, resulted in a significant reduction in cell viability.

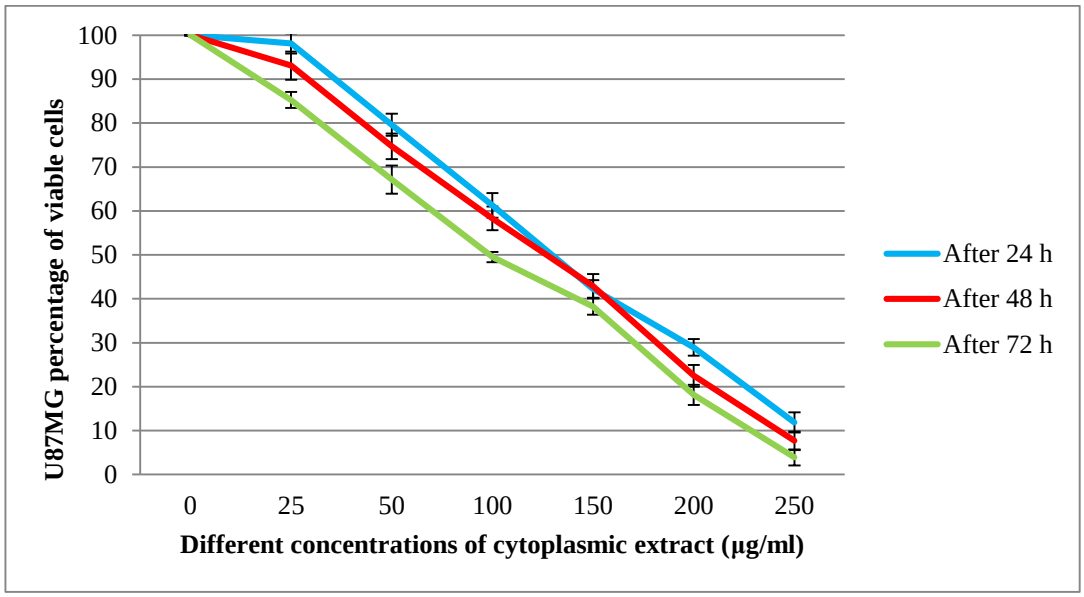

Fig. 3. The viability of U87MG brain tumor cells after treatment with Lactobacillus supernatants isolated from Sistani Yellow Kashk for 24, 48, and 72 hours.

The study indicates that the supernatants derived from Lactobacillus isolated from Sistani Yellow Kashk exhibit cell-killing activity compared to the control group, which did not receive any Lactobacillus supernatants. These findings suggest that the cell-killing activity is dose-dependent, with higher concentrations of Lactobacillus supernatants correlating with increased cancer cell death (Figure

3). The IC50 values of the Lactobacillus supernatants on U87MG cells were as follows: 138.946 µg/mL at 24 hours, 129.587 µg/mL at 48 hours, and 115.446 µg/mL at 72 hours. Additionally, the rate of apoptosis in cancer cells was examined using flow cytometry at an IC50 value of 123.587 µg/mL.

Apoptosis results

The apoptosis results in this study show the relationship between food and the U87MG Glioblastoma Tumor Cell Line and that Sistani Yellow Kashk is capable of apoptosis and cancer treatment.

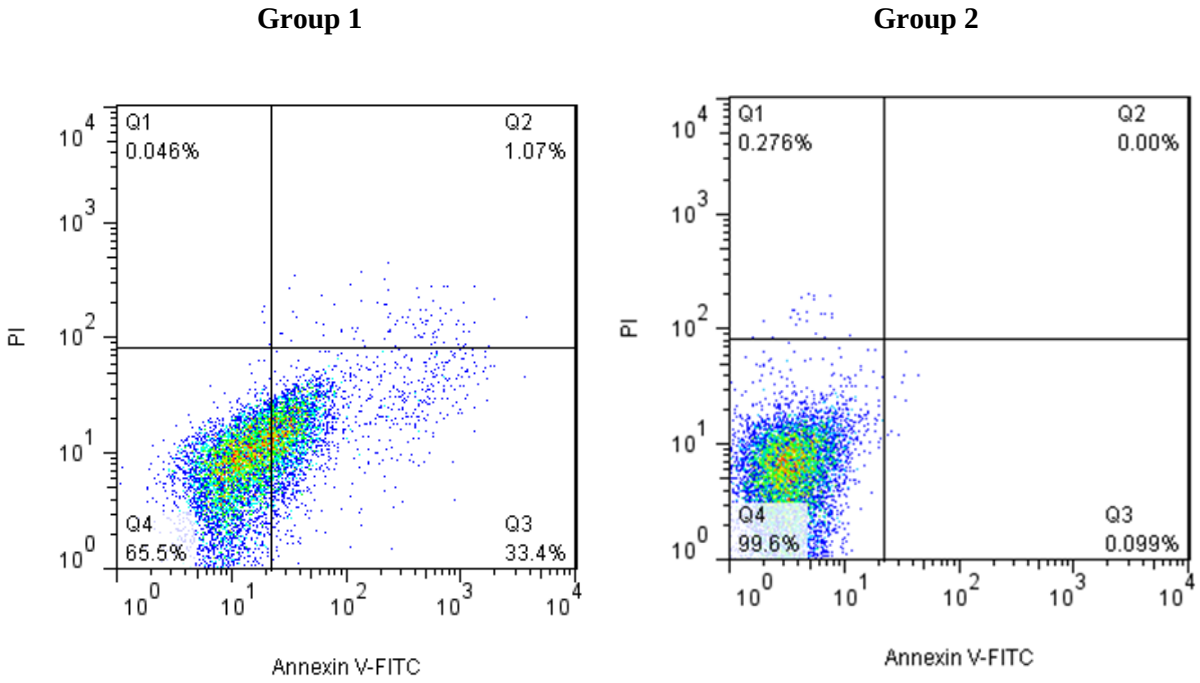

Fig. 4. Effect of Lactobacillus supernatants from Sistani Yellow Kashk on Apoptosis in U87MG Glioblastoma Multiforme Cells. Group 1 (Control): Untreated Cells. Group 2: Cells Treated with 129.587 µg/mL of Lactobacillus supernatants from Sistani Yellow Kashk.

In this study, we show that supernatants derived from Lactobacillus cultures can suppress the growth of U87MG brain tumor cell lines. Our finding showed that the supernatant of lactobacilli derived from Sistani Yellow Kashk significantly inhibited the growth of U87MG Glioblastoma cancer cells in a dose- and time-dependent manner and induced apoptosis (Figure 4) ($p < 0.05$).

Discussion

The LAB such as *L. Acidophilus*, *L. Casei*, and *L. Delborki* are commonly found in fermentation products and probiotics (26). The influence of lactobacillus as modulators of the immune response extends beyond their site of administration, namely the digestive system and mesenteric lymphatic system. In fact, these agents can affect the immune response throughout the entire body (9). These are key components of the Microbiota in the intestines of both humans and animals (27). In this regard, the relationship between dietary components and human health has garnered significant attention from scientists (28).

Diet constitutes a crucial element of microbiota manipulation. Evidence suggests that modifications to dietary patterns can swiftly transform the composition of gut microbial communities (29). Meanwhile, nutritional intervention also contributes significantly to the management of brain tumors (30). Additionally, the regulatory and stimulatory effects of these bacteria on the human immune system have been well established in previous research including the prevention of metastasis and inhibition of tumor growth and cancer treatment (31, 32).

In this context, a study investigated the cellular extracts of kefir microorganisms, utilized as a probiotic agent, exhibit elevated levels of toxicity effects on glioblastoma cancer (33). Another study demonstrated the anti-mutagenic and anti-cancer effects of *Lactobacillus* isolated from tarhana using the MTT method. The anti-cancer efficacy of *Lactobacillus* ranged from a maximum of 60.38% to a minimum of 39.37% (34). The present study confirmed the anti-cancer effects of *Lactobacillus*, consistent with the findings of the aforementioned research.

In another study conducted that *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus bulgaricus* reduced the viability of HT-29 and Caco-2 cells. Research has shown that various bacterial components, including the cell wall, peptidoglycan, cytoplasm, and even heat-killed whole bacteria, have preventive effects against cancer cell lines (35).

In this research, the effects of Lactobacillus supernatant on cancer cells were evaluated, focusing on the rates of necrosis and apoptosis, and the results were satisfactory. Anti-proliferative activity and apoptosis induction in U87MG brain tumor cell lines depend on the time and dose of Lactobacillus supernatant. Induction of apoptosis was observed after 24, 48, and 72 hours compared to the control group, but the amount of apoptosis induction at 48 hours was more remarkable than at 72 hours. Future investigations should focus on studying the potential mechanisms that induce apoptosis.

Conclusion

Given the cytotoxic effects of *Lactobacillus*, the results obtained have been promising. *Lactobacillus* have potential efficacy in cancer treatment, and incorporating them into foods may further enhance therapeutic outcomes. Recent studies highlight the unique properties of this bacterial family, suggesting that, with further research, they could play a significant role in the treatment of currently incurable diseases.

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Ethical approval

Not applicable.

Conflict of Interest statement

The authors declare that they have no conflict of interest.

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