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ORIGINAL ARTICLE

In vitro Cytotoxicity Effect of *Lactobacillus casei* on Kyse-30 Human Esophageal Cancer Cells

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KEYWORDS

Probiotic;
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ABSTRACT: One of the top causes of cancer-related deaths globally is esophageal cancer. In investigations of cell toxicity, the MTT test is one of the most often used cell viability/cytotoxicity assays for cellular metabolic activity. Nowadays, *lactobacilli* with probiotic effectiveness are now acknowledged as a prophylactic agent against cancer. The anti-tumor product of these bacteria have been designated in numerous studies. This investigation examined the probiotic *Lactobacillus casei's in vivo* impact on esophageal cancer. The MTT technique was used in this work to evaluate the cytotoxicity of *L. casei* (supernatant and full cell culture) to 5fu on the cancer cell line Kyse30. *L. casei* was able to decrease cell survival in supernatant and full cell culture (Kyse30). The possible impact of *L. casei*, particularly their supernatant, on esophageal cancer was initially evaluated in this research. As a result, *lactobacilli* species show promise for future research and development as cancer treatments.

INTRODUCTION

The eighth most prevalent cancer in the world is esophageal cancer (EC), a severe tumor of the esophagus tissue [1, 2]. The esophagus, a delicate tube that links the mouth to the stomach, is where this cancer first develops. Esophageal cancer has two primary histological subgroups. a) Squamous cell carcinoma (ESCC), which starts in the squamous cells lining the esophagus, is one example. b) Esophageal adenocarcinoma (EAC); this form develops in the glandular tissue at the base of the esophagus [3, 4]. More than 90% of esophageal cancer cases globally are reported by ESCC [5].

Probiotic microorganisms and the products they produce are important today. They become "Good Guys" via various ways. By influencing digestive enzymes, preventing carcinogens in the body and in laboratory settings, suppressing mutations, cancer-inducing agents, tumors, and the synthesis of good substances, probiotics are useful in the fight against cancer [6, 7]. These characteristics may be used in innovative therapies as an alternative to more harsh forms of care like chemotherapy or radiation [8, 9]. The most well-known genus of *lactic acid* bacteria having a probiotic potential is *Lactobacillus* [10, 11]. Numerous research have shown the anti-

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inflammatory and anti-tumor benefits of this bacteria [12]. This helpful bacteria controls the growth and death of cancer cells [9]. Peptidoglycans, exopolysaccharide extracts, whole-cell components, and probiotic *lactobacilli* supernatants have recently been studied for their considerable impact in the prevention, suppression, and treatment of several malignancies [13]. According to certain research, these bacteria have anti-proliferative properties against cancer in whole cell cultures, heat-killed cells, and supernatants, and their long-term usage may increase the immune system's ability to control the growth of cancer cells [14]. By focusing on the cancer-causing signal molecules, probiotic *lactobacilli* may be beneficial in both the prevention and therapy of cancer. Therefore, in the near future, probiotic bacteria may be effective as biological treatments [8, 15].

One of the most used cell viability/cytotoxicity tests is the MTT assay. The test has wide use as a measurement of cell metabolic activity since it is based on the ability of mitochondrial dehydrogenase enzymes in live cells. The main purpose of cell viability tests is to examine how the cells react to a substance or chemical [16, 17].

The purpose of this research was to examine the cytotoxicity of *Lactobacillus casei* (*L. casei*) whole-cell components and cell-free supernatant (CSF) on the kyse-30 cancer cell lines.

MARERIALS AND MATERIALS

Bacteria strains and culture condition

Lactobacillus casei (PTCC 1608) was obtained from the Persian Type Culture Collection and cultured in Man-Rogosa-Sharpe (Merck, Germany) broth under anaerobic conditions for 48 hours at 37°C. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were obtained from the Islamic Azad University of Damghan and grown in Muller Hinton broth for 24 hours at 37°C.

Cell-Free Supernatant (CFS) preparation

L.casei, a probiotic, was cultivated in MRS broth and kept at 37°C for 24 hours. The whole culture was centrifuged for 10 min at 10,000 rpm and 4°C to extract CFS. The supernatant was then filtered through a 0.2 µm pore size filter [18, 19].

Antimicrobials activity

We evaluated the antibacterial activity of CFS and the whole-cell culture of *L. casei* against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 as follows:

Double-Layer plaque method

The double-layer plaque test was used to assess antimicrobial activity. *L. casei* was cultured for the next day in MRS broth under anaerobic conditions at 37°C. Spotting 10 µl of probiotic *Lactobacillus* culture onto MRS agar and incubating it there for 24 hours at 37°C. Muller Hinton agar that had been melted was then applied on top of the *L. casei* spot plate and let to set. *Staphylococcus aureus* and *Escherichia coli* were each inoculated with 100 µl (0.5 McFarland) by streaking the swab over the whole surface of the agar, and plates were then incubated for 24 hours at 37°C. Each pathogen's clear zone of inhibition was assessed. As a quality check, two antibiotics, such as Cotrimoxazole (10µg) and Nitrofurantoin (10µg), were utilized [20-22].

Well Diffusion method

Probiotic *lactobacilli's* antibacterial properties were assessed using the well diffusion technique on Mueller-Hinton agar (MHA). First, the whole MHA surface was infected with a suspension of pathogen bacteria (10^8 cfu ml^{-1}). Wells with a diameter of 6 mm were then cut, filled with 50µl of CFS, and incubated for 24 hours at 37°C. The diameter of the growth inhibition zones was evaluated after the incubation time. (Experience was performed three times) [23, 24].

Cell line

The Roswell Park Memorial Institute 1640 medium, which contains 10% fetal bovine serum and 1% penicillin-streptomycin, was used to cultivate the KYSE-30 cell line, which was provided by the Pasteur Institute of Iran. The culture was carried out at 37°C in a humidified atmosphere of 95% with 5% carbon dioxide (CO_2). Using an inverted microscope, the morphology, health, and quantity of cells were examined. Trypsin was used to separate the cells from the flask's bottom after cell growth had reached a minimum of 80% [25, 26].

Preparation stock drug

2 mg of 5-FU and *L. casei* (supernatant and whole-cell culture) was individually dissolved in 100 µl of DMSO (dimethyl sulfoxide), then 50 µm of this stock was inoculated into 950 µm of the culture medium, and desired concentrations of 5-FU (0.312-320 mg ml^{-1}) and CFS and whole-cell culture of *L. casei* (2.5-320 mg ml^{-1}) were provided from the second stock.

MTT cytotoxic assay

The cytotoxicity and sensitivity of the KYSE-30 cell line to 5-FU and *L. casei* (supernatant and whole-cell culture) was determined using the MTT assay. KYSE-30 Cells (5 × 10^3/well) were plated into 96-well plates and then incubated for 24 hours. Separate additions of CFS (2.5-320 mg ml^{-1}), whole-cell culture of *L. casei* (2.5-320 mg ml^{-1}), and 5-FU (0.312-320 mg ml^{-1}) were made to the wells, and the plates were then incubated at 37°C with 5% CO_2 for 24, 48, and 72 hours. After incubation at 37°C for 4 hours, 20 µL of 5 mg ml^{-1} MTT (3-4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (DNA biotech) was added to each well. The plate was read at 570 nm after incubation, after which 100µL of DMSO was injected to each well. Measuring the cell viability allowed us to calculate the IC50 concentration, which is the concentration at which 50% of the viability of the cells is, inhibited [16, 19 and 27].

Cell viability (%) = sample OD/Control OD x 100%

Analyses were performed applying GraphPad Prism version 5 software.

Statistical analysis

The results of each experiment were performed in triplicate, and they were presented as mean ±SEM. In order to do the statistical analysis, GraphPad Prism 5.0 was used. For the experimental analysis, a one-way ANOVA with a Tukey's posttest was used. Statistical significance was defined as P values < 0.05 (∗P < 0.05; ∗∗P < 0.01; ∗∗∗P < 0.001).

RESULTS

Antimicrobial activity

Double-Layer plaque method

Producing antimicrobial substances is one of the main probiotic properties for strain selection. *L.casei* indicated high antimicrobial activity against both pathogens than selective antibiotics (Nitrofurantoin against *St.aureus* and Cotrimoxazole against *E.coli*) P < 0.05. The inhibition zone of growth of *St.aureus* and *E.coli* in the presence of *L.casei* was 37.5 mm and 34.3 mm, respectively (*P < 0.05) (Figures 1 & 2).

Well diffusion method

L.casei's supernatant exhibited inhibitory action against the two tested pathogens. In the presence of *L.casei*, *Staphylococcus aureus* and *E. coli* had inhibition zones of growth that were 15 mm and 11 mm, respectively (*P < 0.05). (Figure 3).

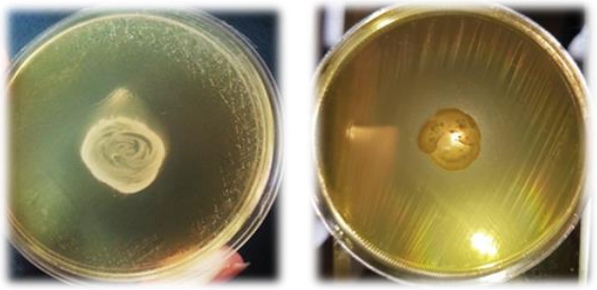

Figure 1. The inhibitory activity of *L. casei* whole-cell culture against *S. aureus* (left side) and *E.coli* (right side)

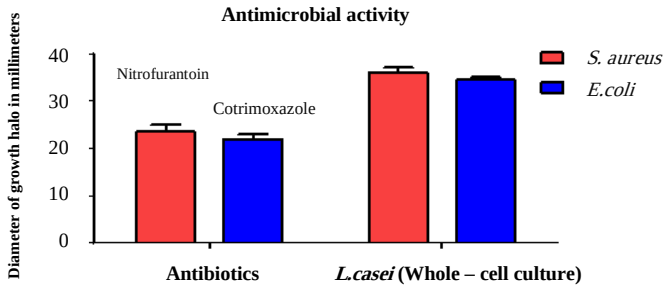

Figure 2. The inhibitory activity of *L. casei* whole-cell culture against *S. aureus* and *E.coli.*

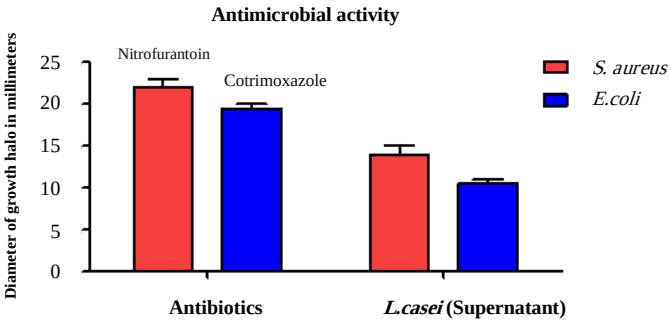

Figure 3. The inhibitory activity of *L. casei* supernatant against *S. aureus* and *E.coli*

MTT cytotoxic assay

Figure 3 summarizes the viability percentage of KYSE-30 cells exposed to different doses of 5-FU and *L. casei* (supernatant and whole-cell culture) (a, b and c). The cytotoxic activity of 5fu alone increased in KYSE-30 cells in a time-dependent way, but as the length of the treatment grew, we also saw a rise in the concentrations of *L. casei* (supernatant and whole-cell culture) bacteria and a decline in the viability of the KYSE-30 cells. The results showed that probiotic bacteria present in supernatant and whole-cell cultures greatly slowed the proliferation of KYSE-30 tumor cells in a dose- and time-dependent manner. Moreover, IC50s (drug concentration that causes 50% mortality) for

24, 48 and 72 h treatment were calculated; they were 7.8, 2.3 and 1.8 (mg ml^{-1}) for 5fu (Figure 4 (a)), 35.74, 71.61 and 126.6 (mg ml^{-1}) for supernatant (Figure 4(b)), 52.88, 107.1 and 211.4 (mg ml^{-1}) for whole-cell culture (Figure 4 (c)). ****P < 0.0001.

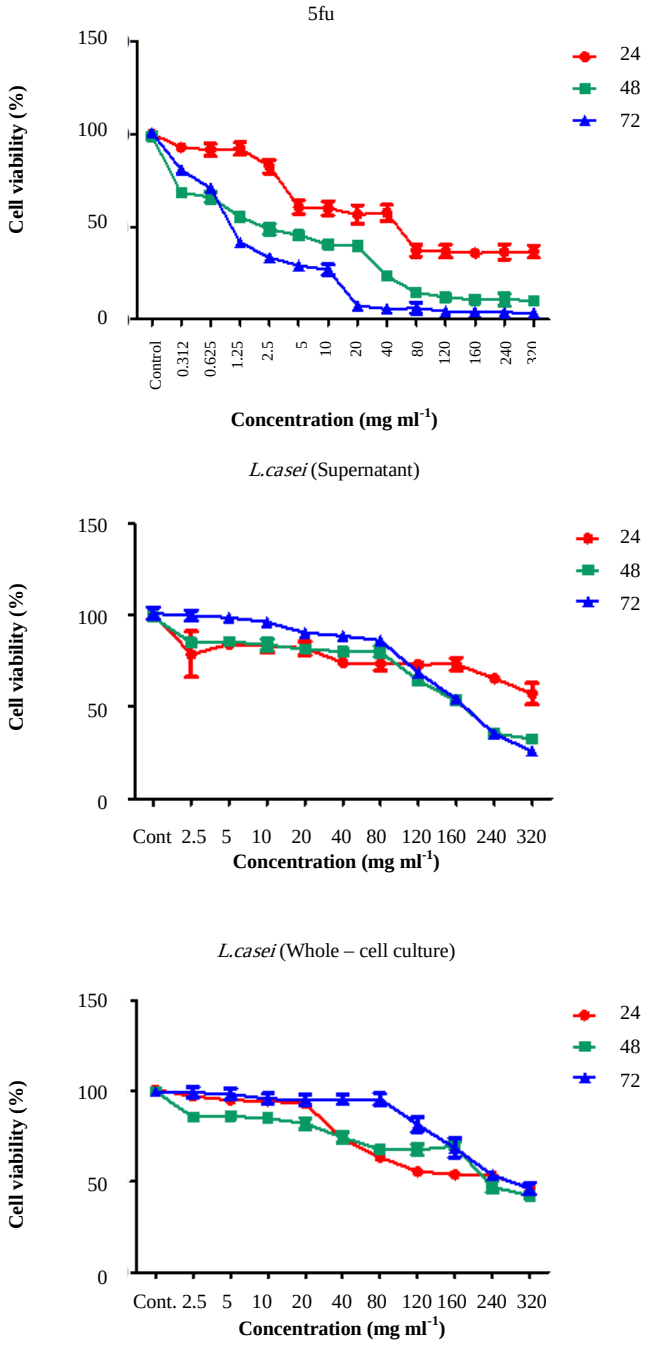

Figure 4. The viability of KYSE-30 cell line treated with 5-FU (a) and *L. casei* (supernatant and whole-cell culture) (b), (c) at 24, 48, and 72 h applying MTT assay. The results are reported as survival percentages compared to the controls in three independent experiments (n = 3; *P < 0.05, **P < 0.01, ***P < 0.001).

DISCUSSION

Esophageal cancer is the third most prevalent gastrointestinal malignancy and the sixth most common cause of cancer-related deaths globally [28]. This disease is treatable but challenging to treat due to a number of factors, including the kind and size of the tumor, its dissemination throughout the body, the patient's overall health, and etc. This malignancy can be treated with nutrition therapy, surgery, chemotherapy, and radiation therapy, but each of these methods has drawbacks [2]. Nowadays, researchers do their best to find newer drugs that target and kill cancer cells or prevent cancer cell proliferation along with other treatments.

In general, there has been an increase in interest in the pharmacological effects of natural substances on cancer prevention and therapy [29]. Due to the advantages that probiotic microorganisms provide for the human body, they are becoming more and more important. Through cytotoxic effects and the inhibition of cancer cell proliferation, they have been shown to have a wide range of anti-tumor activities in a variety of cancer cells. Examples of their products include exopolysaccharide extract, heat-killed bacteria, supernatant, and whole-cell cultures of the bacteria [30, 31]. According to laboratory research, many promising results were obtained that indicate the anticancer effect of these bacteria.

Lactobacilli are the most frequent microorganisms utilized as probiotics, and several research have shown the antibacterial and anticancer effects of these bacteria [11, 32]. In this investigation, *L. casei* inhibited the growth of harmful bacteria [33]. Numerous studies have shown that probiotic *lactobacilli* may suppress the development of pathogenic bacteria by creating antibacterial substances such as short-chain fatty acids, organic acids, hydrogen peroxide, biosurfactant, bacteriocin [34], and, etc. In two different experiments, Lee et al. and Soltani et al. found that *L. casei* showed a potent antibacterial activity against *E.coli* [35, 36]. Numerous research have shown the anti-*staphylococcal* qualities of *Lactobacillus* spp [37]. Sikroska et al. shown in that various species of *Lactobacillus* and *Bifidobacteria* may limit the development of *Staphylococcus aureus* under In-vivo and In-vitro circumstances [38]. This outcome is consistent with our results. Several studies have shown the antitumor characteristics of probiotic bacteria, including alterations in metabolic activity, the binding and destruction of carcinogenic chemicals, immunomodulation, and the inhibition of enzymes that create possible carcinogenic substances [8]. In this work, the inhibitory impact of *L. casei* CFS and whole-cell culture on esophageal cancer cells was investigated at various doses for 24, 48, and 72 hours. In keeping with our earlier investigations, our findings on the toxicity of KYSE-30 cells revealed that supernatant and whole-cell culture of *L. casei* could suppress the proliferation of cancer cells and kill them considerably ($P < 0.05$). Also, it was discovered that the proportion of surviving cells declines with increasing concentration and duration, indicating that the action of *L. casei* is concentration- and time-dependent. *L. casei* and peptidoglycans extracted from its cell wall might suppress the development of human cancer cells in vitro, according to studies by Malik et al [39]. In addition, Lee et al. found that the cytoplasmic extracts of *L. casei* and *Bifidobacterium* had a direct impact on the proliferation of cancer cell lines [30]. Consequently, based on the findings of this study and our earlier research, it seems that cytoplasmic extracts may limit the proliferation of almost all cancer cells to varying degrees, and in some instances, their impact is even larger than that of peptidoglycans. According to our findings, *L. casei* supernatants may have anticancer properties. However, further research is required to identify the mechanisms behind their cytotoxic effects on human cancer cells.

Abbreviations

CFS: Cell-Free Supernatant

ESCC: Esophageal Squamous Cell Carcinoma

DMSO: Dimethyl Sulfoxide

FBS: Fetal Bovine Serum

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ETHICAL CONSIDERATION

This study was approved by the Islamic Azad University, Damghan Branch, Ethical Committee (Approval ID: IR.IAU.DAMGHAN.REC.1398.003). (https://ethics.research.ac.ir/EthicsProposalView.php?id=60331).

Conflict of interests

The authors declare no conflict of interest.

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