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Lactobacillus Plantarum and its Derived Bacteriocin Exhibits Potent Antitumor Activity against Esophageal Cancer Cells

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ABSTRACT

Esophageal cancer presents a challenge in gastroenterology and traditional chemotherapy and radiation therapy have less therapeutic activity with severe side effects. Thus, there is need for effective and safer alternatives. Probiotics, particularly *Lactobacillus plantarum* (*L. plantarum*) and its bacteriocins, might prevent or treat esophageal tumors. We aimed to investigate the use of *L. plantarum* and its bacteriocin as esophageal cancer therapy. First, we obtained 100 isolates of Lactobacillus spp. from dairy product samples. They screened for bacteriocin production and identified by PCR and gel electrophoresis for 16S ribosomal RNA gene. Bacteriocin was partially purified and tested against two different pathogens. Both L. plantarum and its bacteriocin were examined for cytotoxicity in vitro against esophageal cancer cell line (SK-GT4) and normal rat embryo fibroblast (REF) cells by MTT assay. Apoptosis was determined using an acridine orange /propidium iodide assay. The results showed that the isolate gives a high bacteriocin production about (2000AU/ml). In addition to antimicrobial activity, there was significant anticancer activity. *L. plantarum* had an IC_{50} of 51.01 CFU/ml and bacteriocin IC_{50} of 281.9 AU/ml against cancer cells. Both showed no cytotoxicity towards normal REF cells. Furthermore, there was a significant increase in apoptosis induction and in caspase-3 activity in cancer cells treated with L. plantarum and bacteriocin compared to untreated cells. In conclusion, *L. plantarum* and its bacteriocin show potent killing effect against esophageal cancer cells with no effect against normal cells indicating safety and selectivity with activation of apoptosis via caspase-3 induction suggesting potential clinical advantage.

Keywords: *Lactobacillus plantarum*; Bacteriocin; antitumor activity; apoptosis; gastro-intestinal tumors.

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Introduction

Cancer is one of the leading causes of death worldwide, considering that tumors of the gastrointestinal tract, including esophageal carcinoma, are among the most common types of cancer in human malignant tumors (1) and are responsible for almost 15% of cancer-related deaths worldwide and esophageal cancers are considered the sixth most common type of cancer worldwide (1). Whereas gastrointestinal cancer is a complex group of disorders and heterogeneous diseases (2), and the incidence of gastrointestinal cancers is increasing rapidly (3). Together, environmental and genetic risk factors can transform normal tissue into a precancerous lesion leading to malignant cells (2). Despite advances in cancer treatment, the development of resistance to chemotherapy and radiation therapy remains a significant challenge, especially in connection with advances in the development of new diagnostic and therapeutic methods for gastrointestinal tumors with various probiotic strains that are used as dietary supplements. Therefore, the use of probiotics as a potential anticancer therapy has gained considerable attention in recent years. Many bacteria, living in various environments, produce anticancer and antimicrobial substances that are active against other bacteria (4). Antimicrobial peptides can inhibit the growth of other types of bacteria, which are produced by a wide spectrum of bacteria (5). Antimicrobial peptides can be classified into ribosomal or non-ribosomal peptides according to their biosynthetic pathways. Ribosomal synthesized antimicrobial peptides are known as bacteriocins (6).

Lactobacillus spp. is a group of probiotic bacteria that have been shown to possess potent antimicrobial properties. In addition to its direct anticancer effects, *Lactobacillus spp.* also produces bacteriocins, which are antimicrobial peptides that can inhibit the growth of pathogenic bacteria and potentially prevent the development of cancer (6). The potency of *Lactobacilli was applied* with a pure bacteriocin product to esophageal cancer, which is a type of cancer that affects the esophagus, the tube that connects the throat to the stomach. Studies have shown that *Lactobacillus* can help prevent the development of esophageal cancer by inhibiting the growth of cancer cells and reducing inflammation in the esophagus (7). Overall, the aim investigates the use of L. plantarum and its bacteriocin as an anti-esophageal cancer therapy in vitro for potential future clinical application.

Materials and methods

Bacterial Isolates

L. plantarum isolates were selected from 100 samples collected from dairy products during the period January 2022 to March 2022. Overall, isolation of *lactobacillus spp.* from prepared homemade yogurt was done by boiling cow milk for 30 min and then cooled to 40 ºC. An older yogurt was added and then the container was covered and stored at room temperature for 12 h. One gram of this homemade yogurt was added to 10ml of normal saline and serial dilution of 10^{-1} - 10^{-7} were prepared. Then, 100 µL from the last three dilutions were spread on plates of MRS agar and then incubated in an anaerobic jar at 37 ºC for 24-48 hr. After that, they were inoculated on MRS agar at pH 6 and incubated at 37 °C for 24 hours. Subsequently, they were inoculated on MRS agar at 37 °C in an anaerobic jar for 48 hours. Single colonies were then taken from the MRS agar and cultured on separate MRS agar plates under the same conditions. The bacterial

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isolates were identified using biochemical tests and cultural characteristics described in the Manual of Bergey's of Systematic Bacteriology (8).

Identification of *Lactobacillus* isolate

The catalase test

This test involved adding a few drops of 3% H2O2 to a single bacterial colony on a clean slide. Production of gaseous bubbles indicates the bacteria's ability to produce catalase enzymes, resulting in a positive outcome.

The oxidase test

A single bacterial colony was picked up and smeared on filter paper moistened with an oxidase reagent. A positive reaction is indicated by the appearance of a deep purple color within a few seconds (9).

Bacteriocin production by screening of *Lactobacillus* isolates

Lactobacillus isolates were screened to select those producing bacteriocin. Two isolates were used as indicators to detect the production of bacteriocin (*Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia* coli (E. coli). The screening method used the well diffusion technique as described by Abd and Luti (10). The zones of inhibition around the wells were used as a measure to determine the antibacterial activity of each isolate (11).

Genetic identification of *L. plantarum* by PCR

Bacterial DNA was extracted using the Geneaid "presto" (Korea) kit following the manufacturer's protocol. The purified DNA was diluted with TE buffer at a ratio of 1:100, and its concentration and purity were measured using a spectrophotometer to determine absorbance at wavelengths 260 nm. For the PCR, specific primers for the 16S ribosomal RNA gene of *L. plantarum* were used. (12).

A master mix (lyophilized) for PCR (contained optimal concentrations of reaction requirements (MgCl 2 1.5 mM, Taq polymerase 1 U, each dNTP 200 µM) was taken 12.5 µl and mixed with (3µl) bacterial, as well as 1µl from each of designed primer forward (TGGAAACAGRTGCTAATACCG) and reverse (GTCCATTGTGGAAGATTCCC), then completed volume up to 25µl with free nucleases d.dH_2O according to the company's instructions. It was identified as *Lactobacillus plantarum* and used in this study.

Bacteriocin Purification

Precipitation with ammonium sulfate

The bacteriocin was purified by precipitated using different saturation levels of ammonium sulfate (20, 30, 40, 50, 60, 70, 80, and 90%). Ammonium sulfate was slowly added to the cell-free supernatant (CSF) while gently stirring at 4 °C. After that, the precipitate was separated by centrifugation at 10000 rpm for 30 minutes. The resulting precipitates were then redissolved in an appropriate volume of 0.1M phosphate buffer at pH 7.2. The dissolved precipitates were then dialyzed separately in phosphate buffer using dialysis membrane tubes with a 1 kDa MW cutoff. The dialysis bags containing precipitates were placed in 0.5 L of phosphate buffer overnight at 4 °C, and the buffer was replaced four times. Subsequently, the antibacterial activity of the dialyzed protein was determined using the agar well diffusion assay with *P. aeruginosa* and *E. coli* as indicator strains (11, 13S).

Sephadex gel filtration

The bacteriocin obtained from the experiment was loaded onto a Sephadex G-50 gel filtration column

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(1.6 × 90 cm). Protein elution was carried out using a phosphate buffer (0.1 M, pH 7) at a flow rate of 36 ml/hour, and 3 ml fractions were collected. A total of one hundred and seventeen fractions were collected, and their absorption was measured at 280 nm. The antibacterial activity against *P. aeruginosa* and *E. coli* was tested using the well agar diffusion assay. Fractions exhibiting antimicrobial activity were mixed into a single tube, and the concentration of the protein was determined using the Bradford method, along with bacteriocin activity (11, 13S).

The evaluation of potential of antimicrobial *in vitro*

Well Diffusion Assay

Production bacteriocin in isolates was evaluated using the well diffusion assay. Universal tubes were inoculated with 2% of an overnight isolated culture, incubated for 48 hours at 37°C and following this, the tubes were centrifuged at 10000 rpm for 15 minutes to obtain the cell-free supernatant (CFS), which was then filtered under sterile conditions. To neutralize the effect of organic acid and H2O2 activity, NaOH and catalase solution were added to the CFS. Subsequently, the CFS of each isolate was tested for bacteriocin using an agar well diffusion assay. In this assay, 100 µL of an overnight growth culture of the indicator bacterium was mixed with 25 mL of sterile Muller Hinton agar (MHA) and the mixtures were poured into sterile plastic Petri dishes. Circular wells of 5mm diameter were cut and sealed with low melting-temperature MHA. 100 µL of filtered CFS was then dispersed in the wells, and the plates were incubated for 24 hours at 37 °C. The diameter of the growth inhibition zone around each well was measured to determine the bacteriocin activity."

Bacteriocin Activity Determination

Bacteriocin activity was assessed using a critical dilution assay, which is similar to the minimum inhibitory concentration technique (MIC) used for evaluating antibiotics. The method involved the preparation of a series of two-fold dilutions of *Lactobacillus* culture. This culture was then inoculated with 2% of an overnight isolate culture and incubated for 24 hours at 37°C. After incubation, the culture broth was centrifuged at 10000 revolutions per minute (rpm) for 15 minutes, and the cell-free supernatant (CFS) was collected. The CFS was then filtered using a 0.22 µm Millipore filter paper under sterile conditions. To neutralize the effect of organic acid and H2O2 activity, NaOH and catalase solution were added to neutralize the effect of organic acid and H_2O_2 activity. A series of Eppendorf tubes was prepared, each containing 500µl of sterile phosphate buffer. The first two-fold dilution involved 500 µL of *Lactobacillus* culture, followed by a second two-fold dilution. Cell-free supernatant dilutions were then assayed to detect the presence of bacteriocin using the agar-well diffusion assay. The activity of bacteriocin, measured in arbitrary units (AU), was determined using a specific equation.

$$(\mathrm{AU/ml}) = \frac{Reciprocal\ of\ the\ Highest\ Dilution}{volume\ spoted} \times 1000$$

Identification of the Anticancer Effect of *Lactobacillus plantarum* and its Bacteriocin

Preparation of *Lactobacillus plantarum* products (Bacteriocin)

Inoculated 10^8 CFU / mL *L. plantarum* in 100 milliliters of MRS broth medium, incubated for 48 hours at 37 ° C in 5% CO_2. After incubation, the growth culture was filtered using a Millipore filter with a pore size of 0.45 micrometers. And then partial purification by precipitation of ammonium sulfate at different

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saturation levels (20, 30, 40, 50, 60, 70, 80, 90) % at 4 °C, then separated by centrifugation at a speed of 10000 rpm for 30 minutes. Subsequently, the precipitates were dissolved in the appropriate volume of 0.1M phosphate buffer at pH 7 and then dialyzed in 0.5 liters of phosphate buffer overnight at 4 °C using dialysis membrane tubes (3500 kDa MW cutoff) and Sephadex gel filtration chromatography methods, the bacteriocin was loaded onto a 1.6×90 cm column of Sephadex G-50 gel filtration. Elution was carried out with a flow rate of 36 ml/hour using 0.1 M phosphate buffer at pH 7.2, with 3 mL fractions collected in each tube for measurement of absorption at 280nm. Finally, extract was stored at -20 °C until use.

Maintenance of the esophageal (SKGT4) carcinoma cell line

Cell cultures were maintained with RPMI-1640 (Elabscience, China) and DMEM medium (Elabscience, China), with 10% FBS. The cells were incubated at 37°C with 5% CO2 and were passed every 3 to 4 days. During cell subculture, the medium was discarded, and then was added 3 ml of trypsin-EDTA solution to a 75 ml flask. Flask was then returned to the incubator and incubated for 3 minutes. The cell solution was pipetted to separate clumps into single cells, and a quarter volume of the cell suspension was maintained by adding fresh medium.

MTT assay

The cell proliferation and cytotoxicity effect of both Bacteriocin at different concentrations (2000, 1000, 500, 250, 125, 62.5, 31.25, 15.62 AU/mL) and five concentrations (100, 50, 25, 12.5, and 6.25 CFU/mL) of *L. plantarum* was determined using the MTT Cell Proliferation and Cytotoxicity Assay Kit. Briefly, 10^4 cells were seeded in 96-well plates and incubated for 72 hours at 37°C to ensure adherence to the plate surface. After incubation, 100 µg/mL of each bacteriocin concentration was added to the wells, and the cells were cultured for 72 hours. Following the incubation period, the medium was removed, and 150 µL of dimethyl sulfoxide solution (DMSO) from Elabscience, China, was added to each well to dissolve the MTT solution kit (Elabscience, China). The absorbance of each well was then measured at a wavelength of 570 nm using a microplate reader as per the manufacturer's protocol.

Apoptosis Assay

Apoptosis assay to detect Caspase-3 activity in SK-GT4 cells or potential apoptosis at different concentrations of Bacteriocin was used, Caspase-3 Activity Assay Kit. Adherent cells are detached with trypsin and sedimentary cells are collected. After centrifuging the cells at 2000 rpm for 5 minutes, discard the supernatant and gently resuspend them in PBS. Centrifuge again at 2000 rpm for 5 minutes and discard the supernatant. Then, add 50 µL of cold Lysis buffer working solution to each 2×106 cells to resuspend them. Incubate in an ice bath for 30 minutes and oscillate four times during the incubation for testing. Caspase-3 Activity Assay Kit (Elabscience, China) was used to determine the potential for apoptosis, taking Ac-DEVD-pNA and 2× Reaction Buffer, dissolved and put on ice. The preparation of the reaction working solution includes 0.5 µL DTT in each 50 µL 2× Reaction Buffer. Cell lysate or tissue homogenate supernatant (containing 100-200 µg of protein) is added to 45 µL of cell lysate. The reaction was determined by specific calculations:

$$\text{activity of Caspase} - 3 = \frac{(\text{OD } Sample - \text{OD } Blank)}{(\text{OD } Negative\ control - \text{OD } Blank)}$$

Fluorescent assay by Acridine Orange (AO)/Propidium Iodide (PI)

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The selected carcinoma cell lines were treated with IC_{50} concentrations obtained from the MTT assay. The cells were stained with 1 mg/mL of acridine orange (AO) and propidium iodide (PI) and then observed using a fluorescence microscope to carry out the AO/PI fluorescent assay.

Results

Morphological characteristics of *Lactobacillus* colonies grown in MRS agar medium are Gram-positive, rod-shaped bacilli with 3-8 cells, rarely occurring, non-spore-forming, and non-motile, forming clusters in short and long chains (shown in Figure 1).

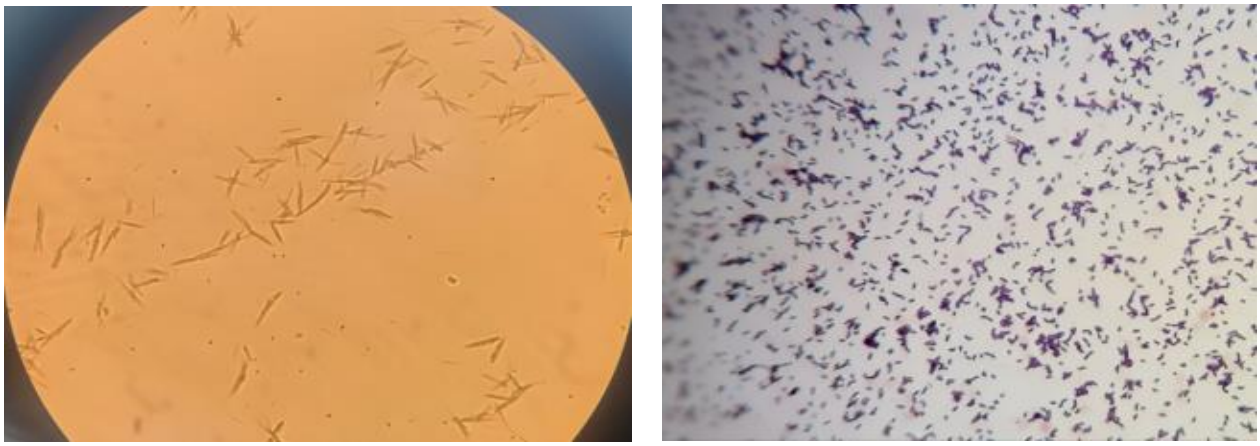

Fig. 1. *Lactobacillus spp*. cells under microscope. First image without stain and the second dyed with gram stain.

Biochemical Tests:

Conducted several biochemical tests to identify the isolate utilized in this study. The results indicated that the isolate tested negative for catalase and oxidase, meaning it is unable to produce the enzyme responsible for converting hydrogen peroxide. Additionally, evaluated the antimicrobial activity of each isolate using the well diffusion agar method, and selected the isolate with the largest inhibition zone diameter for use in this study (Figures 2 and 3).

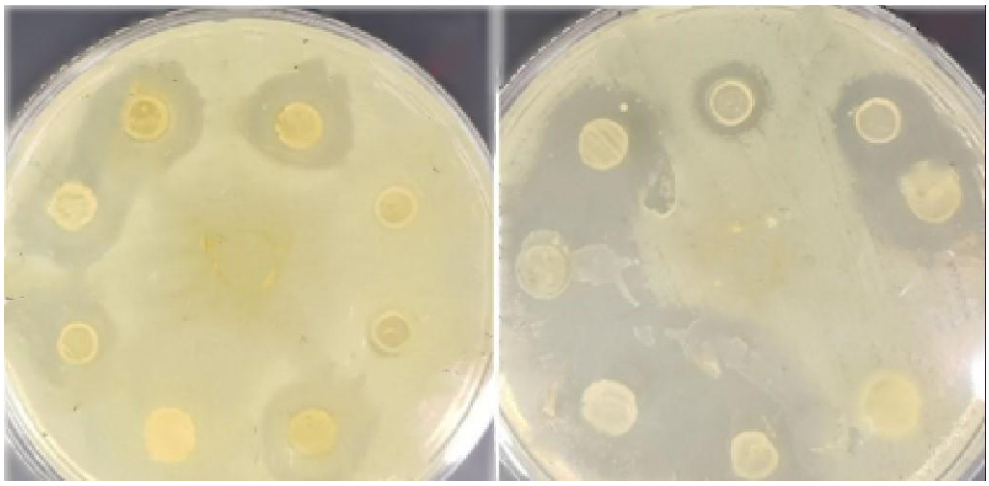

Fig. 2. Primary screening of *Lactobacillus* isolates using the well diffusion method to detect bacteriocin production against the *P. aeruginosa* and *E.coli* indicator.

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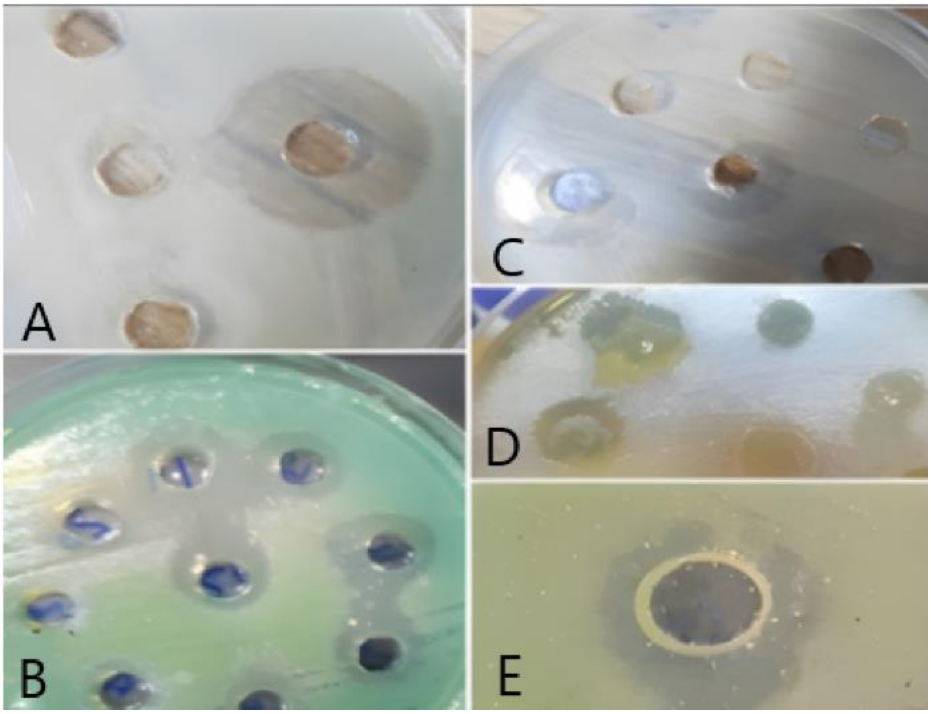

Fig. 3. Secondary screening using a well diffusion method to detect bacteriocin production against two indicators (*P. aeruginosa* and *E. coli*).

The *Lactobacillus* isolate that gave the highest bacteriocin production (2000AU/ml) was further identified by molecular detection using the 16S ribosomal RNA gene. The results from PCR amplification

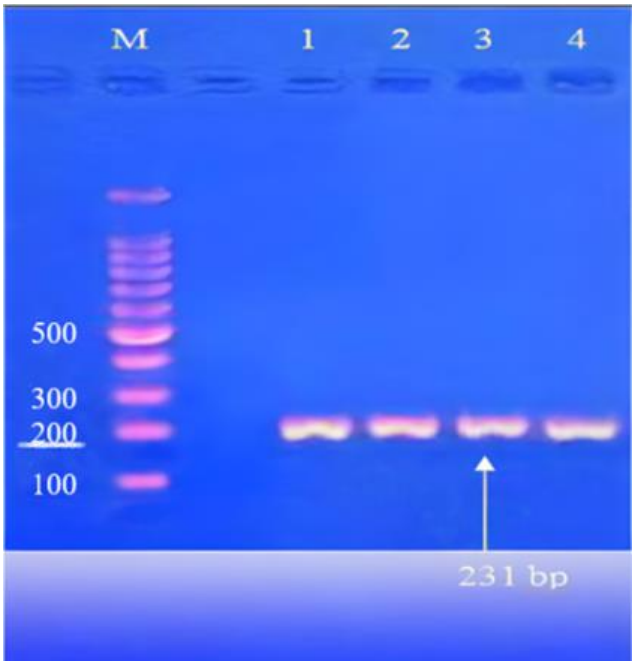

Fig. 4. Gel electrophoresis of amplification of four different isolates (1, 2, 3, 4) of the gene encoded in 16S rRNA in the *Lactobacillus* strain. Electrophoresis was performed on 1% agarose gel and run with a 5v/cm current for 1.5 h. With (1500bp) ladder (M).

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and agarose gel electrophoresis revealed a DNA fragment of approximately 231 bp in length, belonging to *L. plantarum* with 99.57% similarities (Figure 4).

Bacteriocin was partially purified through precipitation with ammonium sulfate and then subsequent separation by Sephadex G-50 gel filtration. The specific activity of the partially purified bacteriocin increased to 2000 AU/mg, as shown in Figure 5.

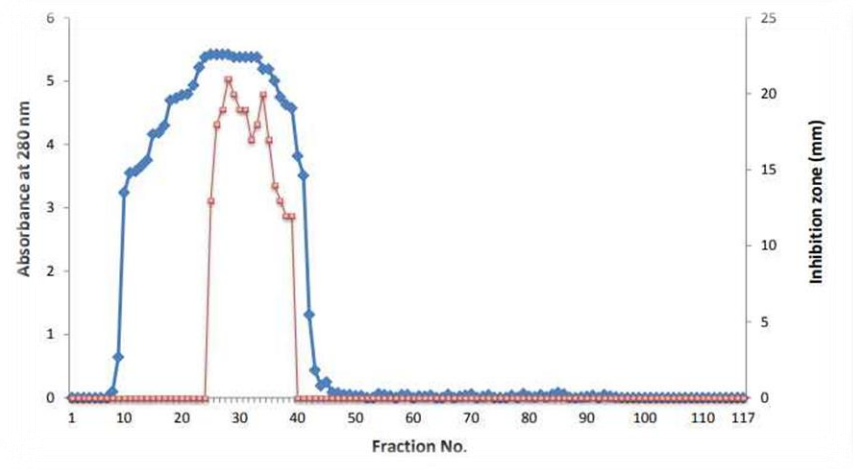

Fig. 5. Purification of bacteriocin produced by Lactobacillus spp. was carried out using a Sephadex G-50 column (1.6 × 90 cm). The column was equilibrated and eluted with sodium phosphate buffer at pH 7, with a flow rate of 0.6 ml/min.

Cytotoxic and cell proliferation effects of *L. plantarum* and its bacteriocin on SK-GT4 cells

The cytotoxicity of *L. plantarum cell proliferation* and its bacteriocin were evaluated using an MTT assay. Therefore, SK-GT4 cancer cells treated with *L.plantarum* at concentrations of 6.25, 12.5, 25, 50, and 100 CFU/mL showed moderate inhibition of cell proliferation which was observed at 72 h of incubation. Cytotoxicity inhibition rates were 96.45%, 84.49%, 71.87%, 53.43%, and 39.89% for a concentration of 100 CFU/mL at 72 h of incubation, respectively, as shown in Figure (6, and 7).

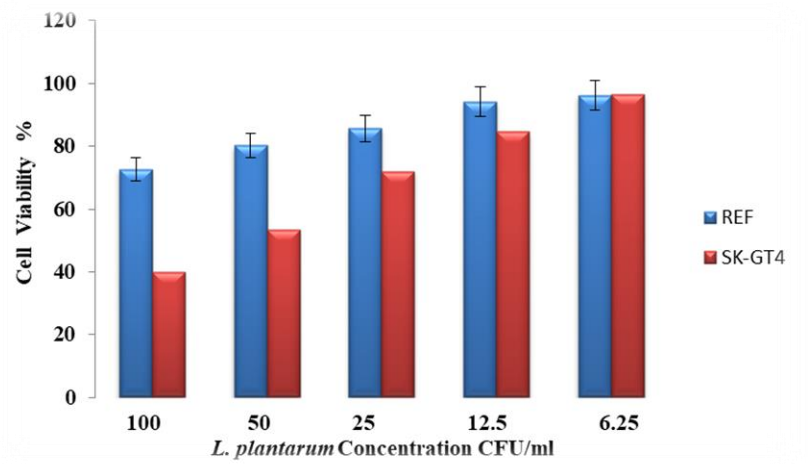

Fig. 6. MTT analysis in SK-GT4 cancer cells. The SKGT4 carcinoma cell line was treated with various indicated concentrations of bacteriocin of *L. plantarum* for time points of 72 h.

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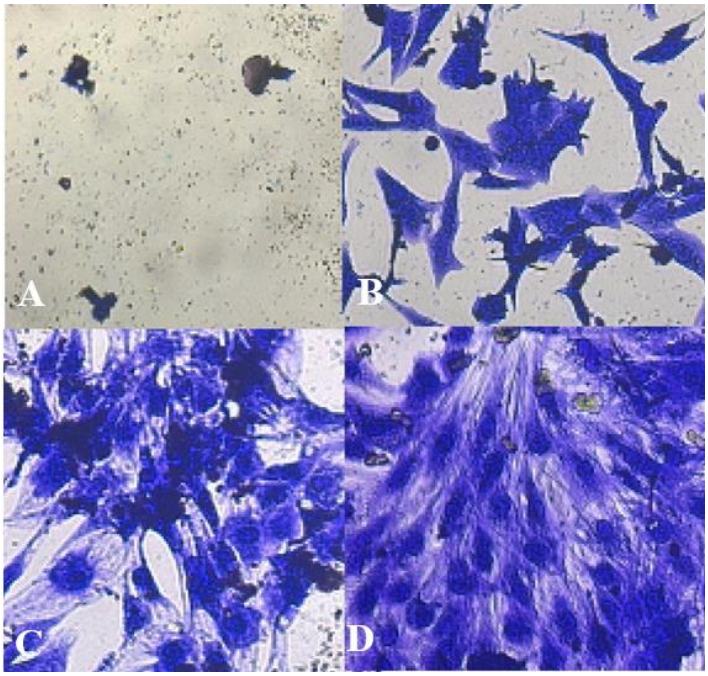

Fig. 7. SK-GT4 cells after and before treatment of bacteria (*Lactobacillus plantarum*). A) *L. plantarum* at concentration 100 CFU/ml. B) *L. plantarum* at concentration 50 CFU/ml. C) *L. plantarum* at concentration 25 CFU/ml. D) control untreated SK-GT4 cells.

While SK-GT4 cells that were treated with bacteriocin at the following concentrations of 15.62 AU/mL, 31.25 AU/mL, 62.5 AU/ mL, 125 AU/mL, and 250 AU/mL, 500 AU/mL, 1000 AU/mL and 2000 AU/mL showed high inhibition of cellular proliferation at 72 h of incubation. Furthermore, the inhibition rates of cytotoxicity were 96.06%, 93.26%, 91.46%, 83.29%, 74.80%, 66.82%, 52.81%, and 41.10% at a concentration of 2000 AU / ml at 72 h of incubation, respectively, shown in Figure (8, and 9).

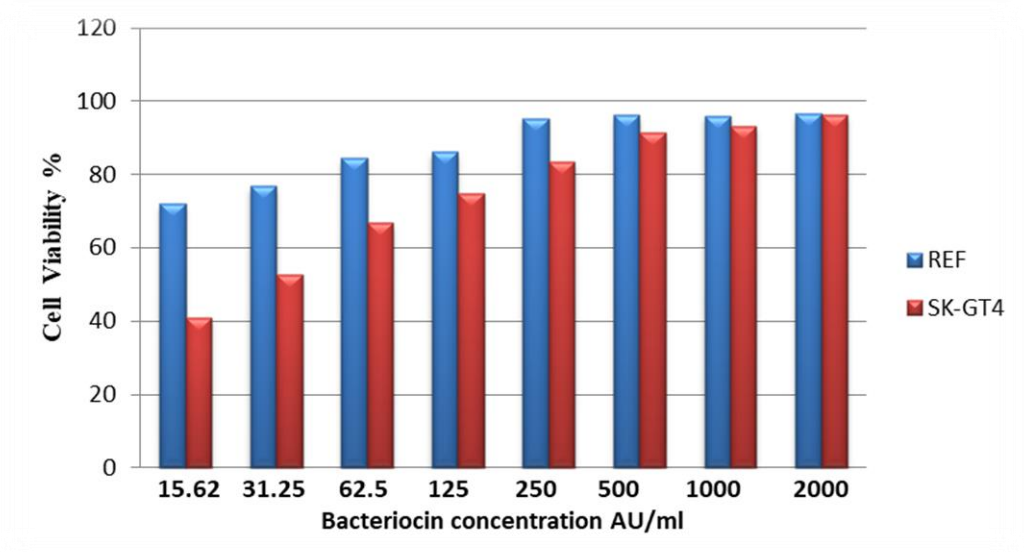

Fig. 8. MTT analysis in SK-GT4 cancer cells. SKGT4 carcinoma cell lines were treated with various indicated concentrations of bacteriocin from *L. plantarum* at time points of 72 h.

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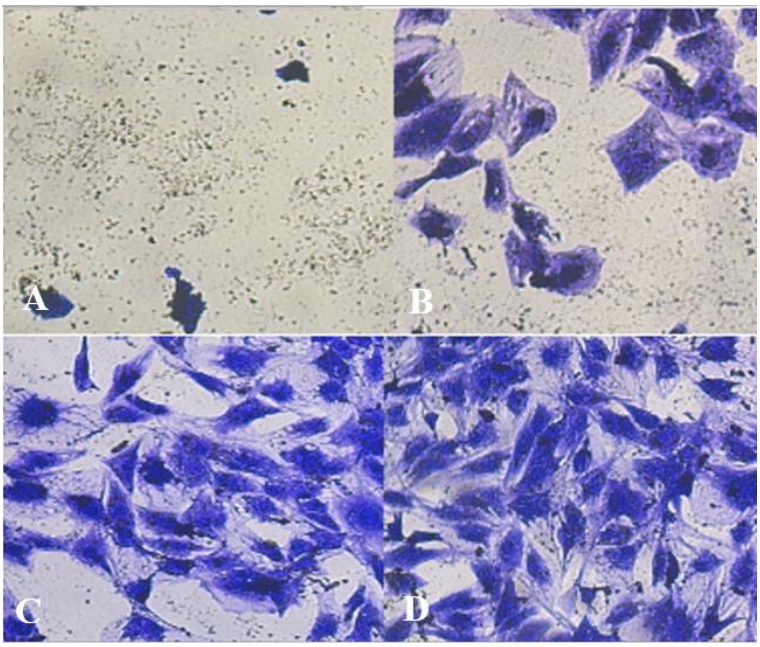

Fig. 9. SK-GT4 cells after and before treatment of bacteriocin A) Bacteriocin at concentration 2000 AU/mL. B) Bacteriocin at concentration 500 AU/mL. C) Bacteriocin at concentration 125AU/mL. D) control untreated SK-GT4 cells.

According to these results, the strongest effect of *L. plantarum* was found at a concentration of 100 CFU/ml and bacteriocin was found at a concentration of 2000 AU/ml at 72 h of incubation.

Furthermore, IC_{50} values for *L.plantarum* and bacteriocin were calculated based on the relationship between the concentrations of each cell viability, as shown in Figures 10 and 11. The calculated IC_{50} values of *L. plantarum* are equivalent to 51.01 CFU/ml, as well as the bacteriocin was equivalent to 281.9 AU/ml respectively. These values refer to the required concentration of samples (µg/mL) to inhibit 50% of cell viability. The result showed that the IC_{50} values of bacteriocin were significantly higher than those of *L. plantarum*. This confirmed that bacteriocin has better inhibition activity against SK-GT4 cancer cells than *L. plantarum*. Furthermore, we assumed that the protein compounds of bacteriocin are one of the bioactive compounds that are responsible for the inhibition properties.

Therefore, the IC_{50} values were calculated using the slope equation. The values indicated the concentration required to bring about 50% inhibition. Cytotoxicity IC50 was found to be the lowest for cells treated with *L.plantarum* (51.01 CFU/mL) and cells treated with bacteriocin indicated that the IC_{50} value of 281.9 AU / ml is the most potent inhibitor in this tested.

Caspase-3 Activity as Apoptosis-indicator for treated cells

The expression of the caspase-3 result was significantly higher in SK-GT4 carcinoma cell lines that were treated with *L. plantarum* compared to the untreated SK-GT4 control at (p= 0.1, p= 0.8, respectively) and REF normal cell lines that were treated with *L. plantarum* compared to the REF control at (p = 1.15, p = 0.9, respectively) show in Figure 12. Treatment of SK-GT4 cancer cells with *L. plantarum* resulted in a significant increase in the percentage of apoptotic cells. In the current study, we observed the activation of Caspase-3 and the occurrence of apoptosis via *L. plantarum* treatment.

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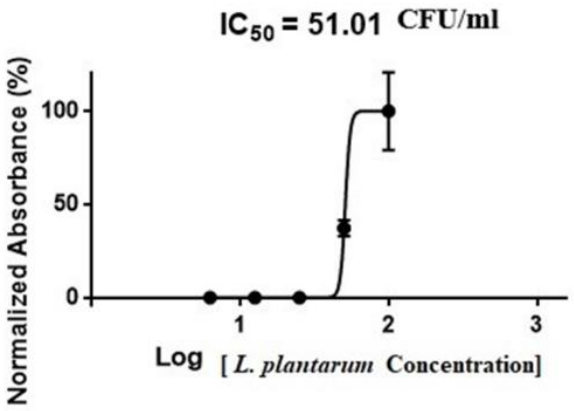

Fig. 10. The IC_{50} values and inhibitory curve in esophageal cancer cell lines with *L. plantarum*. Curves showing the correlation of Growth curve of SK-GT4 cell lines and the IC_{50} values of *L. plantarum*. Data are represented as mean ± SEM of at least three independent experiments.

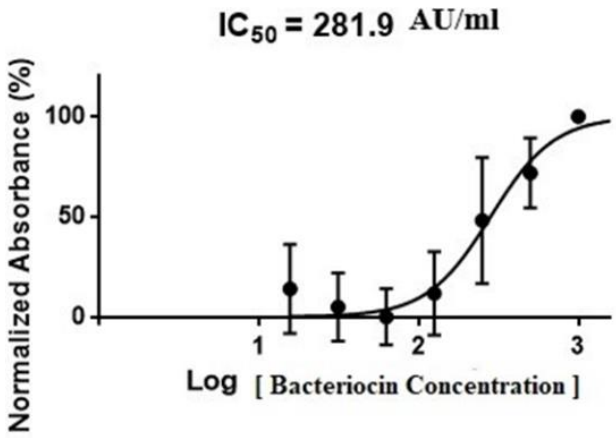

Fig. 11. The IC_{50} values and inhibitory curve in esophageal cancer cell lines with bacteriocin. Curves showing the correlation of Growth curve of SK-GT4 cell lines and the IC_{50} values of bacteriocin. Data are represented as mean ± SEM of at least three independent experiments. The IC_{50} values of *L. plantarum* and its bacteriocin in SK-GT4 cancer cells.

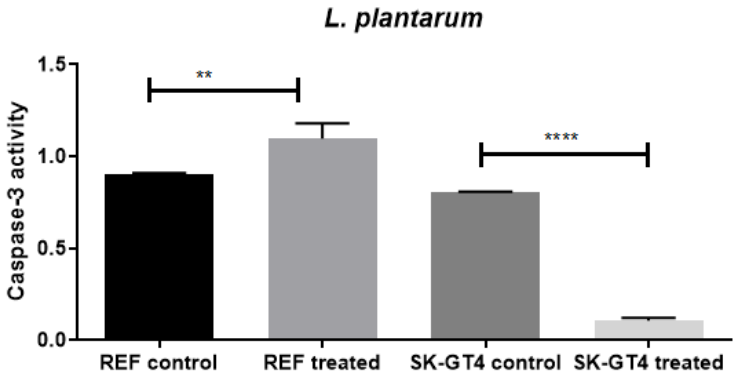

Fig. 12. The analysis of Caspase-3 activity, comparing the proportion of apoptotic cells in untreated cells to treated cells. The REF cells (normal cells) were treated with *L. plantarum*, while SK-GT4 cancer cells were also treated with *L. plantarum*. The cells underwent treatment and were then evaluated using Caspase-3 analysis.

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The expression of the caspase-3 result was significantly higher in SK-GT4 carcinoma cell lines that were treated with bacteriocin compared to the SK-GT4 control at (p = 0.25, p = 0.80, respectively) and REF normal cell lines that were treated with bacteriocin compared to the REF control at (p = 0.98, p = 0.9, respectively) as shown in Figure 13. Treatment of SK-GT4 cancer cells with bacteriocin resulted in a significant increase in the percentage of apoptotic cells. The study observed bacteriocin-induced caspase-3 activation and apoptosis, providing further evidence for the *L. plantarum* product-induced apoptotic response. These results confirm that bacteriocin kills cancer cells to a great extent without harming normal cells. Regarding *L. plantarum*, they have great effectiveness and are effective against caspase-3-specific apoptosis, as they select cancer cells and remove them via caspase-3 in apoptosis. Therefore, these results confirm that bacteriocin gave high toxicity against the esophageal carcinoma cell line, while *L.plantarum is a* high indicator of caspase-3 apoptosis.

Acridine orange and propidium iodide staining

L. plantarum-induced cell death in SK-GT4 cancer cells was quantified using acridine orange (AO) and propidium iodide (PI) staining according to standard procedures and examined under a fluorescence microscope (Leica DM IRB supplied with q-floro software) (Wetzlar, Germany).

In addition to studying morphological changes, apoptotic cells were accounted for using acridine orange (AO) and propidium iodide (PI) staining in SKGT4 cells. The cells were treated with *L. plantarum* and its bacteriocin. A fluorescent microscope was used to study the morphological changes in the bacteriocin-induced cell death, as shown in Figures 14A and 14B.

We used acridine orange (AO) and propidium iodide (PI) staining to differentiate between viable and apoptotic cells. Viable cells were identified by green fluorescence and had round, intact nuclei with intact DNA. In contrast, apoptotic cells exhibited fragmented DNA, which was stained red (Figures 14C).

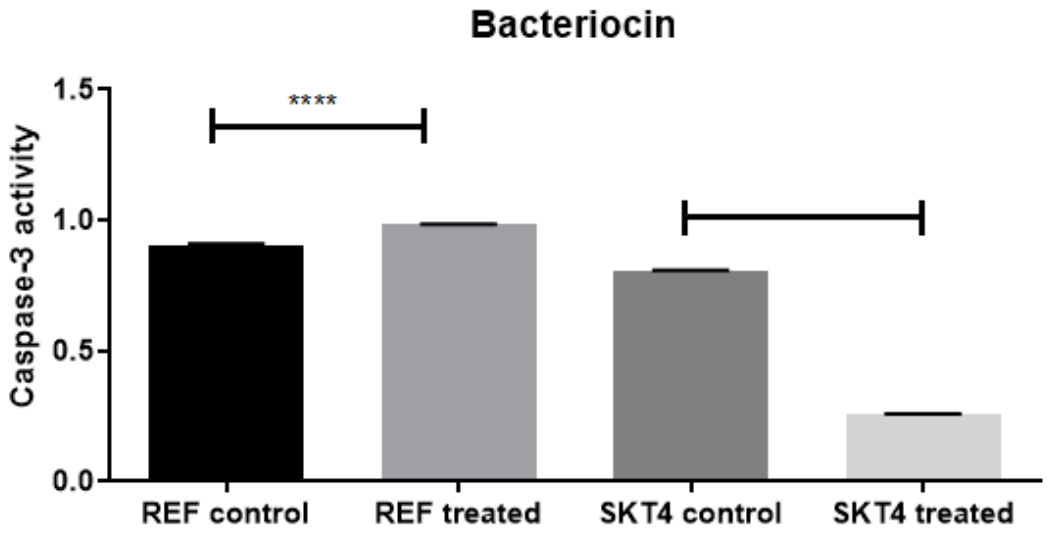

Fig. 13. The Caspase-3 analysis was conducted to determine the proportion of apoptotic cells in untreated REF (normal) cells and SK-GT4 cancer cells, both treated with bacteriocin. SK-GT4 cancer cells were treated with bacteriocin. Cells were treated and evaluated by Caspase-3 analysis.

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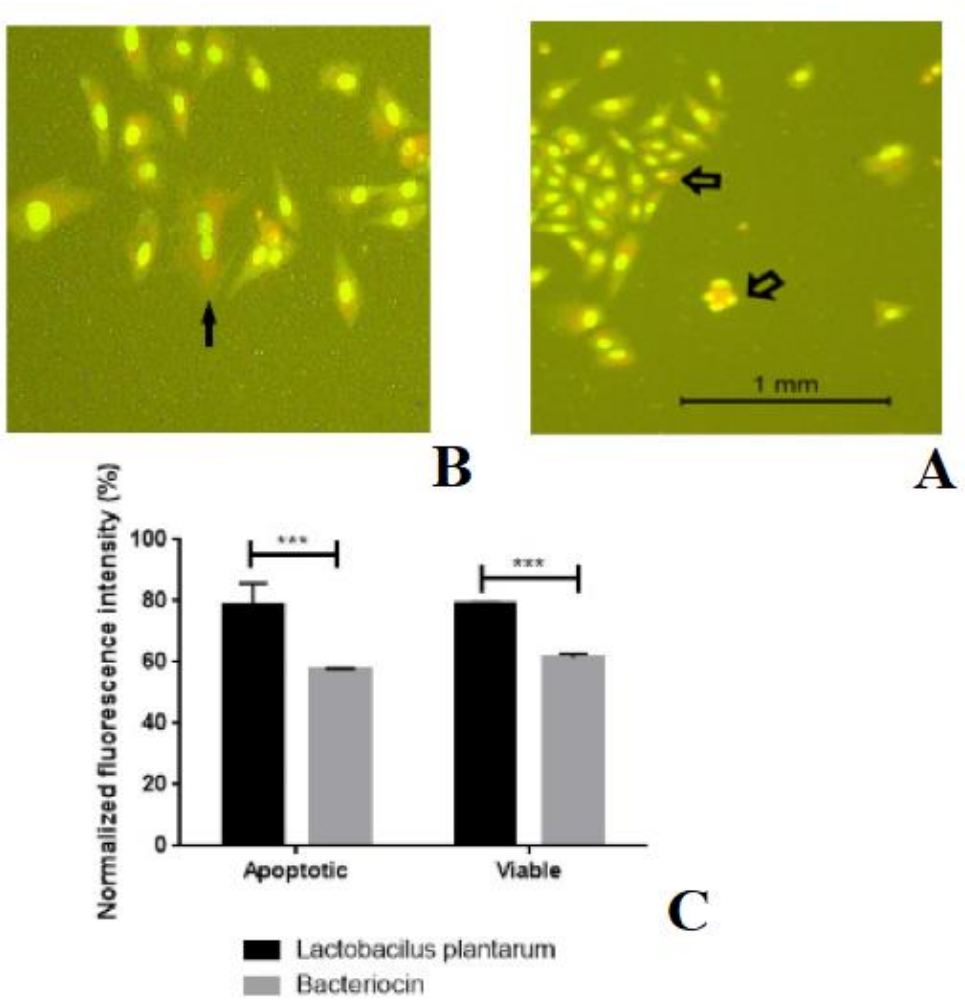

Fig. 14. **A)** Images represent experiments of apoptosis. Fluorescence microscopy examination of the SK-GT4 Carcinoma Cell Line (Magnification 400X). SK-GT4 cells treated with bacteriocin. B) Fluorescence microscopy examination of the SK-GT4 cells were treated with *L. plantarum*. C) The apoptotic cells were accounted for using Acridine Orange (AO) and Propidium Iodide (PI) staining in SK-GT4 cells where they were treated with *L. Plantarum* and bacteriocin.

Discussion

In the current study, we investigated the cytotoxic and apoptotic effects of L. plantarum and its bacteriocin on esophageal cancer cells, providing valuable insights for using them as future cancer therapies. Both, *L. plantarum* and bacteriocin found to possess anticancer activity against cancer cells as well as, low cytotoxicity to normal mammalian cells. A variety of studies have focused on the beneficial effects exerted by probiotics, and this result has been well demonstrated in the reduction of cancer cell viability and tumor size, which is in consistent with the results shown in previous work by Adiyoga and his group.

In this study, we characterized especially *L. plantarum* isolated from 100 sample of homemade yogurt, with the activities of its metabolites produced called bacteriocin for its beneficial characteristics primarily aimed at its antimicrobial and anticancer activity against esophageal carcinoma cells.

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Likewise, (11,15) showed for morphological characteristics that *Lactobacillus* colonies grown on MRS agar medium appear Gram- positive (G+ve), rod-shaped bacilli that form clustered chains containing approximately 3 to 8 cells and rarely single, also they are non-spore-forming and nonmotile and these results were consistent with those documented by previous researchers.

According to (16,17) the results indicated that the isolate has shown negative catalase test due to its inability to produce the catalase enzyme, which is responsible for the conversion of hydrogen peroxide into water and oxygen gas bubbles. Furthermore, negative results for oxidase were obtained because of isolate's inability to produce cytochrome oxidase, which is responsible for the oxidation of tetramethyl phenylenediamine. These results were consistent with our study. Furthermore, the results showed bacteriocin characterized in this study was very stable toward a wide range of temperatures, from 20°C to 100°C. Also, that similarly, with (18) *L. plantarum LPL-1* produced demonstrated wide pH stability (2–10), as well as high thermal stability (121°C, 20 min). Furthermore, according to (11, 19), molecular diagnosis was carried out using PCR analysis to confirm the diagnosis and genetic analysis has advanced the taxonomy of lactobacilli. Genotype-based identification methods have provided more accurate species identification, overcoming the problem of variable phenotype. Meanwhile, our results agree with those reported by (17,20,21) showing that L. plantarum strains can inhibit the growth of certain pathogens in both agar spot tests in primary screening, as well as in the well diffusion method in secondary screening. The bacteriocin produced by the isolate showed wide spectrum activity against two indicator isolates and was chosen for use in this study. Additionally, in researching the importance of probiotics as antimicrobials, the research also focused on the anticancer properties. For example, some probiotics as Lactobacillus strains were found to inhibit the growth of cancer cell lines such as liver, breast, gastric, bladder, and colon cancer. The MTT test showed that the isolated *L. plantarum* and its bacteriocin were cytotoxic to esophageal cancer (Sk-GT4) but did not affect normal cell lines (fibroblast REF), the results agree with those reported by (19, 22).

Meanwhile, in this study, *L.plantarum* and bacteriocin have indicated a significant inhibition of growth in the SK-GT4 carcinoma cell line at low concentrations of IC_{50} values by means of the MTT proliferation assay to determine the growth rate of cells. A linear relationship between the formazan generated and the number of viable cells was demonstrated, where this approach is based on the cells' capacity to convert yellow tetrazolium to blue formazan, and this corresponds to (19), together with time-dependent growth characteristics for the SK-GT4 carcinoma cell line where it was equivalent to 51.01CFU/ml and 281.9AU/ml for each *L.plantarum* and bacteriocin, respectively, which agrees with (23). Treatment with *L.plantarum* and bacteriocin in the SK-GT4 carcinoma cell line resulted in a significant decrease in growth rate compared to the control group. Additionally, there was an increase in the percentage of non-viable cells in the cell lines after the treatment period.

However, the MTT cytotoxic results of *L. plantarum* and bacteriocin in the SK-GT4 carcinoma cell line have been further supported by a morphological study using fluorescent microscopy. Acridine Orange/Propidium Iodide staining assay and caspase-3 were utilized. The characteristics of apoptosis were confirmed, and the percentage of apoptosis was determined under the fluorescent microscope. The calculation of apoptosis cells is described as the percentage of the total population of cells. The percentage of apoptosis cells showed that the percentage of apoptotic cells treated with *L. plantarum* and bacteriocin

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increased over time, were, measure apoptosis by changes in cells by staining them with DNA dyes (24). Apoptosis, due to a change in membrane permeability, showed an increase in uptake of the vital dye, AO/PI, compared to live cells (23, 24). Our result appears to be that the expression of the caspase-3 result was significantly higher in SK-GT4 carcinoma cell lines that were treated with *L. plantarum* compared to the control SK-GT4 at ($p = 0.1$, $p = 0.8$, respectively) and REF normal cell lines that were treated with *L. plantarum* compared to the REF control at ($p = 1.15$, $p = 0.9$, respectively). The results showed that there was a significant difference between the cancer cell line SK-GT4 treated with bacteriocin and the untreated control ($p = 0.25$, $p = 0.80$, respectively). There was no significant difference between the normal cell line REF treated with bacteriocin and the untreated control ($p= 0.98$, $p= 0.9$, respectively). Furthermore, apoptosis was assessed using Acridine orange (AO) and propidium iodide (PI) staining, which showed that in the *L. plantarum*-treated cells, 78.4% were apoptotic and 79.18% were viable. Furthermore, in esophageal cancer, the SK-GT4 cell lines shown in (Figure 11) were treated with bacteriocin where the equivalent apoptotic cell (57.3%) was treated, while the equivalent (57.3 %) while the equivalent viable cell equivalent (61.43 %). The distinct morphological features mentioned are the foundation for the most basic commonly used methods to identify and measure apoptosis. As a result, microscopy and fluorescence microscopy are still considered some of the best ways and effective ways to define apoptosis (25). These techniques are valuable for accurately estimating the amount of apoptosis, and these results agree with those reported (22, 25). Therefore, apoptosis evaluations are conducted using the caspase-3 activity assay. Caspase-3 activation was the central link and executor of cell apoptosis (26, 27).

Notably, *L. plantarum* showed the ability to produce an effective bacteriocin against some common pathogens and therefore this species can be selected as a bacteriocin producer that can be applied against some common pathogens and cancer therapy. Potential of *L. plantarum* and bacteriocin as alternative therapies that possess promising anticancer activity due to their low cytotoxicity to normal mammalian cells and specifically induced apoptosis, resulting in the breakdown of apoptosis cells for the treatment of cancers. The study showed that the *L. plantarum* and its bacteriocins had effective antibacterial activity against *E. coli* and *P. aeroginusa*. Moreover, *L.plantarum* and its bacteriocin can cause cell death in SK-GT4 cell line through direct cytotoxic and apoptotic effects as well as their low toxicity against normal cells suggesting selectivity and high safety. These findings support the future use of probiotics such as L. plantarum and its bacteriocins as an alternative cancer therapy efficacy against esophageal cancer clinically. Moreover, the study recommendations are to conduct further experimental investigations to understand the anticancer mechanism of action and the bacterial-cancer cells interaction.

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