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In vitro Cytotoxicity of Plantaricin MS9 Produced by *Lactiplantibacillus plantarum* MS, Isolated from the Mediterranean Sea, Egypt

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ABSTRACT

The present study evaluates the cytotoxic activity of the bacteriocin produced from *Lactiplantibacillus plantarum* MS, a novel marine lactic acid bacterium. The results showed that the crude bacteriocin displayed a wide antibacterial spectrum against some selected bacterial strains. The purified bacteriocin has a molecular weight of 3.5 kDa detected by SDS-PAGE and a molecular mass of 2995.6 Da quantified by LC/MS. Based on amino acid sequencing, it was concluded that plantaricin MS9 is a new class IId bacteriocin with a bacteriostatic mode of action. Biochemical characterization of plantaricin MS9 confirmed that it is thermostable over a broad range of temperatures (50- 100˚C), retained its activity at acidic and alkaline pH values, and was strongly inhibited by proteolytic enzymes. Furthermore, plantaricin MS9 exhibited strong antiproliferative activity and detrimental effects against three cancer cell lines: colon cancer (Caco-2), liver cancer (HepG2), and breast cancer (Mcf-7), with inhibition rates of 93, 95, and 91%, respectively. In conclusion, the collected data revealed that plantaricin MS9 is of potential interest for future applications in the pharmaceutical industry.

INTRODUCTION

The mid-twentieth century is considered the “golden era” of the discovery and development of antibiotics. Nevertheless, the abuse of unsuitable antibiotics resulted in the emergence of resistance mechanisms to conventional antibiotics. This phenomenon is a threat that haunts scientists, affects the entire world, and necessitates the investigation of natural and safe alternative antibiotics for human consumption **(Maryam *et al.*, 2017)**. Humankind has exploited lactic acid bacteria (LAB) for thousands of years as natural starters in the fermentation of food. Besides, LAB are characterized by hygienic safety, storage stability, and the ability to stop harmful and foodborne deterioration microbes from proliferation **(Sumathi & Reetha, 2012; El Halfawy *et al.*, 2017; Mokoena, 2017)**. Therefore, the US Food and Drug Administration (FDA) awarded the majority of the LAB

the **G**enerally **R**ecognized **A**s **S**afe (GRAS) designation **(Gueimonde *et al.*, 2006**).

Both Gram-positive and Gram-negative bacteria produce bacteriocins, a broad class of ribosomally manufactured antimicrobial peptides, where LAB are the most important producer **(De Vuyst & Leroy, 2007).** Bacteriocins are grouped into three main classes: Class **I**, lantibiotics; Class **II**, nonlantibiotics; Class **III**, large heat-labile bacteriocins **(Alvarez-Sieiro *et al.*, 2016)**. Numerous studies have been conducted on class I and II bacteriocins, some of which have gained significant industrial significance, such as nisins, pediocins, acidophilins, lactacins, and plantaricins **(Sivaraj *et al.*, 2018)**.

For decades, bacteriocins have long been used to prevent bacterial and fungal infections, control foodborne pathogens, and preserve food. However, the development of antimicrobial resistance to traditional antibiotics offers new opportunities for investigating the use of bacteriocins in a variety of medical products where it is necessary to control undesirable and potentially resistant microorganisms **(Cavera *et al.*, 2015; Chikindas *et al.*, 2018; Tiwari *et al.*, 2020)**. Bacteriocins have been reported in several experimental investigations to have the ability to combat different cancer cell lines, which makes them attractive candidates for more research and clinical trials **(Yusuf *et al.*, 2014; Sand *et al.*, 2020)**.

However, to the best of our knowledge, no research has been done on using marine LAB as a potential source of anticancer medications. Thus, the current study explores the effectiveness of a novel bacteriocin produced from the marine lactic acid bacterium, *Lactiplantibacillus plantarum*, isolated from the Alexandrian Mediterranean Sea Coast, Egypt, as a potential anticancer agent against various human cancer cell lines: colorectal adenocarcinoma cells (Caco-2 cells), liver hepatocellular carcinoma cells (HepG2 cells), and breast carcinoma cells (Mcf-7 cells). Additionally, the study investigates the best circumstances for *Lactiplantibacillus plantarum* to produce the most bacteriocin using the Plackett–Burman and Box–Behnken statistical methods.

MATERIALS AND METHODS

Sampling and isolation of marine LAB

Twenty sediment and seawater samples were aseptically collected along the Alexandrian Mediterranean Coast, Egypt, at a depth of 1–4 meters using 50mL sterile Falcon tubes and stored at 4°C. The spread plate technique was used to isolate LAB from the collected samples on de Man, Rogosa, and Sharpe (MRS) agar media (Difco, Detroit, MI, USA), and incubated for 48 hours at 30°C under anaerobic conditions produced by AnaeroPack (Mitsubishi™ AnaeroPack-

Anaero, Oxoid) **(Elkhateeb *et al*., 2022)**. For regular use, Gram-positive isolates with catalase-negative activity were stored on MRS agar slants at 4°C, or for long-term preservation, isolates were kept in 30% glycerol at -80°C.

Screening of antimicrobial activity

Preparation of crude cell-free extracts

Ten milliliters of LAB isolates were cultivated on 100mL MRS broth under anaerobic conditions for 48 hours at 30°C. The culture was centrifuged at 6,000 ×g for 15 minutes, and the cell-free supernatants were filtered through Sartorius 0.2µm pore size filters (Göttingen, Germany) and adjusted to pH 7.0 using 1N NaOH **(Rajaram *et al*., 2010)**.

Agar well diffusion method

Using agar well diffusion method, the antimicrobial activity of the crude extracts was evaluated against a wide range of bacterial strains, including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus coagulans* JCM 2257, *Enterococcus faecalis* JCM 5803, and *Latilactobacillus sakei* JCM 1157 **(Hamdan *et al.*, 2022)**.

Identification of the potent LAB isolate

Out of the encountered isolates, the potential LAB which exhibited strong antimicrobial activity, were identified using the API 50 CHL test kit (BioMérieux, Lyon, France), according to the manufacturer's instructions, and incubated for 24 and 48 hours at 30°C. The software API-WEBTM V.5.0, a computer-aided database, was used to help interpret the fermentation profiles. Additionally, the chosen isolate was sequenced using the 16S rRNA gene using the methodology outlined by **Ameen *et al.* (2020)**. The BLAST algorithm online tool was used to conduct a similarity search in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Extraction and purification of crude bacteriocin

The selected LAB isolate was inoculated in 500 milliliters of MRS broth, and incubated for 48 hours at 30ºC. Cells were harvested by centrifugation (6,000 ×g for 15 minutes, 4°C), and the supernatant was filter-sterilized and adjusted to pH 7.0. Cell-free supernatant was saturated with 70% ammonium sulfate and the mixture was continuously stirred for 24 hours at 4°C to precipitate out the proteins. Then, it was centrifuged at 8,000 ×g for 15 minutes at 4°C. Using a tubular cellulose acetate membrane (1000 Da cut-off, Sigma-Aldrich, Taufkirchen, Germany), the collected precipitate was dialyzed against the same

buffer for 12 hours at 4ºC after being resuspended in 0.1 M potassium phosphate buffer (pH 7.2) **(Rajaram *et al.*, 2010)**. The protein extract was freeze-dried and stored for additional purification.

The lyophilized crude protein was purified by RP-HPLC (Agilent 1200 Series Technologies Inc., USA) using a C18 reverse-phase column (BioBasic-18; Thermo Scientific, Rockford, IL, USA), in accordance with **Mahrous *et al.* (2013)**. The column was prewashed with acetonitrile containing 0.1% trifluoroacetic acid (TFA) and conditioned with Milli-Q water containing 0.1% TFA. The protein was initially eluted from the column using a Milli-Q water/acetonitrile gradient containing 0.1% TFA at a flow rate of 1.0 ml min^{-1} in the following order: 10%–20% (v/v) for 0–10 minutes; 20%–100% (v/v) for 10–65 minutes; and 100% (v/v) acetonitrile for 65–70 minutes.

Eluted fractions were screened for antibacterial activity by the spot-on-lawn assay, according to **Oliveira *et al.* (2017)**. Briefly, double-layered Muller-Hinton agar plates inoculated with 1% overnight culture of *E. coli* ATCC 25922 were spotted with 10 µL of each protein fraction and plates were incubated for 24 hours at 37ºC to assess for the development of inhibitory zones surrounding each protein spot. The antimicrobial activity was expressed as an arbitrary unit (AU/mL) using the following formula **(Hamdan *et al.*, 2016)**:

$$\text{Arbitrary Unit (AU/mL)} = \frac{\text{Zone of inhibition (mm)}}{\text{Volume of the sample loaded (µL)}} \text{ x } 1000$$

Where; AU/mL is the reciprocal of the maximum dilution at which the growth of the indicator was clearly inhibited.

Measurement of bacteriocin concentration and molecular weight

At each purification step, the Bradford method was conducted to determine the bacteriocin concentration using bovine serum albumin (BSA) as a protein standard **(Bradford, 1976)**. Furthermore, in accordance with **Ge *et al.* (2016)**, 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to measure the molecular weight of the bacteriocin. The molecular weight of the protein band was compared with the standard ladder marker (2.5–47 kDa).

Determination of the molecular mass of pure bacteriocin

Selected bacteriocin band was excised from the SDS-PAGE gel and eluted in phosphate buffered saline (PBS) using the procedure outlined by **Castellanos-Serra *et al.* (1997)**. According to **Zendo *et al.* (2006)**, Liquid Chromatography/Mass Spectrometry (LC/MS) (6420 triple Quadrupole LC/MS, Agilant Technologies Inc.) was used to detect the molecular mass of the purified bacteriocin. In brief, 10µL of the protein sample was directly injected into C18 reverse-phase column (75 µm x 150 mm). The column was eluted by acetonitrile:

formic acid (0.2%) with a 1:1 ratio (v/v), and detection was performed with a mass range from 100 to 1000 Da for 35 minutes.

Determination of the amino acid sequence of the bacteriocin

Edman degradation analysis was used to sequence the bacteriocin using an Applied Biosystems (ABI) model 477A protein sequencer with an online ABI model 120A phenylthiohydantoin amino acid analyzer **(Cornwell *et al*., 1988).** The online tool BLAST was used to compare the retrieved amino acid sequences with those in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Optimization of bacteriocin production by response surface methodology (RSM)

Plackett-Burman design (PBD)

Plackett–Burman experiment **(Plackett & Burman, 1944)** was performed to select the most significant factors required for maximum bacteriocin production. Seven different factors were examined at two levels; high level (+1) and low level (-1), including: initial pH (X_1), incubation temperature (X_2), incubation time (X_3), rate of shaking (X_4), glucose concentration (X_5), peptone concentration (X_6) and inoculum size (X_7) as shown in Table (1). The Plackett–Burman design follows the first-order model equation:

$$Y = \beta_0 + \Sigma \beta_1 X_i$$

Where "Y" is the measured response (Bacteriocin activity, mm inhibition zone), "β_0" is the model intercept, "β_1" is the linear coefficient and "X_i" is the level of independent variables **(Ameen *et al*., 2020)**.

Table 1. Independent variables for maximum bacteriocin production by the selected LAB isolate using Plackett–Burman design

Independent Variable	Codes	Experimental levels	
		-1	+1
Initial pH	X_1	5.0	9.0
Incubation Temperature (°C)	X_2	20	40
Incubation Time (hr)	X_3	24	48
Rate of shaking (rpm)	X_4	90	150
Glucose conc. (g/L)	X_5	10	30
Peptone conc. (g/L)	X_6	5	15
Inoculum size (%)	X_7	0.5	2

-1 low level, **+1** high level.

Box–Behnken design

Based on the results of PBD, three significant variables, initial pH, incubation temperature (°C), and glucose concentration (g/l) were selected for further optimization using the Box–Behnken design **(Box & Behnken, 1960).** In triplicate sets, each variable was tested in 15 experimental trials at three different levels: low (-1), middle, (0) and high (+1), as shown in Table (2). The interaction effects between bacteriocin activity and the significant independent variables were estimated using the following second-order polynomial equation **(El-Naggar *et al.*, 2018)**:

$$Y= \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1X_1 + \beta_{22} X_2X_2 + \beta_{33} X_3X_3 + \beta_{12} X_1X_2 + \beta_{23} X_2X_3 + \beta_{13} X_1X_3$$

Where "Y" is the response (Bacteriocin activity, mm), "β_0" is the regression coefficient, "β_1", "β_2" and "β_3" are the linear coefficients, "β_{11}", "β_{22}" and "β_{33}" are the quadratic coefficients, "β_{12}", "β_{23}" and "β_{13}" are the interaction coefficients and "X_1" "X_2" and "X_3" are the independent variables.

Table 2. The levels of variables selected for the Box-Behnken optimization design

Variable	Codes	-1	0	+1
Initial pH	X_1	8	9	10
Incubation temperature (°C)	X_2	30	40	50
Glucose conc. (g/L)	X_3	20	30	40

Effect of temperature, pH and proteolytic enzymes on bacteriocin stability

In this set of experiments, the sensitivity of the bacteriocin against different temperatures, pH values, and proteolytic enzymes was tested following **Hu *et al.* (2013)**, with slight modifications.

The pure bacteriocin was incubated at 50 and 100°C for 10, 20, and 30 minutes. In addition, the pH of the bacteriocin was adjusted to 4.0 and 9.0, with 1N HCl and 1N NaOH, respectively, and incubated for 1 hour at room temperature. Then, the bacteriocin was readjusted to pH 7.0. Furthermore, the effect of 0.25mg/ mL of the following proteolytic enzymes, trypsinogen (from bovine pancreas), proteinase, pepsin A (from porcine stomach mucosa), and α-chymotrypsin (from bovine pancreas type 2), on bacteriocin activity was detected. The mixture was incubated at 30°C for 3 hours. Bacteriocin without treatment was used as a control. All tests were done in triplicate. Moreover, after each treatment, the bacteriocin activity was determined by the spot-on-lawn method using *E. coli* ATCC 25922 as the indicator bacterium. Furthermore, inhibition zone diameters were recorded in mm.

Mode of action of bacteriocin using scanning electron microscopy (SEM)

In accordance with **Sharma *et al.* (2018)**, scanning electron microscopy (SEM) was used to examine the bacteriocin's antibacterial capability, being bacteriostatic or bactericidal. Briefly, 5mL of exponentially growing cells of *E. coli* ATCC 25922 was treated with 50µL of the bacteriocin and incubated at 30°C for 24hrs. Viable cell count (CFU) was recorded every 2 hours for 24 hours **(Zhu *et al.*, 2014)**. Additionally, *E. coli* ATCC 25922 cells that had not been treated with the active compound served as a negative control. Cells from treated and untreated samples were then separated by centrifugation (6,000 ×g for 15 minutes, 4°C), and the pellets were fixed for the night at 4°C using 2.5% glutaraldehyde. **Jiang *et al.* (2017)** recommended additional processing for cells, and they were examined for morphological alterations using a SEM (JEOL JSM-5400, Tokyo, Japan).

Cytotoxic activity of the bacteriocin

Using microculture tetrazolium (MTT) assay, the cytotoxic activity of the pure bacteriocin was evaluated *in vitro* against three human cancer lines: human epithelial colorectal adenocarcinoma cells (Caco-2 cells), human liver hepatocellular carcinoma cells (HepG2 cells), and human breast carcinoma cells (Mcf-7 cells) **(Hamdan *et al.*, 2022)**.

Briefly, cells were seeded at a density of 1.0×10^5 cells/well in a 96-well microplate and incubated for 24 hours at 37°C with 5% CO_2. Following treatment with varying concentrations of the compound (5– 60µg/ mL), cells were incubated for 24 hours at 37°C. After treatment, each well received 20µL of MTT solution (5mg/ mL), which was then incubated for 4 hours at 37°C. Following that, each well received 200µL of dimethyl sulfoxide (DMSO) to completely dissolve the purple MTT-formazan crystals. A microtiter plate reader (ELISA reader) was used to test the absorbance of each well at 560 nm. The following formula was used to determine the percentage of cell viability of each well:

$$\text{Cell viability (\%)} = \frac{\text{Ac - At}}{\text{Ac}} \text{ x } 100$$

Where, "Ac" is the absorbance of the untreated cells, and "At" is the absorbance of the treated cells.

RESULTS

Screening of bacteriocin-producing marine LAB strains

In the preliminary experiment, out of the 15 LAB isolates, successfully isolated from the collected marine samples, the crude cell-free extract of LAB 9 isolate demonstrated an impressively wide range of antibacterial activity against the

selected bacterial indicators (Table 3). Thus, it was selected as the potential bacteriocin-producing strain for further biochemical and molecular analyses.

Table 3. Antibacterial activity of LAB 9 isolate

Indicator bacterium	Antibacterial activity (mm)
Lactilactobacillus sakei JCM 1157	40
Staphylococcus aureus subsp. *aureus* ATCC 25923	28
Pseudomonas aeruginosa ATCC 27853	23
Enterococcus feacalis JCM 5803	40
Escherichia coli ATCC 25922	21
Bacillus coagulans JCM 2257	50

Identification of LAB 9 isolate

LAB 9 was a rod-shaped, nonspore-forming, Gram-positive bacterium. There was 99.9% similarity between the API 50 CHL sugar fermentation profile and *Lactiplantibacillus plantarum*. The 16S rRNA gene sequence analysis further verified the identity of the LAB 9 isolate, showing 99.7% sequence similarity with the *Lactiplantibacillus plantarum* BS28-19 strain (accession number HG798511.1) that was obtained from the GenBank databases. Accordingly, LAB 9 was identified as *Lactiplantibacillus plantarum* MS with an accession number of LC439411.1 in the NCBI database.

Characterization of the bacteriocin produced by *Lactiplantibacillus plantarum* MS

According to the purification protocol, proteins were firstly precipitated by ammonium sulfate followed by fractionation by RP-HPLC (Fig. 1). The eluents were collected and tested against *E. coli* ATCC 25922. One fraction corresponded to peak number 11 demonstrated considerable antibacterial activity, while other fractions exhibited no antimicrobial activity (Data not shown). Additionally, the Bradford method was used to assess the protein concentration at each purification step, and both total and specific activity were noted (Table 4). The yield of the pure bacteriocin was 16%, and its final specific activity was 33 times higher than that of the cell-free supernatant. It was estimated that the pure bacteriocin had a protein content of 0.6 mg/ml.

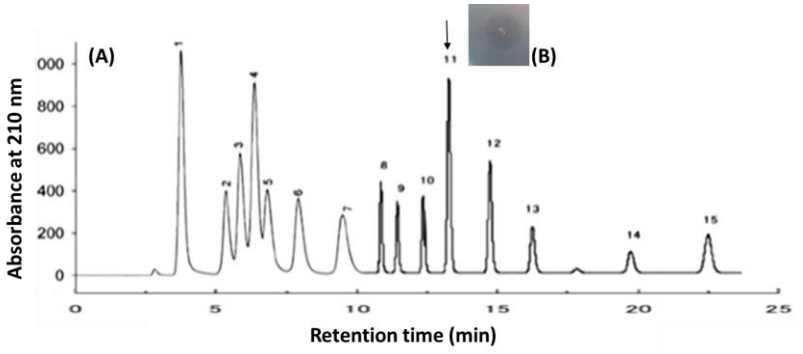

Fig. 1. (A) RP-HPLC chromatogram of the crude protein extract from *L. plantarum* MS, and (B) Inhibitory activity of fraction no. 11 against *E. coli* ATCC 25922 using spot-on-lawn assay

Table 4. Stepwise purification of bacteriocin produced by *L. plantarum* MS

Purification step	Volume (mL)	Total activity (AU)	Protein content (mg/ml)	Specific activity (AU/mg protein)	Purification fold	Yield (%)
Cell-free supernatant	1000	220000	0.62	354.8	1	100
Ammonium sulphate precipitation	20	102500	2.64	1941.28	5.47	46.5
RP-HPLC	5	35000	0.6	11666.6	33	16

Furthermore, the molecular weight of the HPLC-purified bacteriocin was detected by SDS-PAGE. As shown in Fig. (2), a single band was observed with approximately 3.5 kDa compared with the corresponding standard protein marker. The protein band was cut out from the gel, and amino acid sequences were analyzed by Edman degradation. Moreover, 30 amino acids were determined to be KQLKQLLQFLKALQTQLFKVQLFGYTLQKQ. Similarity search within the databases available in the GenBank online tool revealed that the sequenced bacteriocin produced by *Lactiplantibacillus plantarum* MS has no apparent homology with other known bacteriocins or proteins. Thus, this novel bacteriocin was designated as plantaricin MS9. LC/MS analysis showed that the molecular mass of the plantaricin MS9 was 2995.6 Da, with peaks around 30 minutes (Fig. 3).

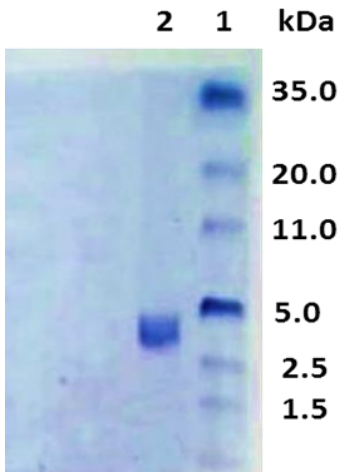

Fig. 2. SDS-PAGE of bacteriocin active fraction

lane 1; protein marker ranging from 2.5 to 47.0 kDa. *lane* 2; pure bacteriocin.

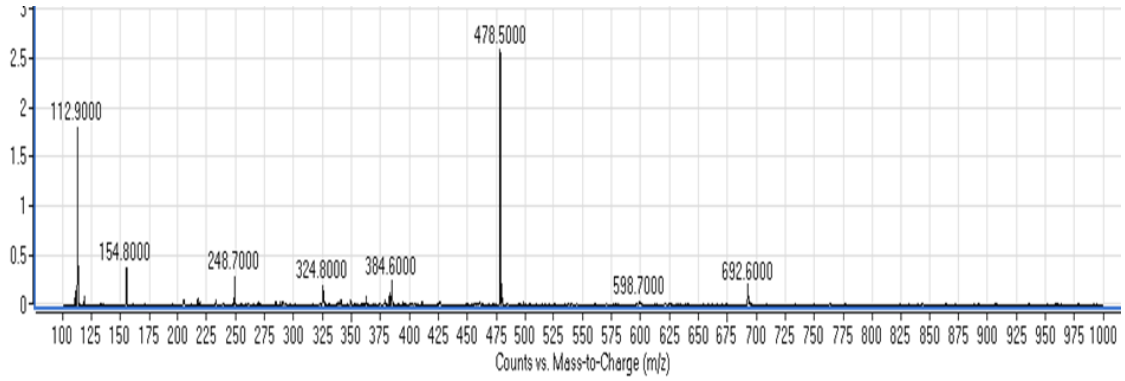

Fig. 3. LC/MS spectrum of plantaricin MS9

Optimization of the culture conditions for plantaricin MS9 production using Plackett-Burman design

Attempts have been made to investigate the medium components and culture conditions that affect bacteriocin production by *L. plantarum* MS using the Plackett-Burman statistical design (Table 5a). According to the findings, trial no. 2 had the highest bacteriocin activity (15mm). Additionally, the difference between the total of responses at the high level (+1) and at the low level (-1) of each factor was used to determine the major effect of each variable on the generation of bacteriocin (Fig. 4). According to the statistical analysis, the most important variables (P<0.05) that significantly impacted the synthesis of bacteriocin were the initial pH, incubation temperature (°C), and glucose concentration (g/l) (Table 5b). Thus, the increase in initial pH, incubation temperature, and glucose concentration could positively affect bacteriocin production, whereas other factors (incubation time, shaking rate, peptone concentration, and inoculum size) negatively influenced bacteriocin production.

Table 5a. Screening of significant factors affecting bacteriocin production by *L. plantarum* MS

Trial	Initial pH	Incubation temp. (°C)	Incubation time (hr)	Shaking rate (rpm)	Glucose conc. (g/l)	Peptone conc. (g/l)	Inoculum size (%)	Bacteriocin activity (mm)
1	+1	-1	-1	+1	-1	+1	+1	5
2	+1	+1	-1	-1	+1	-1	+1	15
3	+1	+1	+1	-1	-1	+1	-1	12
4	-1	+1	+1	+1	-1	-1	+1	10
5	+1	-1	+1	+1	+1	-1	-1	14
6	-1	+1	-1	+1	+1	+1	-1	13
7	-1	-1	+1	-1	+1	+1	+1	10
8	-1	-1	-1	-1	-1	-1	-1	12

Table 5b. Regression statistics and analysis of the experimental results of Plackett–Burman design

Variable	Coefficients	*t*-test	*P*-value
Initial pH	171.5	14	0.0432
Incubation temp. (°C)	171.5	14	0.0455
Incubation time (hr)	112.5	9	0.0704
Shaking rate (rpm)	- 87.5	-7	0.0903
Glucose conc. (g/l)	162.5	13	0.0488
Peptone conc. (g/l)	- 137.5	-11	0.0577
Inoculum size (%)	- 137.5	-11	0.0577
R^2 = 0.998		Adjusted R^2 = 0.987	

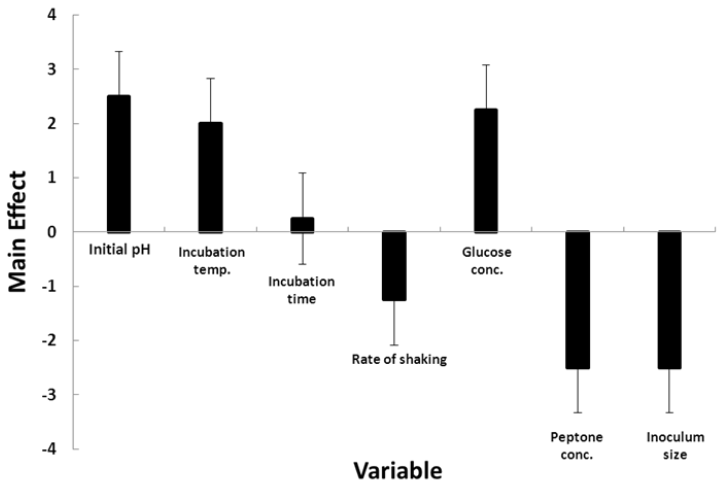

Fig. 4. Main effects of different independent variables on bacteriocin production by *L. plantarum* MS

Box-Behnken design for statistical optimization of plantaricin MS9 production

Based on Plackett–Burman design results, the three significant factors, initial pH, incubation temperature, and glucose concentration, were tested on three levels, as shown in Table (2). Moreover, the interaction between these factors on plantaricin MS9 production by *L. plantarum* MS is illustrated in Fig. (5). The relationship between the independent variables and the value of the bacteriocin activity "Y" was expressed in the following polynomial equation:

$$Y= 3.2 – 32.5\ X_1 – 73.75\ X_2 + 8.75\ X_3 + 13.75\ X_1X_1 + 46.25\ X_2X_2 + 41.25\ X_3X_3 + 10\ X_1X_2 - 5\ X_2X_3 – 12.5\ X_1X_3$$

By analyzing the results, it is conceivable that lower pH values and incubation temperature with high glucose concentration promote plantaricin MS9 production by *L. plantarum* MS. Furthermore, beyond these values the activity of plantaricin MS9 declined sharply.

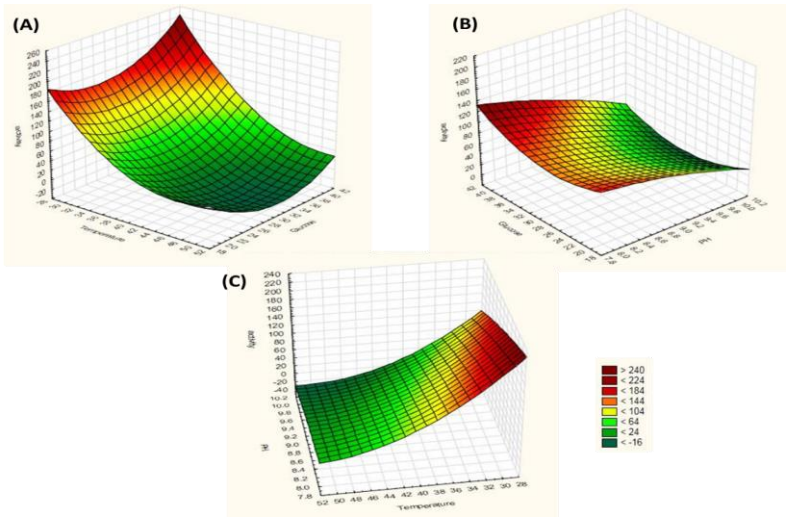

Fig. 5. 3-D surface plots showing the effect of the interaction between **(A)** Incubation temp. and glucose conc., **(B)** Glucose conc. and initial pH and **(C)** Initial pH and incubation temp., on plantaricin MS9 production by *L. plantarum* MS

Sensitivity of plantaricin MS9 to different temperature, pH levels and proteolytic enzymes

The effects of temperature, pH, and proteolytic enzymes on plantaricin MS9 activity were examined using *E. coli* ATCC 25922 as the indicator organism (Table 6). The results have revealed that plantaricin MS9 retained stability upon exposure to pH 4.0 and 9.0, with almost 90 and 60% of the bacteriocin activity remaining after the treatment, respectively. On the other hand, it was observed that almost 20 and 30% of the bacteriocin activity were lost after heat treatment at 50 and 100°C for 30min, respectively. Furthermore, the results have shown that plantaricin MS9 was completely inactivated upon treatment with trypsinogen, proteinase K, pepsin A, and α-chymotrypsin.

Table 6. Effects of pH, temperature and proteolytic enzymes on the activity of plantaricin MS9

Indicator bacterium	Antibacterial activity (mm)								
	Control	pH		Temperature		Proteolytic enzymes			
		4.0	9.0	50°C	100°C	trypsinogen	proteinase K	pepsin A	α-chymotrypsi
L. sakei JCM 1157	21	19	12	17	15	0	0	0	0

Mode of action of plantaricin MS9

In order to investigate the effect of plantaricin MS9, *E. coli* ATCC 25922 was exposed to 50µL of plantaricin MS9 and viable cell counts (CFU) of both control cells and bacteriocin-treated cells were recorded. The addition of plantaricin MS9 to a 4-hour-old culture of *E. coli* ATCC 25922 led to an immediate stop in the cell growth, which remained stable until the end of the experiment (Fig. 6). In contrast, the CFU counts of the untreated cells increased during the experimental period. Furthermore, an attempt was made to study the effect of plantaricin MS9 on *E. coli* ATCC 25922 using SEM. No morphological changes were observed in *E. coli*-treated cells compared with the untreated cells. Thus, the results suggest that plantaricin MS9 has a bacteriostatic mode of action (Data not shown).

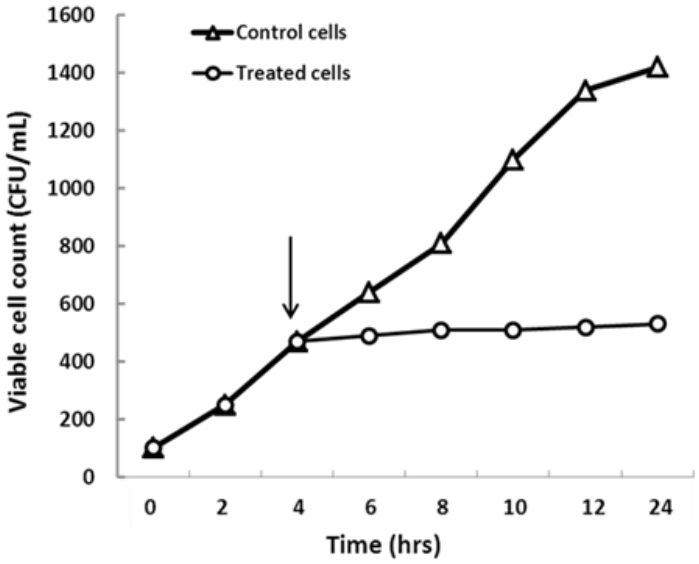

Fig. 6. Mode of action of plantaricin MS9 on *E. coli* ATCC 25922

* Arrow represents the time of addition of plantaricin MS9 to the treated cells.

Cytotoxic activity of plantaricin MS9

Using MTT assay, the cytotoxic potential of plantaricin MS9 was tested against various cancer cell lines, Caco-2, Mcf-7, and HepG2 (Fig. 7). Plantaricin MS9 exposure caused apoptosis, which resulted in damage to the cell nucleus. In addition, the results showed that plantaricin MS9 exhibited IC_{50} values of 15, 7.5, and 10µg/ mL against Caco-2, Mcf-7, and HepG2 cell lines, respectively. Furthermore, compared with untreated cancer cells, plantaricin MS9 (60 µg/mL) exhibited the strongest cytotoxic activity with approximately 95, 98, and 90% inhibition rates against Caco-2, Mcf-7, and HepG2 cell lines, respectively (Table 7).

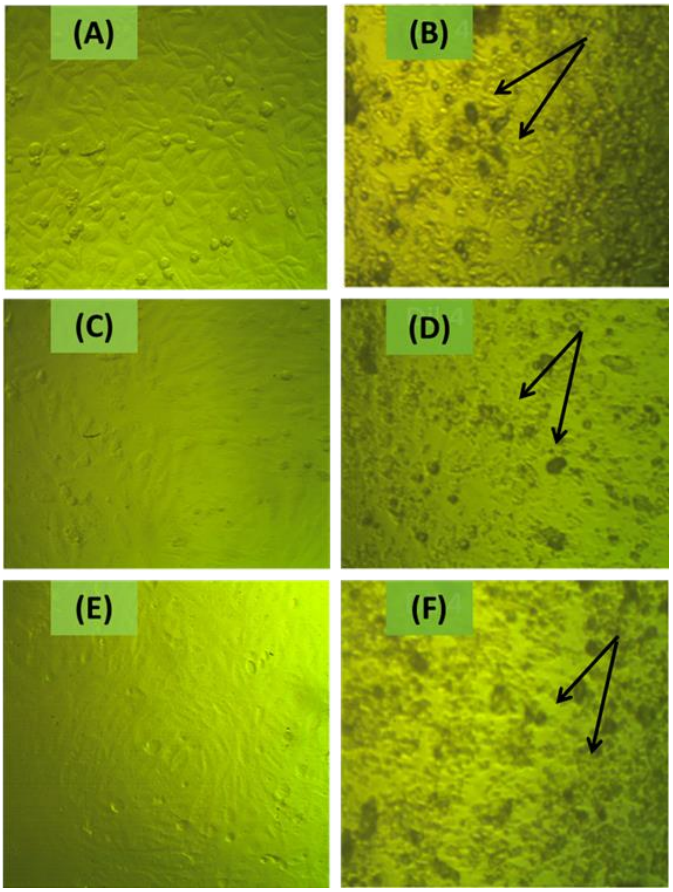

Fig. 7. Effect of plantaricin MS9 exposure on the viability of various cancer cell lines

(A) Untreated Caco-2 cells (B) Treated Caco-2 cells
(C) Untreated Mcf-7 cells (D) Treated Mcf-7 cells
(E) Untreated HepG2 cells (F) Treated HepG2 cells

❖ Arrows show the induction of apoptosis on cancer cell lines after treatment with plantaricin MS9.

Table 7. Toxicity (%) and IC_{50} of plantaricin MS9 against various human tumerous cell lines

Tumor Cell line	Toxicity (%)	IC_{50} (µg/ml)
Caco-2	95	15
Mcf-7	98	7.5
HepG2	90	10

Where; Caco-2 (colorectal adenocarcinoma cells), Mcf-7 (breast carcinoma cells), HepG2 (hepatocellular carcinoma cells) and IC_{50} (the concentration that inhibits 50% of cell growth).

DISCUSSION

An intense search for novel and efficient antimicrobial compounds has been conducted to address the issues associated with the rising proliferation of pathogenic microbes resistant to antibiotics **(Bush *et al.*, 2011)**. The potential use of LAB-producing bacteriocins as safe antibacterial agents in the food and pharmaceutical industries have drawn a lot of interest lately **(Cotter *et al.*, 2013)**. In the current investigation, the screening approach focused on the isolation of marine LAB, which successfully demonstrated an exceptionally wide range of antibacterial activity against specific bacterial markers. Therefore, *Lactiplantibacillus plantarum* MS was chosen for additional research as a potential bacteriocin-producing strain.

The bacteriocin recovered from RP-HPLC displayed a single band with a low molecular weight (<5 kDa) on SDS-PAGE electrophoresis, comparable to the molecular weight of other bacteriocins generated by strains of *Lactiplantibacillus plantarum* **(Amortegui *et al.*, 2014; Elyass *et al.*, 2017)**. According to **Bush *et al.* (2011)**, the bacteriocin of *L. plantarum* MS may be a member of class II nonlantibiotics, which contain small thermostable peptides that act on membrane structures. Furthermore, the findings indicate that *L. plantarum* MS generates plantaricin MS9, a new bacteriocin with a molecular mass of 2995.6 Da, as determined by LC/MS spectroscopy.

Several studies have documented the critical effects of nutritional factors and culture conditions, especially pH, temperature, and carbon and nitrogen concentrations, on bacteriocin production **(Mahrous *et al.*, 2013)**. The Plackett–Burman and Box–Behnken statistical design trials were used to optimize the production of plantaricin MS9 by *L. plantarum* MS. According to **Cheigh *et al.* (2002)**, the initial pH has a significant impact on the synthesis of bacteriocin because it influences the aggregation of the cells that produce it as well as the adsorption of bacteriocin to their surface. Additionally, the results of this investigation are consistent with those of **Elyass *et al.* (2017)**, who found that 30°C was the ideal temperature for *L. plantarum*, which was isolated from fermented beef, to produce the most bacteriocin. On the other hand, **Miller and McMullen (2014)** have reported the detrimental effects of high incubation temperature on bacteriocin production by *Carnobacterium maltaromaticum* UAL26, where high temperature led to conformational changes in proteins involved in bacteriocin transport and alteration of their function.

In addition, a substantial increment of bacteriocin production in the presence of 30–50 g/L glucose was also reported by **Todorov (2008)**. Conversely, **Ooi *et al.* (2015**) have noted that bacteriocin production was unexpectedly lower at 40g/ L

of glucose than at 10 or 15g/ L. Moreover, **Khay *et al*. (2013)** have reported that the reduction of the inhibitory activity might be attributed to substrate inhibition at high glucose concentration in the production medium.

On the other hand, the results have revealed that plantaricin MS9 showed stability and retained its activity upon exposure to high temperature (100°C) and acidic pH value (pH 4.0). Similarly, **Marie *et al*. (2012)** have documented that 80% of the antibacterial properties of the bacteriocin produced by *L. plantarum* Lp6SH could be detected after 30 min exposure to 121°C and a wide pH range (2.0–12.0). In addition, **Sifour *et al*. (2012)** have suggested that the heat stability of bacteriocins is considered an important criterion where many food preservation procedures involve heating.

Furthermore, complete inactivation of plantaricin MS9 was observed upon treatment with proteolytic enzymes, such as trypsinogen, proteinase K, pepsin A, and α-chymotrypsin, thus asserting its proteinaceous nature **(Zhou *et al*., 2014; Elyass *et al*., 2017**). In this respect, plantaricin MS9 shares similar thermal stability and resistance features with bacteriocins produced by various strains of *L. plantarum* at several pH ranges **(Milioni *et al*., 2015)**.

In the present study, the addition of plantaricin MS9 to a 4-hour-old culture of *E. coli* ATCC 25922 resulted in an immediate stop in viable counts. Nonetheless, cells remained metabolically active until the end of the experiment, indicating the bacteriostatic mode of action of plantaricin MS9. This phenomenon was previously reported by **Faye *et al*. (2000)**, who suggested that propionicin T1, the bacteriocin produced from *Propionibacterium thoenii*, inhibited septum formation by interfering with the biosynthesis of essential macromolecules. Moreover, **Milioni *et al*. (2015)** have detected a bacteriostatic effect of plantaricin LpU4 produced by *L. plantarum* LpU4, where CFU counts of the target microorganism, *Enterococcus faecalis* JH2-2, remained unchanged until the end of the incubation.

Furthermore, SEM micrographs showed that cells of plantaricin MS9-treated bacterium had intact surface cell membranes with no morphological changes. On the contrary, **Jiang *et al*. (2017)** have reported disruption and deformation of the cell membrane with cavities observed on the cell surface of pentocin JL-1-treated cells. Moreover, electron micrographs of bacteriocin-treated *Bacillus cereus* showed shrinkage of the cells indicating water loss from the cells **(Goh & Philip, 2015)**.

In the present study, as detected by the MTT assay, plantaricin MS9 showed detrimental effects on various cancer cell lines, Caco-2, Mcf-7, and HepG2. The lethal effect of LAB-produced bacteriocins on mammalian cancer cells has been reviewed by **Delesa (2017)**. Furthermore, the bacteriocin that was isolated from *Pediococcus acidilactici* has shown a lethal effect on human colon cancer cells, as reported by **Villarante *et al.* (2011)**. Furthermore, 50% suppression of Caco-2

cell proliferation was shown by a crude bacteriocin extract from *L. plantarum* (500µg/ mL) **(Er *et al.*, 2015)**.

In conclusion, the current investigation collectively shows that plantaricin MS9, the novel bacteriocin produced by the marine candidate *L. plantarum* MS, is a potentially effective chemotherapeutic product for tumor treatment. Further studies on the mode of action of plantaricin MS9 and the validity of applying this novel bacteriocin on nontumorigenic cells lines for future application in the pharmaceutical industry are warranted. Finally, the results indicate that the Egyptian territorial coast is a rich source of microbial-derived secondary metabolites with potential biotechnological use.

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