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Antibacterial and Cytotoxic Effect of Bacteriocin Produced by *Lactiplantibacillus plantarum*

[1]Shahlaa M. Khairullah, [2]Hutaf A.A. Alsalim

[1]Department of Biotechnology, College of Science, University of Baghdad, Baghdad – Iraq

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Abstract : *Lactobacillus* spp. was isolated from 253 children's stool samples to achieve the bacteriocin antibacterial activity and cytotoxic effect. *Lactobacillus* spp. isolates were identified by morphological and biochemical tests, then screened primarily and secondarily for bacteriocin production using pathogenic bacterial isolates gained from diarrheal patients as indicators (*Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophilia*). Molecular detection for the *16S rRNA* gene responsible for this bacterium, sequencing, and BLASTN analysis were performed, the sequences were submitted to GenBank. The bacteriocin produced under the optimum conditions was extracted and purified using ammonium sulfate, ion exchange, and gel filtration chromatography (Sephadex G-150) methods. Minimum inhibitory concentration (MIC) and MTT assay were determined to investigate bacteriocin antibacterial activity against the three pathogenic indicators and anticancer activity against colon cancer cells (Caco-2), respectively. One hundred and eleven *Lactobacillus* spp. isolates were obtained based on morphological and biochemical tests; of them, ninety-seven (87.4%) isolates exhibited antibacterial activity in primary screening; however, only eighteen of the ninety-seven isolates (18.6%) demonstrated bacteriocin production effectiveness in secondary screening. The molecular diagnosis of these18 isolates revealed that only 15 belonged to *Lactobacillus* spp. Their sequencing analysis showed that two isolates displayed 100% similarity with reference *Lactiplantibacillus plantarum*, four isolates showed 100% similarity with reference *Limosilactobacillus fermentum*, and 2 isolates exhibited 100% similarity with reference *Lacticaseibacillus rhamnosus*. *Lactiplantibacillus plantarum* (SMHA16) isolate showed the most bacteriocin production activity (160AU/mL) and higher production at pH 6.0, 32°C, and 48 hrs. of incubation. The bacteriocin extracted from this isolate was purified, and the final purification fold was 21-fold with a 48.1% yield. Bacteriocin antibacterial activity (MIC) against *Shigella* spp. was 256 µg/mL, while against *Salmonella* typhi and *Aeromonas hydrophila* was 512 µg/mL. The cancer cells (Caco2) revealed less viability at 400 µg/mL of bacteriocin concentration (IC50 value of 135.9 µg/ml), while the effect was less on the normal cells (IC50 value of 157.9 µg/ml), which indicated the anticancer activity of bacteriocin against colon cells. This work indicates the promoting role of bacteriocin as antibacterial and anticancer agent.

Keyword: *Lactiplantibacillus plantarum*, bacteriocin production, bacteriocin purification, MIC, MTT

Corresponding author :(Email:Shahlaa.khair2306@sc.uobaghdad.edu.iq)

Introduction

Bacteriocins are short-chain peptides synthesized by bacterial ribosomes, that are frequently utilized as antimicrobial agents in food and pharmaceutical applications. In addition to their antimicrobial properties, they are increasingly being considered as potential anticancer therapeutic agents (1). Bacteriocins are categorized into four primary classes based on their chemical characteristics, genetic structure, mode of action, and molecular weight. These include Class I (lantibiotics), Class II (small heat-stable

proteins), Class III (large heat-labile proteins), and Class IV (complex bacteriocins containing lipid or carbohydrate moieties (2). Foodborne pathogens, such as *Salmonella* spp. and *Shigella* spp., *E. coli*, and *Staphylococcus aureus*, are significant public health concerns, often transmitted through contaminated food or water. It was found that bacteriocins produced by *Lactobacillus* spp. show antimicrobial activity against many of these foodborne pathogens (3). Bacteriocins and other antimicrobials work synergistically to enhance antibacterial action and reduce the risk of developing resistance, indicating that bacteriocins possess potential applications in combating pathogen infections (4). Cancer is a leading cause of morbidity and mortality among non-communicable diseases. The bacteriocins show cytotoxic activity against the development of cancer cells and thus have potential anticancer properties. Recent investigations showed the selectivity of bacteriocins against cancer cells compared to normal cells (5). Colicins and pediocin PA-1 have demonstrated efficacy in inhibiting HT29 CRC cell and colorectal cancer cell line SW480 viability in vitro, respectively (6,7). *Lactobacillus plantarum* is a native bacterium of the human gut, primarily benefiting gastrointestinal health, such as reducing gas and abdominal pain in individuals with irritable bowel syndrome (IBS1) (8). *Lactiplantibacillus plantarum* is a distinct species within the genus *Lactobacillu*s, widely known for its unique functional and probiotic properties. It has shown an impressive ability to create bacteriocins that can fight off a wide range of harmful bacteria, including *Salmonella* spp. and *Shigella* spp., which are two main causes of stomach infections. It's Generally Recognized as Safe (GRAS) classification enhances its use in the food and pharmaceutical industries, in addition to its antimicrobial efficacy (4). Previous study by Liu *et al.*, reported that *L. plantarum* Q7 produced plantaricin Q7 with a bacteriocin activity of 400 AU/mL against *Listeria monocytogenes*, indicating strong inhibitory and bactericidal effects(9). One of the key mechanisms through which *Lactobacillus* spp. exert their beneficial effects is the production of specific bioactive compounds, including hydrogen peroxide, organic acids, inhibitory enzymes, and bacteriocins (10). This study aimed to estimate the inhibitory activity of the purified bacteriocins against intestinal pathogens as well as the anticancer properties for potential future clinical applications. To achieve this aim, *Lactobacillus* spp. was isolated from fecal samples, and after optimizing the better production conditions, the bacteriocins were extracted and purified by several strategies and then tested as antibacterial and anti-colon cancer cell therapies.

Materials and methods

Collection of samples

Clinical samples were collected between October 1, 2023, and April 30, 2024, from Childe Hospital in the city of Medicine, Ibn Al Baladi, and Fatima Alzahraa hospitals in Baghdad city. Two hundred fifty-three stool samples were collected from children (90 healthy and 163 diarrheal patients) with ages between 1 day and 11 years. The samples were collected in a plastic container and transferred to the laboratory for *Lactobacillus* spp. and pathogenic bacteria detection.

Isolation and identification of *Lactobacillus* spp.

The samples were mixed and cultured in test tubes containing 9ml of

normal saline to form a 10^{-1} dilution. Serial dilutions were prepared, 10^{-2} to 10^{-3}, by passing 1 ml from each test tube to the next. One milliliter of the 10^{-1} ,$10^{-2}$and 10^{-3}dilutions were cultured in test tubes with 9 ml of MRS broth and incubated for 48 hrs. at 37°C. Loopful of MRS broth culture was streaked onto MRS agar and incubated for 48 hrs. at 37°C. The suspected colonies were purified by re-culturing on MRS agar containing 1% $CaCO_3$ and incubated anaerobically at 37 ºC for 48 hrs. Bacterial isolates were identified by colony morphology, cellular microscopic properties (100X magnification with oil immersion lenses), and biochemical tests, including catalase, oxidase, blood hemolysis and indole tests, and then *16SrRNA* gene determination by molecular detection.

Isolation and identification of the pathogenic bacteria

Patients' stool samples were cultured on MacConkey agar, Xylose Lysine Deoxycholate agar, Hekaton enteric agar, and Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar, followed by overnight incubation at 37°C. The growing colonies were re-cultured on the same media, and their morphological characters were recorded, and then biochemical tests were conducted, including catalase, oxidase, indole, citrate utilization, motility, Triple Sugar Iron (TSI), and urease tests. Finally, The Vitek 2 compact system was used to confirm the identification of the pathogenic isolates.

Antibiotic sensitivity determination

The antibiotic sensitivity test was conducted for pathogenic isolates using 13 antibiotic discs: Amikacin (AK), Ampicillin (Amp), Aztreonam (AZM), Cefepime (CPM), Cefotaxime (CFM), Ceftazidime (CAZ), Ceftriaxone (CRO), Ciprofloxacin (CIP), Chloramphenicol (C), Imipenem (IMI), Meropenem (MRP), Piperacillin tazobactam (PIT), and Trimethoprim-sulfamethoxazole (COT), Kirby-Bauer method. The inhibition zone sizes were compared with those of (11).

Primary screening

The agar plug diffusion method was used to select the best bacteriocin-producing *Lactobacillus* spp. isolates by cultivating the isolates on MRS agar plates and incubating anaerobically at 37ºC for 48 hrs. Plugs from each isolate with 7 mm in diameter were formed using a sterile cork borer. These plugs were placed upside down on Muller-Hinton agar (MH) that was streaked with 100 µl of indicator culture (1×10^8 cells/ml). The plates were incubated overnight at 37ºC, and then inhibition zones around the bacterial plugs were measured to determine the antibacterial activity of each isolate (12).

Secondary screening

The active isolates in the primary screening were subjected to secondary screening using the agar well diffusion method to recognize the most bacteriocin producers by culturing in MRS broth under, anaerobic conditions at 37ºC for 48 hrs. The cultures were centrifuged at 6000 rpm for 15 minutes at 4ºC, and then the cell-free supernatant (CFS) was collected. The activated pathogenic indicators (*Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophilia*) were cultured on MH agar by spreading 0.1 ml of 10^8 cell/ml. Wells (7 mm diameter) were formed using a sterile cork borer and then filled with 100 µl of CFS. The plates were kept at room temperature for 2 hrs. and then incubated at 37°C for 18-24 hrs. The inhibition zones formed around the wells were compared with the control group, which contained MRS broth only (13).

DNA Extraction

Lactobacillus spp. genomic DNA was extracted and purified by using the ABIOpure TM kit and following its protocol. DNA concentration was measured by the Quantus Fluorometer to assess sample quality for further applications. Diluted Quant fluor dye (200 µl) was added to 1 µl of DNA, and DNA concentration values were recorded after five minutes of incubation at room temperature.

PCR amplification

16SrRNA gene-specific primer was used to confirm *Lactobacillus* spp. identification, targeting the gene for *Lactobacillus* spp. in general (Table 1). This primer amplified the region 345bp, of the *16SrRNA* gene. The reaction solution (25µl) included 12.5µl of master mix, 1.5µl of each primer (forward and reverse), 4.5µl of nuclease-free water, and 5µl of the purified DNA template. The used program is shown in Table 2. The products of PCR were analysed by 1.5% w/v agarose gel electrophoresis, formed from 1X TAE buffer with 1µl of ethidium bromide, then powered at 100 v/m Amp for 60 minutes. The UV transilluminator was used for the detection of stained bands (14). The negative control reaction included all components except the DNA template to ensure that any contaminating DNA present in the reaction would be amplified and subsequently detected on an agarose gel.

Table (1): The primer used in this study (from Macrogen® /Korea).

gene	Sequence primer 5′→3′	Size product (bp)	Reference
16SrRNA	F-GCAGTAGGGAATCTTCCA	345	(15)
	R-ATTYCACCGCTACACA		

Table (2): PCR program for amplification of *16SrRNA* gene.

Steps	Temperature	Time (m:s)	Number of cycles
Initial Denaturation	95°C	05:00	1
Denaturation	95°C	00:30	30
Annealing *16SrRNA*	55°C	00:30	
Extension	72°C	00:30	
Final extension	72°C	07:00	1
Hodi	10°C	10:00	

Gene sequencing

The PCR products of *16S rRNA* were sent to Sanger sequenced using the ABI 3730XL automated DNA sequencer by Macrogene Company in Korea. Sequencing analysis confirms the genus and species identification, provides accurate information on genetic composition, and advances our knowledge of evolutionary links across species. Consensus sequences were generated utilizing Geneious version 2023.0.7. BLASTN analysis was employed to compare the *16S rRNA* gene sequences of the 15 local isolates with a reference strain in the GenBank database, National Center Biotechnology Information (NCBI).

Determination of bacteriocin activity

To assess the bacteriocin activity for the eight local *Lactobacillus* spp. isolates, a double dilution series of the isolates' culture was prepared according to (16). The cell-free supernatant (CFS) was collected and filtered using a 0.22 µm Millipore filter. Catalase solutions

and NaOH were added to CFS to neutralize the effects of organic acid and H_2O_2 activity. Eppendorf tubes were filled with 500 µl of sterile normal saline, then 500 µl of the collected filtrate CFS was added to the first Eppendorf tube and thoroughly mixed; this represents the initial two-fold dilution followed by a second, third and fourth. Plates containing MHA were streaked with the pathogenic indicators; wells were then made, and 100 µl of each filtered dilution was added. The plates were incubated for 24 hrs. at 37°C. The bacteriocin activity was evaluated by the maximum dilution of an inhibitory zone (DF), measured in arbitrary units (AU). It was determined using a specific equation (12).

AU/ml = 1/(DF) × 1000 / (volume spotted in µl)

Optimization of optimal conditions for bacteriocin production

Several optimization experiments were performed to determine the optimal pH, incubation period, and temperature for bacteriocin production for the selected *Lactobacillus* spp. isolates based on their activity against the pathogenic indicators, *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophila*.

Determination of optimal pH

MRS broth was prepared in various pH values (4, 5, 6, 7, and 8), autoclaved, and inoculated with *Lactobacillus* spp. isolate (2%) and then incubated at 37ºC for 48 hrs. Bacteriocin production optimal pH was determined by the agar well diffusion method.

Determination of best incubation period

MRS broth was prepared with the optimum pH value that was optimized in the previous experiments and inoculated with *Lactobacillus* spp. isolate (2%) and incubated at 37ºC. Samples were incubated for 24, 48, and 72 hrs., and the agar well diffusion method was performed to determine the best incubation period.

Determination of optimal temperature of incubation

MRS broth was prepared with the optimum pH value and incubation period, then inoculated with *Lactobacillus* spp. isolate (2%) and incubated at several incubation temperatures (27, 32, 37, and 42ºC) to detect the optimum temperature that supports maximal bacteriocin production. The agar well diffusion method was performed to determine the optimal temperature for bacteriocin production.

Extraction of bacteriocin

The cell-free supernatant (CFS) was prepared according to (17) with some modifications, and as follows: 100ml of MRS broth medium were inoculated with 5 ml of *Lactobacillus* spp. broth culture and incubated under anaerobic conditions for 48 hrs. at 32°C, then centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was filtered through Millipore filters (0.22 µm in diameter), while the precipitate was discarded. The obtained cell-free supernatant was stored at 4°C until needed and used as a basic bacteriocin. Protein concentration (mg/ml) for each sample at various stages of the purification process was determined by the Bradford method. Specific activity was calculated by dividing the total bacteriocin activity (AU) by the total protein (mg). Each stage of purification had a different recovery value obtained from the difference between the total activity after purification and the total activity before purification, multiplied by 100. The level of purification of bacteriocins was obtained from the difference between the specific activity

after purification and the specific activity before purification (18).

Purification of bacteriocin

The bacteriocin was purified using a three-step method: ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography. The antimicrobial assay for each purification step was performed using *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophila* indicators.

1.Ammonium sulphate precipitation

The CFS of crude bacteriocin was transferred to the small beaker, which was placed in an ice bath on a slow magnetic stirrer. Ammonium sulfate ($(NH_4)_2 SO_4$) was added gradually with continuous mixing on ice at different saturation ratios (60%, 70%, and 80%) and then centrifuged at 6,000 rpm for 15 min at 4°C. The supernatant was discarded, and the precipitate formed at each saturation level was dissolved in a suitable amount of phosphate buffer solution. The protein activity was assayed to determine the optimal saturation ratio (19).

2.Dialysis

The ammonium sulfate precipitate at saturation ratios obtained from the previous step (60%) was re-dissolved in phosphate buffer. The dissolved precipitates were dialyzed in a dialysis tube with a 3500 Da MW cutoff against phosphate buffer pH 7 for 24 hrs. under cooling conditions (4°C), then the antibacterial activity of the dialyzed protein was determined by agar well diffusion assay (19).

3.Ion exchange chromatography

Diethyl-amino-ethyl cellulose (DEAE-cellulose) was prepared according to Whitaker (1972) as follows: DEAE-cellulose resin (20 g) was suspended in 1L of D.W., then beads were left to settle down and washed several times with D.W. until getting a clear appearance. The suspension was filtered by Whatman No. 1 filter paper using Buchner funnel until discharged. The resin was re-suspended in sodium hydroxide (0.25 M) solution then in sodium chloride (0.25 M) solution. The suspension was filtered again and washed several times by hydrochloric acid (HCl; 0.25M) and next by D.W. before it was equilibrated with potassium phosphate buffer (pH 7). Then the resin packed into the column (2×20 cm) and the sample solution was added to the DEAE-cellulose column. After that, the column was washed with an equivalent volume of the same buffer, and the attached proteins were stepwise eluted with gradual concentrations of sodium chloride (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 M). The absorbance of each fraction was measured at 280 nm using a UV-visible spectrophotometer. Protein activity and concentration were determined for each peak (20).

4.Gel filtration chromatography

Sephadex G-150 was prepared by diluting 15 g in 500 ml of D.W., as recommended by Pharmacia Fine Chemicals Company, and subjected to heating using a water bath at 90°C for 5 hrs. to ensure beads swelling, then degassed and packed in a glass column (1.5 × 35 cm) and equilibrated with potassium phosphate buffer (pH 7). A purified sample obtained from the ion exchange step was applied onto the matrix, the fraction was collected using potassium phosphate buffer, and the absorbance was measured for each fraction at 280. Protein activity and concentration were determined for each peak (20).

Minimum inhibitory concentration (MIC)

The MIC of purified bacteriocin produced by *Lactiplantibacillus plantarum* was tested against

Salmonella typhi, *Shigella* spp., and *Aeromonas hydrophilia* indicators. The pathogenic bacterial isolates were reactivated on BHI broth at 37°C for 24 hrs., then 1×10^8 CFU/ml suspension was prepared by transferring a small touch of the culture into 5 ml normal saline. Subsequently,10 µl of the bacterial suspension was transferred into 90 µl of Muller-Hinton broth; the mixture was then added (100 µl) to all microtiter plate wells except the negative control. Bacteriocin concentrations were prepared using the two-fold dilution method by additions and removals from one tube to another while discarding the final volume. The inoculum was transferred to each well, except the negative control, to achieve a final volume of 200 µl per well, then incubated at 37°C for 18 hrs., then resazurin indicator (30 µl) was added to every well and incubated for 4 hrs. at 37°C (21).

Cytotoxic effect of bacteriocin

The cytotoxic effect of bacteriocin on colon cancer cells (Caco-2) and normal human dermal fibroblast (HdFn) cells were evaluated in vitro using the MTT assay (3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide).

Cell line maintenance

The subsequent protocol (Freshney, 2015) was performed after the formation of a confluent monolayer by the cells in the vessel: phosphate buffer saline was used to wash the cell sheet after the removal of the growth media. Trypsin/versine solution (2 to 3 ml) was added to the cells with gentle rocking; the vessel was flipped over to cover the monolayer completely. The vessel was incubated for 1 to 2 minutes at 37 °C, until the cells detached from the container. After adding 15-20 ml of fresh complete RPMI-1640 medium, the cells were pipetted into the growth medium from the wetting surface (22).

The cell concentration was counted by the hemocytometer using the following formula: Total cell count/ml = cell count × dilution factor (sample volume) × 10^4.

MTT Assay

The effect of purified bacteriocin was determined at different concentrations (25, 50, 100, 200, and 400 µg/ml) on the cells proliferation and cytotoxicity using the MTT Cell Proliferation and Cytotoxicity Assay Kit. The cells (10^4) were seeded in 96-well plates in a final volume of 200 µl complete culture medium per well and incubated for 24 hrs. at 37°C in a 5% CO_2 atmosphere, then the medium was removed to ensure adherence to the plate surface. After medium removal, 200 µl of a two-fold serial dilution of each bacteriocin concentration was added to the wells, and the cells were cultured for 48 hrs. at 37°C, 5% CO_2. After the exposure period, 10 µl of the MTT solution was added to the wells, and the plates were incubated for an additional 4 hrs. under the same conditions. The media were then carefully aspirated, and 100 µl of solubilization solution was added to the wells. Absorbance was measured after 5 minutes using an ELISA reader at a wavelength of 575 nm. The viability percentage was calculated using the formula described by (21): Viability (%) = Optical density of sample / Optical density of control × 100.

Statistical analysis

The Statistical Packages of Social Sciences-SPSS (2019) program was used to detect the effect of different factors on the studied parameters. Least significant difference (LSD) and T-test was used to significantly compare between means. The Chi-square test was used to significantly compare between percentages (0.05 and 0.01 probability) in this study.

Result and discussion

Isolation and identification of *Lactobacillus* spp.

One hundred and eleven isolates (43.9%) of 253 stool samples were able to grow on MRS agar after anaerobic incubation at 37 ºC for 48 hrs. Their colonies appeared single, small, circular, rough surface, white to creamy with a regular edge, slight mucoid, round form, convex, and with a strong odor. Microscopically, their cells appear as gram-positive bacilli or coccobacilli in single, paired, or chain arrangements. The isolates were non-hemolysis on blood agar and reacted negatively for catalase, oxidase, and indole tests. Though these isolates were determined as *Lactobacillus* spp.(23,24).(25) found that 75% of the infants' stool samples were positive for *Lactobacillus* spp., while (26)found 87.5% of the infants' stool samples were positive for *Lactobacillus* spp. It is regarded as a key component of a healthy gut microbiota, particularly in early life (25). Based on bacterial therapy, the gastrointestinal tract constitutes a healthy microbiota throughout life which plays an important role in the prevention and treatment of diarrhea (27). *Lactobacillus* spp. is a normal gastrointestinal tract flora and acts to regulate luminal pH, enhance barrier function by increasing mucus production, and secrete antimicrobial peptides(28).

Isolation and identification of pathogenic bacteria

The incidence of numerous bacterial colonies in the patient stool samples were detected by morphological, cultural, and biochemical tests, such as *Escherichia coli*, *Proteus* spp., *Klebsiella* spp., *Staphylococcus* spp., *Acinetobacter* spp., *Salmonella* spp., *Shigella* spp., *Aeromonas* spp., and others. Some of them were regarded as gut normal flora, thus, *Salmonella* spp., *Shigella* spp., and *Aeromonas* spp. were picked (Table 3) and used as indicators in the bacteriocin production test. These bacteria are the most common intestinal pathogens that cause diarrhea (29). The Vitek 2 compact system showed that the probability of those isolated organisms being *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophila* were 95%, 99%, and 97%, respectively.

Table (3): The biochemical test for *Salmonella* spp., *Shigella* spp., and *Aeromonas* spp.

Biochemical test		*Salmonella* spp.	*Shigella* spp.	*Aeromonas* spp.
MacConkey agar		Pale colonies	Transparent or colorless colonies	Pale colonies
XLD agar		Red colonies with black center	Pure pink colonies without black center	----------
HEA gar		Transparent green or blue-green colonies with or without black center	Transparent green or blue-green colonies without black center	----------
TCBS agar		----------	----------	Yellow colonies
Catalase test		+	+	+
Oxidase test		-	-	+
Urease test		-	-	-
Simmon citrate		-	-	+
Indole test		-	-	+
Motility test		+	-	+
(TSI)	Slant/butt	K/A	K/K	K/A
	H_2S	+	-	-
	Gas	-	-	+

+: Positive. -: Negative. K: Alkaline. A: Acidic

Antibiotic sensitivity test

Salmonella typhi and *Shigella* spp. isolates were resistant to ampicillin, cefotaxime, and ceftriaxone antibiotics, whereas they were sensitive to aztreonam, ciprofloxacin, trimethoprim-sulfamethoxazole-chloramphenicol, and meropenem antibiotics, while *Shigella* spp. was also sensitive to imipenem. On the other hand, *Aeromonas hydrophila* isolate was resistant to cefotaxime, ciprofloxacin, cefepime, ceftazidime, and piperacillin-tazobactam antibiotics, while it was sensitive to imipenem, meropenem, and amikacin antibiotics only. In general, *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophila* isolates were sensitive to 55.56%, 66.67%, and 37.50% of the tested antibiotics, respectively.

Statistical analysis results revealed highly significant P-values for *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophilia* isolates; P-values were 0.0098**, 0.0082**, and 0.0089**, respectively (Table 4). *Aeromonas hydrophila* exhibited more resistance than *Shigella* spp. and *Salmonella typhi*; meanwhile the three tested pathogens have high rates of antibiotic resistance, with multidrug resistance observed against some critical antibiotics, such as cefotaxime, ceftriaxone, and ciprofloxacin, this result indicating caution with antibiotic treatments. Many studies mentioned that *Salmonella* spp., *Shigella* spp., and *Aeromonas hydrophila* isolates have high rates of antibiotic resistance (30). The extensive use of antibiotics by humans is more relevant for antibacterial resistance development among enteropathogenic bacteria; also, self-medication and using antibiotic drugs without a prescription are frequently observed in the developing world (31).

Table (4): Antibiotic susceptibility test of the pathogenic indicators.

Antibiotic	*Salmonella typhi*	*Shigella* spp.	*Aeromonas hydrophilia*
Ampicillin (AMP)	R	R	---------
Aztreonam (AZM)	S	S	---------
Cefotaxime (CTX)	R	R	R
Ceftriaxone (CRO)	R	R	
Ciprofloxacin (CIP)	S	S	R
Trimethoprim- sulfamethoxazole (COP)	S	S	---------
chloramphenicol (C)	S	S	---------
Imipenem (IMI)	I	S	S
Meropenem (MRP)	S	S	S
Cefepime (CPM)	---------	---------	R
Ceftazidime (CAZ)	---------	---------	R
Piperacillin tazobactam (PIT)	---------	---------	R
Amikacin (AK)	---------	---------	S
P-value	0.0098 **	0.0082 **	0.0089 **
** (P<0.01)			

Primary screening

Seventy-nine *Lactobacillus* spp. isolates (87.4%) showed antibacterial activity against the *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophila* pathogens by forming zones ranging from 9 to 22 mm. *Lactobacillus* spp.'s high rate of antimicrobial activity may be related to their production of antimicrobial agents, including hydrogen peroxide (H_2O_2), organic acid, diacetyl, inhibitory enzymes, bacteriocin, and carbon monoxide (CO), as well as other substances such as bio-

emulsifiers (3). (32) found only 26.7% of *Lactobacillus* spp. isolates inhibited the growth of *Staphylococcus aureus, E. coli*, and *Streptococcus mutans*. (33) mentioned that *Lactobacillus* spp. isolated from the feces of healthy individuals have ability to inhibit the growth of enteropathogenic *E. coli* (EPEC), *enteroaggregative E. coli* (EAEC), *Salmonella typhi*, and *Shigella dysenteriae* using the agar-plug diffusion method. The primary screening statistical analysis showed high significant differences in *Lactobacillus* spp. isolates activity (P-value = 0.0001 **), L.S.D. values were 6.027 **, 5.941 **, and 5.602 ** for *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophila*, respectively (Table 5). In general, *Salmonella typhi* revealed a greater sensitivity against *Lactobacillus* spp. isolates (96.4%), followed by *Shigella* spp. (91.0%), then *Aeromonas hydrophila* (88.3%), with a highly significant difference between them (P-value = 0.0001 **).

Secondary screening

Eighteen *Lactobacillus* spp. isolates (18.6%) exhibited bacteriocin effectiveness against *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophila*, with inhibition zones ranging from 11 to 16 mm. The secondary screening statistical analysis showed high significant differences in *Lactobacillus* spp. isolates activity, the P-value was 0.0001 ** for *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophila*, (Table 5). In general, the bacteriocin produced by *Lactobacillus* spp. isolates showed higher activity against *Shigella* spp. (44.3%), followed by *Aeromonas hydrophila* (37.1%), then *Salmonella typhi* (36.1%). (33) showed that 40% of *Lactobacillus* spp. isolates have antimicrobial activity against one or more of *Enteropathogenic E.coli* (EPEC),Entero aggregative *E. coli* (EAEC), *Salmonella typhi*, and *Shigella dysenteria*e, while only 17.4% of them were active against all the indicators. Lactic acid bacterial cell-free supernatants have the ability to inhibit gram-positive and gram-negative bacteria, such as *Salmonella* spp., *E. coli*, *S. aureus*, and *P. aeruginosa* (17).

Table (5): Primary and secondary screening for *Lactobacillus* spp. against pathogenic indicators.

The indicators	The isolates number			
	Primary screening No.=111	L.S.D. (P-value)	Secondary screening No. = 97	P-value
Salmonella typhi	107 (96.4%)	6.027 ** (0.0001)	35 (36.1%)	0.0001 **
Shigella spp.	101 (91.0%)	5.941 ** (0.0001)	43 (44.3%)	0.0001 **
Aeromonas hydrophila.	98 (88.3%)	5.602 ** (0.0001)	36 (37.1%)	0.0001 **
The three indicaters	97 (87.4%)		18 (18.6%)	

P-value * (P<0.05) ** (P<0.01)

***Lactobacillus* spp. identification by PCR technique**

Eighteen bacterial isolates were subjected to DNA extraction, and then the gene*16SrRNA* was amplified by specific primers (Table 2), in order to diagnose *Lactobacillus* spp. The results of PCR product's gel electrophoresis revealed that fifteen isolates had bands at 345 bp, thus they belonged to *Lactobacillus* spp. (Figure 1).

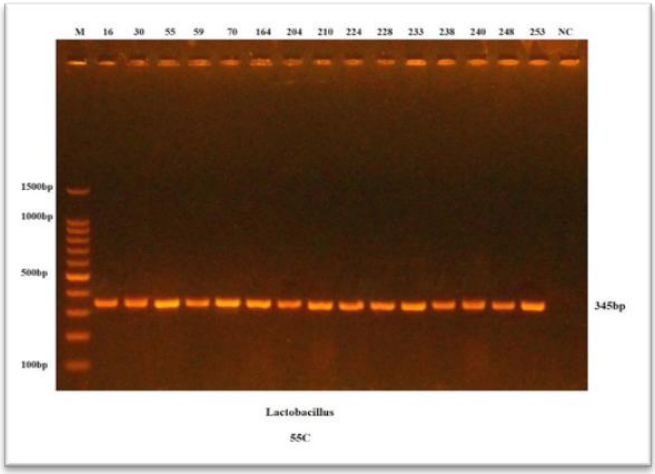

Figure (1): Amplification product of *16S rRNA* gene in *Lactobacillus* spp. isolates after electrophoresis on 1.5% agarose gel in the presence of DNA Ladder (1500 bp).

Lactobacillus spp. sequencing

The PCR products sequence analysis of fifteen local isolates (*Lactobacillus* spp.) showed that eight isolates were identified as *Lactobacillus* species. According to NCBI (BLASTN) alignment, two isolates were found to be *Lactiplantibacillus plantarum* (identical to the Nigerian isolate ID: PP978768.1), four isolates were *Limosilactobacillus fermentum* (identical to the Malaysian isolate ID: MT645492.1), and two isolates were matched *Lacticaseibacillus rhamnosus* (identical to the Chinese isolate ID: MT645513. The eight *Lactobacillus* strains were submitted at the gene bank (NCBI), *Lactiplantibacillus plantarum* (SMHA16), *Limosilactobacillus fermentum* (SMHA30), *Lactiplantibacillus plantarum* (SMHA208), *Limosilactobacillus fermentum* (SMHA210), *Limosilactobacillus fermentum* (SMHA224), *Lacticaseibacillus rhamnosus* (SMHA228), *Limosilactobacillus fermentum* (SMHA240), and *Lacticaseibacillus rhamnosus* (SMHA253) accession number were PQ 309568, PQ 309569, PQ 309570, PQ452299.1, PQ45200.1, PQ 309571, PQ452301.1, and PQ452302.1, respectively.

Limosilactobacillus fermentum strain UL 16S ribosomal RNA gene, partial sequence

Sequence ID: MT645492.1 Length: 1045 Number of Matches: 1

Range 1: 187 to 503 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
586 bits(317)	1e-162	317/317(100%)	0/317(0%)	Plus/Plus

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Query  1    TCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTC  60
Sbjct  187  ............................................................  246

Query  61   GTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACTGTTCATACGTTGACGGTA  120
Sbjct  247  ............................................................  306

Query  121  TTTAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA  180
Sbjct  307  ............................................................  366

Query  181  GCGTTATCCGGATTTATTGGGCGTAAAGAGAGTGCAGGCGGTTTTCTAAGTCTGATGTGA  240
Sbjct  367  ............................................................  426

Query  241  AAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAAACTGGATAACTTGAGTGCAGAAGAGG  300
Sbjct  427  ............................................................  486

Query  301  GTAGTGGAACTCCATGT  317
Sbjct  487  .................  503
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Figure (2): Sequences of *Limosilactobacillus fermentum* isolate (No. 224-Lacto) with *Limosilactobacillus fermentum* isolate strain UL. No found differences in the nucleotides of this study query and the subject.

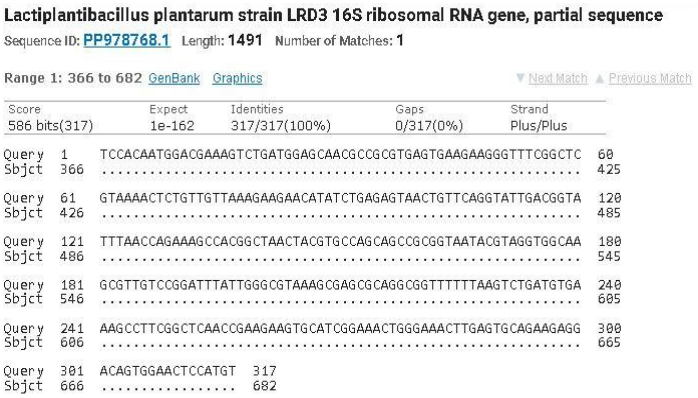

Figure 3: Sequences of *Lactiplantibacillus plantarum* isolate (No.16-Lacto) with *Lactiplantibacillus plantarum* isolate strain LRD3. No found differences in the nucleotides of this study query and the subject.

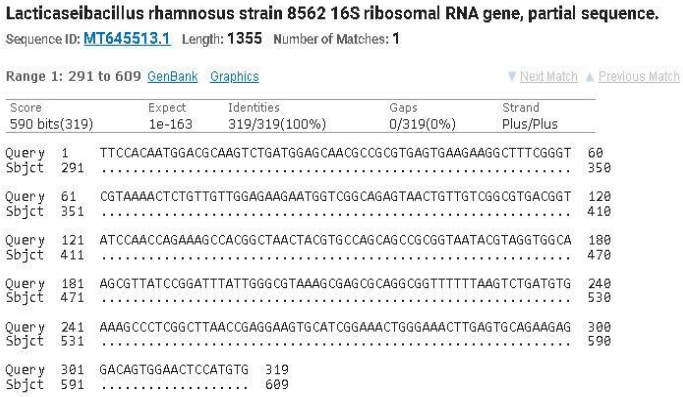

Figure 4: Sequences of *Lacticaseibacillus rhamnosus* isolate (No. 228-Lacto) with *Lacticaseibacillus rhamnosus* isolate strain 8562. No found differences in the nucleotides of this study query and the subject.

Determination of bacteriocin production optimal conditions

Lactiplantibacillus plantarum (SMHA16) isolate was found to be the most bacteriocin producer according to the bacteriocin activity experiment (160 AU/mL). This isolate was subjected to optimization to determine optimal conditions for enhanced bacteriocin production.

Medium pH

The maximum production of bacteriocin was observed at pH 5.0-6.0 and quite a bit less at pH 4, while it had completely disappeared at pH 7 and 8 (Figure 2- A). . (19,32) reported that the optimal pH for bacteriocin production by *Lactobacillus acidophilus* was at pH 6.4 and for *Lactobacillus parabucheri* N14 was at pH 7, respectively. The pH affects enzymatic activity, growth rate, and metabolite production, including bacteriocins. The acidification of the medium is required to stimulate the bacteriocin production sometimes; consequently, a buffered process would negatively affect their production. However, high acidity can impair nutrient transport and essential cytoplasmic processes, leading to reduced cell growth and bacteriocin synthesis (34).

Incubation period

Maximum production of bacteriocin was observed after 48 hrs. of incubation, and less production was observed after 24 and 72 hrs. of incubation (Figure 2- B) (19,12,32)

observed that *Lactobacillus acidophilus* HT1, *Lactobacillus crispatus* IS30, and *Lactobacillus parabucheri* N14, respectively, reached the maximum level of bacteriocin production after a 24-hour incubation period. Bacteriocins are considered primary metabolites and are mainly produced during the exponential growth phase of *Lactobacillus* spp. However, some LAB produce bacteriocins during the stationary growth phase, at conditions such as lower pH and reduced nutrient availability (34).

Incubation temperature

The higher production of bacteriocin was detected at 32°C, while less production was at 37°C and 27°C, and it completely disappeared at 42°C (Figure 2-C). Thus, 32°C can be considered the optimum temperature for bacteriocin production by the isolate *Lactiplantibacillus plantarum* (SMHA16). This result contradicts the findings of previous studies conducted by (32,19) which mentioned that 37°C was the optimum temperature for the production of bacteriocin by *Lactobacillus parabucheri* N14 and *Lactobacillus acidophilus* HT1, respectively. Bacteriocin production is highly sensitive to temperature due to its direct influence on enzymatic activity and cell growth rates of microorganisms (34).

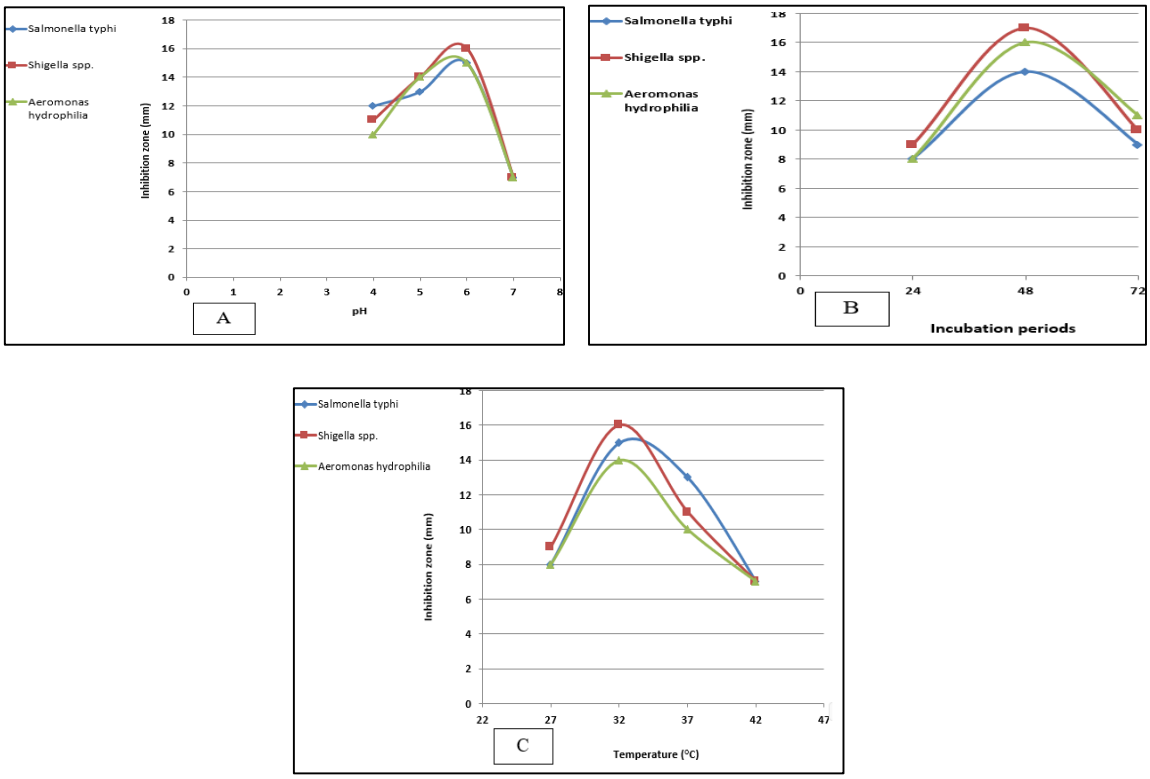

Figure (5) :The effect of(A) medium pH, (B)Incubation period and (c) temperature on bacteriocin production by *Lactiplantibacillus plantarum* against *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophila* pathogenic indicators.

Extraction and purification of bacteriocin

The crude extract of bacteriocin obtained from *Lactiplantibacillus plantarum* (SMHA16) had a protein concentration of 0.7 mg/ml, a specific activity of 190 units/mg, a purification fold of 1, and a yield of 100% (Table 6).

Ammonium sulfate

The maximum bacteriocin precipitation was obtained at a 60% saturation level of ammonium sulfate which has 443.3 AU/ml bacteriocin activity and a specific activity of 554.3 AU/ml (Table 6). The salt and impurities were then removed by dialysis for further purification of bacteriocin. (19) obtained maximum bacteriocin precipitation (*L. acidophilus* HT) at a 70% ammonium sulfate saturation level and charactarised by 1280 AU/ml bacteriocin activity and 4266.6 AU/mg specific activity. (35) mention that the maximum antibacterial activity of plantaricin UG1, extracted from *Lactobacillus plantarum*, was 22880 AU/ml at a 60% ammonim sulfate saturation level and with a pH range of 4.0 to 6.0.

Ion exchange

Crude bacteriocin obtained by dialysis was loaded into an ion exchange chromatography column. Two peaks were obtained from the washing step and one peak from elution by gradient concentrations of sodium chloride (Figure 3). All peaks were assayed by the agar well diffusion method to detect the activity of bacteriocin. The eluted peak had more bacteriocin activity at fractions numbered 55 to 60, which was 400 AU/ml. Enzyme specific activity was 1333 AU/mg, purification fold 7, and 60.1% yield (Table 6).

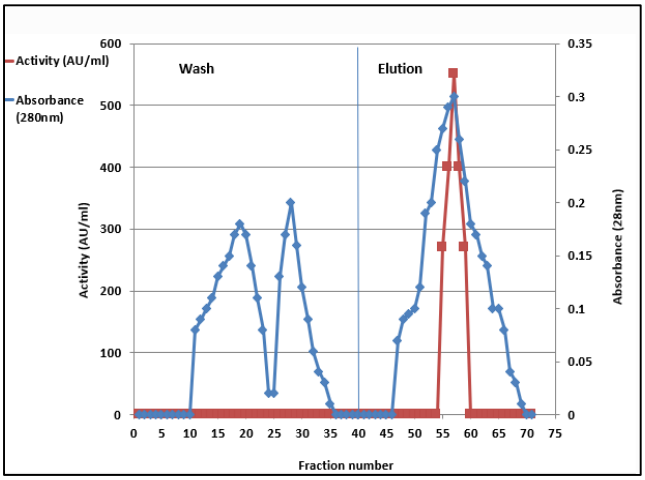

Figure (6): Ion exchange chromatography of bacteriocin using DEAE-cellulose column (2 × 20 cm).

Gel filtration chromatography

The concentrated bacteriocin from ion exchange was loaded in Sephadex G-150. Figure 4 illustrated the measurement of the collected fractions at 280 nm and the bacteriocin activity for the active fractions, 22, 23, 24, and 25. The specific activity of the purified bacteriocin was increased to 4000 AU/ml, resulting in 21-fold purification with 48.1% yield **(**Table 6). Purification of bacteriocin using gel filtration chromatography is a common method and is based on bacteriocins molecular masses. This method is conducted using various gel columns, such as Superdex, Sephadex, and Sepharose. Sphadex G-150 gel was chosen in this study because it permits the passage of 5 to 300 kDa proteins (36). Using a Sephadex G50 column, (18) found 2560 AU/ml activity, 12800 U /mg specific activity, 20% yield, and 10 purification fold when purified the bacteriocin extracted from *Lactobacillus acidophilus* HT1. Another study obtained bacteriocin from *Lactobacillus plantarum* W3-2 using a Sephadex G-25 gel chromatography column (1).

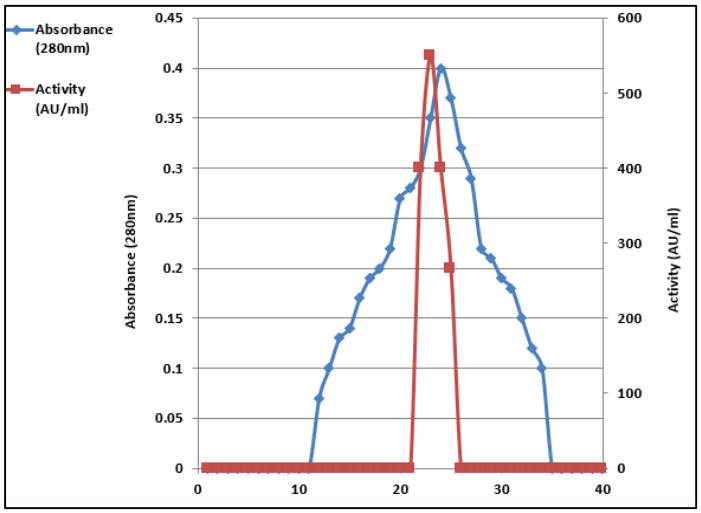

Figure (7): Purification of bacteriocin by sephadex G-150 gel filtration chromatograph, equilibrated and eluted with phosphate buffer.

Table (6): The purification steps of bacteriocin extracted from *Lactiplantibacillus plantarum* (SMHA16).

Purification step	Protein (bacteriocin)						
	Volume (ml)	Activity (AU/ml)	Concentration (mg/ml)	Specific activity (AU/mg)	Total activity (AU)	Purification (folds)	Yield (%)
Crude supernatant	75	133	0.7	190	9975	1	100
Ammonium sulphate precipitation (60%)	15	443.3	0.8	554.13	6650	2.92	66.67
DEAE-cellulose	15	400	0.3	1333	6000	7	60.1
Sephadex-G150	12	400	0.1	4000	4800	21	48.1

Antibacterial effect of bacteriocin (MIC)

The activity of purified bacteriocin was tested against *Salmonella typhi*, *Shigella* spp., and *Aeromonas hydrophila* with different concentrations (1024, 512, 256, 128, 64, 32,16, 8, 4, 2, and 1 µg/ml). *Shigell*a spp. exhibited greater sensitivity to bacteriocins than *Salmonella typhi* and *Aeromonas hydrophila.* The MICs for *Shigella* spp. was 256 µg/ml, thus the sub-MIC value was 128 µg/ml, while the MICs for *Salmonella typhi* and *Aeromonas hydrophila* was 512 µg/ml, thus the sub-MIC value was 256 µg/ml (Table 7). These results indicate that *Salmonella typhi* and *Aeromonas hydrophila* require higher concentrations of bacteriocin compared with *Shigella* spp., indicating greater sensitivity of *Shigella* spp. to the bacteriocin. Thus, they are consistent with the antibiotic susceptibility patterns, where *Shigella* spp. showed the highest sensitivity (66.67%), followed by *Salmonella typhi* (55.56%) and *Aeromonas hydrophila* (37.50%). The relation between the antibacterial activity of bacteriocins and conventional antibiotics supports the potential use of bacteriocins as effective antimicrobial agents. (9) found that the MIC of Plantaricin FB-2 derived from *Lactobacillus plantarum* against

Staphylococcus aureus ATCC6538 was 256 µg/ml. (37) reported that the MIC of bacteriocin extracted from *Lactobacillus plantarum* against treatment-resistant *Escherichia coli* was 13.38 µg/ml and the minimum bactericidal concentration (MBC) was 26.76 µg/ml.

Table (7): The MIC and sub-MIC of *Lactiplantibacillu plantarum* bacteriocin against *Shigella* spp., *Aeromonas hydrophila* and *Salmonella typhi* isolates.

Pathogenic bacterial	Bacteriocin MIC (µg/ml)	Bacteriocin Sub MIC (µg/ml)
***Shigella* spp.**	256	128
Aeromonas hydrophila	512	256
Salmonella typhi	512	256

Cytotoxic effect of bacteriocin (MTT)

The viability of colon cancer cells was reduced with increasing the bacteriocin concentrations. The decrease in the cells ($Caco_2$) viability was noted at 400 µg/ml of bacteriocin concentration, reaching 51.929 ± 1.67884%, within the IC50 value of 135.9 µg/ml. However, the normal cells (Hdfn) viability revealed less effect, reaching 73.49533±0.6945 at 400 µg/ml of bacteriocin concentration, within IC50 value of 157.9 µg/ml (Fig.5, Table 8). Many researchers worldwide have studied the effect of bacteriocins on many types of cancer cells, but its effect on colon cancer cells has not been studied, particularly in Iraq.

The bacteriocins have the ability to stop cancer cells from multiplying, maybe due to enhancing the fluidity of cell membranes and creating ion channels on the membranes of cancer cells. This mechanism subsequently leads to an increased release of LDH. The presence of bacteriocins can also induce the accumulation of intracellular reactive oxygen species, resulting in an increase in apoptotic activity (38). (22) reported the anticancer potential of bacteriocin derived from *L. plantarum* against esophageal cancer cells (SK-GT4), within an IC50 value of 281.9 AU/ml, and mentioned that it has no cytotoxic effects on normal rat embryo fibroblast cells (REF), emphasizing its high selectivity and safety profile.

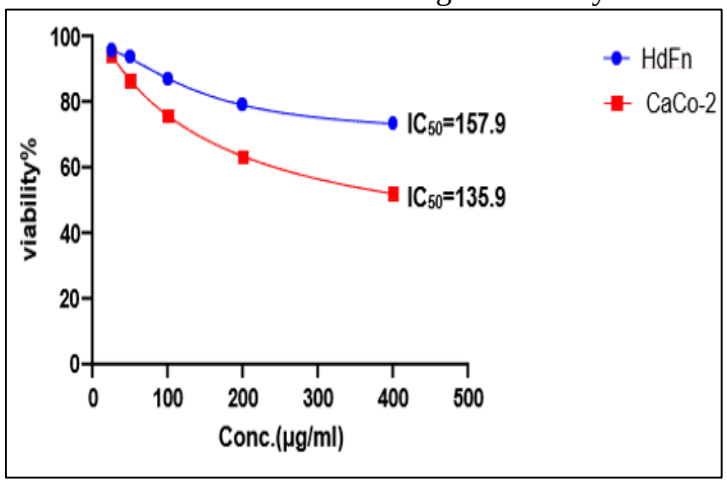

Figure (8): Cytotoxic effect of Bacteriocin on Caco2 and Hdfn cells after 24 hours incubation at 37°C.

Table (8): Cytotoxic effect of bacteriocin on Caco2 and Hdfn cells after 24 hours incubation at 37ºC.

Bacteriocin Concentrations (µg/ ml)	Viable cell count of Hdfn cell line Mean± S.D.	Viable cell count of Caco2 cell line Mean± S.D.
400	73.49533±0.6945 [d]	51.929 ± 1.67884 [e]
200	79.398± 0.579 [c]	63.31033 ± 1.518276 [d]
100	87.037± 0.463 [b]	75.84867 ±2.058566 [c]
50	93.78867± 0.240513 [a]	86.38133± 1.678828 [b]
25	95.87167± 0.467819 [a]	94.4056±7 0.547066 [a]

Different letters in the row indicate a significant difference ($p \leq 0.05$) between the mean values.

Conclusion

Limosilactobacillus fermentum was more prevalent in fecal samples and exhibited a higher production of bacteriocins, thus possessing the ability to inhibit diarrhea-associated bacteria, including *Salmonella typhi*, *Shigell*a spp., and *Aeromonas hydrophila*, which emerged as the most prevalent. Bacteriocin extracted from *Lactiplantibacillus plantarum* can be considered as an alternative antibiotic due to its ability to inhibit pathogenic bacteria. Moreover, it can serve as an anticancer agent due to its cytotoxic effects against colon cancer cells. This selective cytotoxicity specifically targets colon cancer cells with little effect on normal cells, making them a potential candidate for cancer therapy .

Ethical clearance

The research ethical committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq (CSEC/0724/0050)

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