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Implications of Bio-Preservation Using Pediococcus Pentosaceus HPM1-Derived Bacteriocin for the Shelf-Life Enhancement of Food

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Abstract

The growing demand for effective, natural food preservation methods necessitates exploring novel biocontrol agents. In this study, *Pediococcus pentosaceus* HPM1, a potential bacteriocin-producing strain, was isolated from the traditional fermented food beuli dal bari, which is more popular in village areas. The cell-free supernatant of HPM1 demonstrated broad-spectrum antimicrobial activity against all selected foodborne pathogens, and quantitative analysis confirmed HPM1 as a highly efficient bacteriocin producer. Optimal growth was observed at temperatures between 35°C and 40°C and pH 6.5 to 7.0. Purification then MALDI-TOF MS analysis identified the antimicrobial peptide as a Class I bacteriocin with the molecular weight of 1231.49 Da. The bacteriocin has shown a remarkable stability, retaining its bioactivity under extreme thermal stress at 121°C for 15 minutes and across a broad pH range of 4.0-9.0. Application of this bacteriocin to food matrices, including vegetables, fruits, meat, and fish, extended shelf life under both ambient and refrigerated storage without interfering with the nutritional parameters ($P > 0.05$). Moreover, the biocompatibility of the bacteriocin was investigated with the help of safety assessments using the MTT assay on the cell lines of RAW264.7 macrophage, which confirmed its biocompatibility. These results define the *P. pentosaceus* HPM1-derived bacteriocin as a powerful, multifunctional, and commercial bio-preservative that provides a promising opportunity to improve food supply safety and quality in the global food chain.

Introduction

The use of fermented foods in human diets is not a new phenomenon, as we have seen throughout our history, and the traditional Indian practice of utilising local crops is so diverse. These traditional fermented foods offer special environments which rich in diverse microorganisms [1]. Because of this, the exploration and isolation of beneficial Lactic Acid Bacteria (LAB) present in fermented foods have become an attractive area of microbiological research. Groups of LAB, such as *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Leuconostoc*, etc., are valuable because they are already known to play key roles in various bioprocesses [2]. Specifically, the escalating global demand for safe, high-quality, eco-friendly food products mandates the development of viable alternatives to synthetic chemical preservatives. LABs are widely employed in bio-preservation processes [3].

Among the beneficial isolates, strains belonging to the genus *Pediococcus* have shown efficacy in controlling major food-borne pathogens, including *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa* and various other species, alongside common spoilage fungi such as *Aspergillus*, *Penicillium*, *Fusarium*, and *Candida albicans* [4]. This antagonistic activity significantly contributes to improved food quality, flavour development, and enhanced shelf life. The high regard for *Pediococcus* species is further supported by their Generally Recognised As Safe (GRAS) status and their natural capacity to suppress undesirable microbes [5]. This suppression mainly occurs by the secretion of specialised antimicrobial agents called bacteriocins [6, 7].

Bacteriocins are defined as ribosomally synthesised antimicrobial peptides that exert potent, typically narrow-spectrum, and inhibitory effects, vital for stabilising both traditional fermented foods and modern food matrices [8, 9]. Moreover, in addition to their preservative effects, certain strains, including *Pediococcus pentosaceus*, have been widely reported to have major functional probiotic characteristics, including anti-inflammatory, anti-cancer, antioxidant, detoxification and cholesterol-lowering properties [4, 10].

However, despite this wide range of reported benefits, the current application of high-potential LAB strains, including *P. pentosaceus*, frequently fails to adequately address the numerous and multifaceted technological challenges faced in modern and large-scale food production and food preservation [11]. Consequently, the need remains to continually discover and characterise novel strains adapted to diverse environmental niches. Building upon the known competitive exclusion mechanisms mediated by *Pediococcus* species, the present study intends to isolate *Pediococcus pentosaceus* from an indigenous source: beuli dal bari, a traditional fermented food sample that is collected from the Midnapore town area of West Bengal, India. This research aims to look into new strains that can satisfy the demand for strong biopreservatives through their innate antimicrobial and functional attributes.

Methodology

Isolation and Enumeration of Bacteriocin-Producing Lactic Acid Bacteria

Seventeen diverse food samples from the Midnapore district of West Bengal, including rice, milk, pickle, and flour products, were collected and refrigerated (4–8°C). Amongst this entire collection, it was the traditional bengali fermented food, beuli dal bori, that was selected as the main focus of the subsequent stages of this research. This sample was subjected to isolation of lactic acid bacteria (LAB) through a serial dilution (up to 10^{-3}) in the peptone buffer, and triplicate aliquots were spread out on the de Man, Rogosa, and Sharpe (MRS) agar plates. The incubation of the plates was done in aerobic conditions at 37°C up to 48–72 hours. Isolates of presumptive LAB were propagated in pure cultures through successive subcultures on new MRS agar. Phenotypic characterisation was first done using the macroscopic morphology on solid media. Further observations were made whereby the shape, colour, consistency, surface texture and the opacity of the colony were recorded. Isolated LAB was abbreviated as HPM1 [12, 13].

Assessment of Antibacterial Potential of Isolated HPM1

In order to ascertain the antimicrobial properties of the LAB-derived cell-free culture supernatant (CFCS), the agar well diffusion assay was used. CFCS was prepared by incubating all the isolated LAB in MRS broth with a 1% inoculum, at 37°C for 48 hours in an aerobic environment. The preparation of CFCS involved culturing all the isolated LAB in MRS broth with a 1% inoculum, incubated aerobically at 37°C for 48 hours. Following incubation, bacterial cells were removed from the broth by centrifugation at 10,000 rpm for fifteen minutes at 4°C, then filtered through a 0.22 µm pore-size membrane filter. This assay utilised a group of gram-positive and gram-negative bacteria, commonly responsible for food spoilage, as indicator organisms. Specifically, *Bacillus cereus* MTCC 13193, *Staphylococcus aureus* MTCC 1430, *Clostridium botulinum* ATCC 3502, *Enterococcus faecalis* MTCC 439, *Escherichia coli* MTCC 1567, *Vibrio cholerae* MTCC 15025, *Pseudomonas aeruginosa* MTCC 424 and *Klebsiella pneumoniae* MTCC 109 were used. Nutrient agar plates were prepared and then evenly spread with 100 µL of an overnight culture of each indicator strain. A volume of 100 µL of prepared CFCS was then carefully dispensed into individual wells. Sterile distilled water was added to separate wells, which served as a control, and then the inoculated plates were incubated at 37°C for 24 hours aerobically [14]. Post-incubation, the diameter of any clear inhibition zone surrounding each well was precisely observed, and measurements were recorded in millimetres.

Determination of Antifungal Activity of Isolate HPM1

To assess the antifungal potential of the isolate HPM1, its cell-free culture supernatants, prepared as previously described, were tested against the fungal strains *Aspergillus niger* MTCC 12988, *Aspergillus fumigatus* MTCC 9389, *Aspergillus flavus* MTCC 3682 and *Candida albicans* MTCC 227. Antifungal activity was assessed using the agar well diffusion method on Potato Dextrose Agar (PDA) plates. Following incubation, the diameter of the resulting inhibition zone surrounding each well was measured precisely [15, 16].

Confirmation of Bacteriocin Production by Isolate HPM1

To unequivocally confirm bacteriocin production by isolate HPM1, a series of confirmatory assays was performed. Two reference strains were purchased from the Microbial Type Culture Collection (MTCC): *Lactobacillus plantarum* MTCC 9495, known for its bacteriocin-producing capabilities [17], served as the positive control, while *Listeria monocytogenes* MTCC 657, recognised for its sensitivity to bacteriocins [18], was employed as the negative control. The activity of the CFCS derived from the selected LAB isolate, and the positive control (*L. plantarum* MTCC 9495) was evaluated against the previously established agar well diffusion method against food-spoilage indicator strains, along with the negative control (*L. monocytogenes*). Is there any similarity in the activity of the selected isolate and the positive control was observed.

To be more sure about the proteinaceous nature of the substrate, proteinase K was utilised. Proteinase K was dissolved in 0.2 mol/L sodium phosphate buffer (pH 7.5) and added to aliquots of CFCS from both the selected isolate and the positive control to achieve a final concentration of 1 mg/mL. Following enzymatic treatment, the treated CFCS (with proteinase K), untreated CFCS (without proteinase K), and sterile distilled water (as a control) were subjected to the agar well diffusion assay by a previously established protocol. The plates were incubated aerobically at 37°C for 24 hours [19, 20]. Post-incubation, the plates were examined for the presence or absence of a zone of inhibition, and the diameter of any clear zone surrounding each well was then precisely measured.

Examination of Cell-Free Culture Supernatant for Bacteriocin Assay

The CFCS of isolate HPM1 were prepared using a standard, previously established method. Bacteriocin activity was assayed using the agar well diffusion method against the panel of food-borne and human pathogens, including S. *aureus*, *L. monocytogenes*, *C. botulinum*, *B. cereus*, *E. coli*, *V. cholerae*, *E. faecalis*, *P. aeruginosa* and *K. pneumoniae*. For this assay, 100 µl of CFCS of the isolate HPM1 was loaded into 8 mm wells in nutrient agar plates. Conforming 24 h of incubation at 37°C, the diameter of inhibition zones was measured. Bacteriocin activity was then quantified in Arbitrary Units per millilitre (AU/ml), defined as the area of inhibition (mm^2) per unit volume of bacteriocin added (ml), calculated as the following formula [20, 21]:

$$Arbitrary\frac{Unit}{ml} = \frac{L_z - L_s}{V} \quad (1)$$

L_z = clear zone area (mm^2)

L_s = well area (mm^2)

V = volume of sample (ml)

Assessment of Phenotypic and Biochemical Characteristics of Isolate HPM1

Isolate HPM1 is preliminarily characterised by standard microscopic techniques, including Gram staining to ascertain cell morphology and endospore staining to differentiate isolates based on endospore production capabilities. After these initial microscopic studies, a comprehensive biochemical characterisation was done according to established protocols to confirm species identity [22, 23].

Identification of Isolate HPM1 Using 16S rRNA Gene-Based Molecular Method and Phylogenetic Analysis

Molecular identification of the selected isolate HPM1 was performed by Barcode Biosciences Pvt. Ltd. (Bangalore, Karnataka, India), starting with genomic DNA extraction, the integrity of which was confirmed via 1.0% agarose gel electrophoresis, revealing a single, high-molecular-weight DNA band. Amplification of the 16S ribosomal RNA (rRNA) gene was achieved using the forward primer 16SrRNA-F and the reverse primer 16SrRNA-R. The polymerase chain reaction (PCR) product, expected to be approximately 1500 bp, was visualised on a separate agarose gel, where a single, discrete amplicon band was observed. The purified PCR amplicon, free from reaction contaminants, was then prepared for sequencing. DNA sequencing was performed on both the forward and reverse strands of the PCR amplicon using the 16SrRNA-F and 16SrRNA-R primers, employing a BDT v3.1 Cycle sequencing kit on an ABI 3730xl Genetic Analyser. The resultant forward and reverse sequence data were subsequently integrated to generate a consensus sequence for the 16S rRNA gene using designated aligner software. For phylogenetic analysis, the consensus 16S rRNA gene sequence was compared against the 'nr' (non-redundant) nucleotide database within the NCBI GenBank using the Basic Local Alignment Search Tool (BLAST). The ten most homologous sequences, exhibiting the highest identity scores, were selected. These sequences, along with the isolate's sequence, were aligned using the Clustal W multiple alignment program. Finally, phylogenetic relatedness was assessed through the construction of a distance matrix and a phylogenetic tree utilising the MEGA 10 software [24, 25].

Optimum Growth Condition Determination

The optimal growth temperature and pH for the HPM1 isolate were determined. For temperature optimisation, cultures were inoculated into MRS broth (1% v/v) and incubated aerobically at a range of temperatures: 4°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. Concurrently, for pH optimisation, 1% (v/v) fresh overnight cultures were inoculated into MRS broth adjusted pH to varying values of 3, 5, 6, 6.5, 7, 8, 9 and incubated under aerobic conditions at the previously determined optimal temperature. Bacterial growth in all conditions was quantified turbidimetrically by measuring the optical density at 560 nm against the respective uninoculated broth blanks at 12-hour intervals up to 72 hours of incubation [22].

Purification of Bacteriocin

Purification of the bacteriocin produced by HPM1 began with the preparation of the CFCS, utilising the standard protocol described previously. Initial purification involved salting in the proteinaceous components: solid ammonium sulfate was added to the CFCS to achieve 90% saturation [26], and the solution was placed at 4°C for overnight. The resulting precipitated material was collected via centrifugation (10,000 rpm, 4°C, 20 min). The crude precipitate was dissolved in HPLC-grade water and prepared for chromatographic separation. Further purification was achieved using C-18 Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). Fractions demonstrating antimicrobial efficacy against the indicator strain *L. monocytogenes* (as confirmed by the agar well diffusion assay) were collected [27]. These active fractions were concentrated through lyophilisation. The final step determines the molecular mass of the purified bacteriocin using Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry [28, 29].

Thermal Stability of Bacteriocin Activity

The thermal stability of the HPM1 produced bacteriocin was assessed by the previously defined standard agar well method. The antimicrobial efficacy of the CFCS was tested after exposure to various thermal treatments. Specific treatments included 60°C and 80°C for durations of 40 and 60 minutes, as well as exposure to 100°C for 40 and 60 minutes, and sterilisation conditions at 121°C for 15 min. Following each heat treatment, the retained bacteriocin activity (mm^2 /ml) of the treated CFCS was quantified alongside an untreated CFCS of HPM1, which was measured precisely. The thermal stability was determined by comparing the bacteriocin activity in heat-treated CFCS to the untreated CFCS [29, 30].

pH Stability of Bacteriocin Activity

To ascertain the influence of pH variation on the bacteriocin activity of isolate HPM1 CFCS, the supernatant was adjusted across a range of pH values, specifically from 4 to 9, using sterile 0.1M sodium hydroxide for alkalinization and 0.1M hydrochloric acid for acidification. These pH-adjusted samples were then incubated at 37°C for 2 hours. Subsequently, the retained antimicrobial activity of each pH -treated CFCS was evaluated via the agar well diffusion method. The pH range for bacteriocin stability and activity was determined by comparing the observed bacteriocin activity (mm^2 /ml) across the different pH treatments with those of the unadjusted CFCS [21, 31, 32].

Assessment of the Food Preservation Property of Bacteriocin Produced by HPM1

The potential of bacteriocin produced by isolate HPM1 as a food preservative was assessed using vegetables, fruits, meat and fish. Fresh cultures of HPM1 (1% v/v inoculum of overnight culture) were grown in 100 mL of broth under optimal conditions. Cell-free supernatants, serving as the source of bacteriocin and acting as a biofilm coating agent, were prepared according to the previously established standard protocol. Fruits and vegetables were divided into two treatment groups: coated and uncoated. For the coated group, products were dipped in the CFCS and allowed to coat. The control group consisted of uncoated products dipped in sterile distilled water. After treatment, all samples were air-dried for 1 hour to remove surface moisture [33]. Both coated and uncoated products were stored at room temperature and freezing temperatures and observed for spoilage until deterioration was evident. To assess the impact of the preservation treatment on nutritional quality, key parameters (weight, pH, carbohydrate

concentration, protein concentration and fat percentage) were analyzed of the food samples (vegetables, fruits, meat and fish) in their fresh state and after the preservation experiment, for both control and bacteriocin-treated (HPM1 CFCS) samples. Weight was measured using a standard weighing machine, pH was determined using pH paper, carbohydrate concentration was quantified by the 'Anthrone method' [34], protein concentration was determined according to the 'Lowry method' [35], and fat percentage was measured using the 'Soxhlet method' [36].

Cell Cytotoxicity Test of Bacteriocin by MTT Assay

The cytotoxicity of the bacteriocin produced by isolate HPM1 was evaluated in RAW 264.7 murine macrophages using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Macrophages were seeded at a density of 1×10^5 cells per well in 96-well tissue culture plates and treated with HPM1 concentrations ranging from 10 µg/mL to 100 µg/mL, followed by incubation in a CO^2 incubator. After 44 hours, 10 µL of MTT reagent (5 mg/mL) was added to each well, and the plates were incubated for an additional 4 hours at 37°C. Subsequently, the culture media were removed, and 200 µL of acid-isopropanol (0.04 M HCl in isopropanol) was added to each well to solubilise the formazan crystals, followed by a 30-minute incubation at room temperature. Absorbance was measured at 570 nm using a multimode plate reader (Synergy H1, Biotek, USA), and the percentage of cell viability was determined using the following formula [37]:

$$\%\ of\ cell\ viability = \frac{OD\ of\ test}{OD\ of\ control} \times\ 100 \quad (2)$$

Statistical Analysis

Statistical analysis of all the collected data was conducted using Analysis of Variance (ANOVA) to ascertain significant differences in the zone of inhibition. This analysis investigated the application of supernatant treatment of the selected isolate, and, crucially, changes in physical and nutrient parameters observed during the preservation study. Post-hoc pairwise comparisons between treated and untreated groups were performed using Tukey's Honestly Significant Difference (HSD) test. The significance level of α = 0.05 was adopted for all statistical evaluations. The statistical calculation was performed through IBM SPSS version 25.

Results

Isolation and Enumeration of Lactic Acid Bacteria

The LAB was successfully isolated from the traditional fermented food, beuli dal bari, using MRS agar medium. Primary observation showed that all the isolated colonies had morphological similarities; they were small, circular, smooth, and convex with entire margins, and their appearance was opaque with either white or creamy colouration. Following this initial identification, a colony was randomly selected and sub-cultured in MRS broth, and then subjected to a streak plate procedure to ensure purity. The resulting single-type bacterial colony was abbreviated as isolate HPM1 for all subsequent investigations.

Assessment of Antimicrobial Potential of Isolated HPM1

Initial qualitative antimicrobial activity assessments indicated substantial variability in the antimicrobial activity of isolate HPM1. Quantitative analysis, however, demonstrated that isolated HPM1 possessed statistically significant ($p > 0.05$) antibacterial activity compared to the control (sterile deionised water) (Table S1 and Fig. 1a). This efficacy extended against all the selected indicator strains, including both gram-positive strains (*Bacillus cereus* MTCC 13193, *Staphylococcus aureus* MTCC 1430, *Clostridium botulinum* ATCC 3502, *Enterococcus faecalis* MTCC 439) and gram-negative strains (*Escherichia coli* MTCC 1567, *Pseudomonas aeruginosa* MTCC 424, *Vibrio cholerae* MTCC 15025, and *Klebsiella pneumoniae* MTCC 109). In addition to its established antibacterial capabilities, isolate HPM1 also demonstrated significant ($p > 0.05$) antifungal potential (Table S2) against a panel of fungal pathogens (Fig. 1b), including *Aspergillus niger* MTCC 12988, *Aspergillus fumigatus* MTCC 9389, *Aspergillus flavus* MTCC 3682 and *Candida albicans* MTCC 227.

Confirmation of Bacteriocin Production by Isolate HPM1

To evaluate whether the strong inhibitory activity shown by the compound produced by isolate HPM1 is a bacteriocin, the efficacy of its cell-free culture supernatant was assessed in comparison to the positive control (*L. plantarum*). As shown in Fig. 2, HPM1 demonstrated antibacterial efficacy that was statistically indistinguishable from the positive control for the majority of the tested indicator strains, including *B. cereus*, *S. aureus*, *V. cholerae*, and *K. pneumoniae* ($p > 0.05$). A notable exception was observed in the inhibition of *C. botulinum*, where HPM1 (19.67 mm) exhibited significantly superior activity ($p < 0.05$) compared to *L. plantarum* (16.33 mm). The positive control maintained a statistically higher inhibition than HPM1 against *E. faecalis*, *P. aeruginosa*, *E. coli*, and *L. monocytogenes* ($p < 0.05$). Overall, the antimicrobial efficacy of both isolate HPM1 and *L. plantarum* (Table S3) remained significantly higher than the sterile control ($p < 0.05$). This functional equality across most strains strongly suggests that HPM1 produces antimicrobial compounds similar in nature to those found in *L. plantarum*, likely bacteriocins.

To more definitively establish the proteinaceous nature of the antimicrobial substrate produced by isolate HPM1, its CFCS was subjected to enzymatic degradation using proteinase K. Prior to the enzymatic treatment, both the positive control (*L. plantarum*) and isolate HPM1 demonstrated statistically significant differences in antimicrobial activity compared to the control (sterile dH_2O). Following incubation with proteinase K, complete

abrogation of antimicrobial activity was observed for both the positive control and isolate HPM1 (Table S4 and Fig. 3). Specifically, the inhibition zones in the proteinase K-treated samples were no longer statistically distinguishable from the control ($p > 0.05$), a finding that contrasts sharply with the significant inhibition observed prior to enzymatic degradation ($p < 0.05$). This complete loss of inhibitory capacity after treatment with the protein-degrading enzyme provides evidence that the antimicrobial activity of isolate HPM1 may be mediated by produced bacteriocins.

Examination of Cell-Free Culture Supernatant for Bacteriocin Assay

The bacteriocin activity of the isolated LAB HPM1 was evaluated against selected foodborne and human pathogens. The inhibitory activity was quantified based on the difference between the total well area and the inhibition zone area, expressed as an arbitrary unit (mm²/ml), as detailed in Table 1. The results demonstrate that LAB isolate HPM1 exhibits very potent bacteriocin activity against the selected panel of pathogens.

Table 1
Bacteriocin Activity of isolate HPM1 against selected indicator strains

Indicator strain	Well area (mm^2)	Inhibition zone area (mm^2)	Inhibitory activity (mm^2 /ml)
L. monocytogenus	50.27	226.98	1767.10
B. cereus	50.27	254.47	2041.99
S. aureus	50.27	226.98	1767.10
C. botulinum	50.27	303.77	2535.05
E. faecalis.	50.27	263.98	2137.11
E. coli	50.27	209.53	1679.83
P. aeruginosa	50.27	368.70	3195.66
V. cholerae	50.27	245.13	1949.54
K. pneumoniae.	50.27	314.15	2638.89

Assessment of Phenotypic and Biochemical Characteristics of Isolate HPM1

Phenotypic and biochemical characterisation was performed to determine the taxonomic identity of isolate HPM1. Microscopically, HPM1 was found to be an immotile, non-sporing, gram-positive, coccoid bacterium. Biochemically, HPM1 is negative in any of several tests, including catalase, methylred, indole, urease, nitrate reduction, citrate utilisation and H_2S production. About carbohydrates, it is negative in case of lactose fermentation, positive in case of glucose fermentation and sucrose fermentation (Table S5).

Identification of Isolate HPM1 Using 16S rRNA Gene-Based Molecular Method and Phylogenetic Analysis

The molecular identification of the potent bacteriocin-producing isolate HPM1 was achieved through 16S rRNA gene sequencing and subsequent phylogenetic analysis (Fig. 4a, b). Bidirectional sequencing of the amplified 16S rRNA gene product yielded high-quality data suitable for taxonomic determination. BLAST analysis with the NCBI GenBank 'nr' database revealed a high degree of nucleotide homology for isolate HPM1. Specifically, HPM1 demonstrated 99.85% sequence similarity to *Pediococcus pentosaceus* (accession number PP340500). Phylogenetic analysis corroborated this finding, unequivocally identifying isolate HPM1 as *Pediococcus pentosaceus* HPM1. The sequencing data for isolate HPM1 have been assigned the NCBI accession number PP341216.

Optimum Growth Condition Determination

The optimum growth conditions for enhanced bacteriocin production by *P. pentosaceus* HPM1 were determined through a systematic evaluation of physicochemical parameters. Temperature testing revealed that the optimal growth temperature range was determined between 35°C and 40°C, where initial growth within the first 24 hours was observed to be slow, followed by a maximal growth rate between 36–48 hours of incubation (Table S6 and Fig. 5a). pH analysis indicated that Growth rates were insufficient at lower and higher pH ranges, suggesting these conditions are not conducive to maximal growth for HPM1. *P. pentosaceus* HPM1 attained its optimal growth performance at a pH range of 6.5-7 (Table S7 and Fig. 5b). Similar to temperature optimisation, growth kinetics at the optimal pH exhibited slow initial development within the first 24 hours, a significant acceleration in growth rate between 36–48 hours, and a later decrease in growth rate.

Purification of Bacteriocin

After confirming its antimicrobial potential, the bacteriocin from *P. pentosaceus* HPM1 was isolated and purified. For that, the active components from the CFCS were concentrated via 90% ammonium sulfate precipitation and separated by C18 reversed-phase HPLC. The subsequent HPLC yielded thirteen fractions, of which fractions F5 through F9 exhibited potent antibacterial activity against *L. monocytogenes* (MTCC 657) in an agar

well diffusion assay. Further analysis using MALDI-TOF mass spectrometry (Fig. 6) confirmed the low molecular mass of 1231.492 Da, indicating its classification as a Class I bacteriocin.

Thermal Stability of Bacteriocin Activity

The thermal stability of the bacteriocin produced by *P. pentosaceus* HPM1 was examined in selected thermal conditions. The untreated CFCS showed antimicrobial activity, with efficacy ranging from 1679.83 mm^2 /ml (*E. coli*) to 3195.66 mm^2 /ml (*P. aeruginosa*). Data in Table 2 explain that exposure to thermal stress at 60°C, 80°C, and 100°C for 40 minutes showed minimal variation in bacteriocin activity compared to the untreated. Extending the treatment time to 60 minutes at these temperatures induced a slight, gradual decline in activity across all indicator strains. Notably, even after autoclaving at 121°C for 15 minutes, the bacteriocin retained functional activity, with values such as 1767.10 mm^2 /ml against *P. aeruginosa* and 1591.71 mm^2 /ml against *B. cereus*, demonstrating that the antimicrobial peptide remains highly effective at severe heat exposure.

Table 2
Thermal stability of bacteriocin activity produced by *P. pentosaceus* HPM1

Temperature /time		**Bacteriocin activity (mm^2 /ml)**								
		L. monocytogenus	***B. cereus***	***S. aureus***	***C. botulinum***	***E. faecalis.***	***E. coli***	***P. aeruginosa***	***V. cholerae***	***K. pneumoniae.***
HPM1(Untreated)		1767.10	2041.99	1767.10	2535.05	2137.11	1679.83	3195.66	1949.54	2638.89
60 ° c	40 min	1679.83	2041.99	1767.10	2234.95	1856.08	1591.71	3070.62	1949.54	2431.93
	60 min	1507.92	1856.08	1679.83	2136.15	1679.83	1343.06	2536.08	1856.08	2234.95
80 ° c	40 min	1591.71	1949.54	1591.71	2136.15	1591.71	1425.84	2852.91	1856.08	2136.15
	60 min	1264.45	1767.10	1343.06	1856.08	1264.45	1110.11	2332.59	1679.83	1949.54
100 ° c	40 min	1507.92	1767.10	1507.92	2136.15	1507.92	1343.06	2743.42	1679.83	1949.54
	60 min	1110.11	1679.83	1110.11	1425.84	1036.68	824.62	1949.54	1425.84	1679.83
121° c	15 min	824.62	1591.71	892.87	1264.45	758.09	824.62	1767.10	1507.92	1343.06

pH Stability of Bacteriocin Activity

The impact of pH on the antimicrobial efficacy of the bacteriocin produced by *P. pentosaceus* HPM1 was determined across a range of pH 4.0 to 9.0, using the untreated CFCS at pH 6.5 as the baseline. Data in Table 3 shows that the bacteriocin exhibited optimal inhibitory activity at pH 6.0, with levels (2743.42 mm^2 /ml against *P. aeruginosa* and 2332.59 mm^2 /ml against *C. botulinum*) that were highly comparable to the untreated CFCS. Activity remained relatively high at pH 7.0, though a slight decline was noted compared to the value observed at pH 6.0. Exposure to more acidic (pH 5.0 and 4.0) and alkaline (pH 8.0 and 9.0) conditions resulted in a marked reduction in bacteriocin potency. The most significant loss of activity occurred at pH 4.0 and pH 9.0; for instance, the activity against *S. aureus* decreased from 1767.10 mm^2 /ml in the untreated sample to 447.63 mm^2 /ml at pH 4.0 and 691.33 mm^2 /ml at pH 9.0, demonstrating that the antimicrobial metabolite is most stable in the near-neutral pH to slightly acidic pH environment range. In case of other pH ranges, the antimicrobial activity is low but also remains potent.

Table 3
pH stability of bacteriocin activity produced by *P. pentosaceus* HPM1

pH	**Bacteriocin activity (mm^2 /ml)**								
	L. monocytogenus	***B. cereus***	***S. aureus***	***C. botulinum***	***E. faecalis.***	***E. coli***	***P. aeruginosa***	***V. cholerae***	***K. pneumoniae.***
HPM1(Untreated)	1767.10	2041.99	1767.10	2535.05	2137.11	1679.83	3195.66	1949.54	2638.89
pH4	758.091	628.273	447.632	1343.056	758.091	447.632	1036.680	1110.106	758.091
pH5	964.965	1187.547	1036.680	1679.834	1343.056	824.623	2041.990	1591.713	1264.446
pH6	1767.101	2136.151	1856.078	2332.587	1949.540	1679.834	2743.421	1949.540	2332.587
pH7	1507.919	1949.540	1856.078	2136.151	1856.078	1591.713	2536.075	1679.834	2136.151
pH8	1425.837	824.623	1110.106	1591.713	1264.446	1036.680	1679.834	1591.713	1767.101
pH9	1110.106	505.507	691.332	758.091	758.091	566.925	1187.547	1036.680	1036.680

Assessment of the Food Preservation Property of Bacteriocin Produced by P. pentosaceus HPM1

This assessment demonstrated the substantial potential of the *P. pentosaceus* HPM1 bacteriocin as a biocontrol agent for extending the shelf life of diverse food commodities, including vegetables, fruits, meat, and fish, under two distinct storage regimes: ambient temperature and freezing conditions. The application of the bacteriocin-containing cell-free culture supernatant prolongs shelf life across all tested items (Table 4) compared to controls. Under ambient storage, increases in freshness were recorded for vegetables; specifically, the shelf life of tomato extended from 7 to 15 days, cucumber from 9 to 16 days, bitter gourd from 7 to 10 days, and pointed gourd from 5 to 11 days. Fruits also showed considerable benefit: apples extended from 12 to 22 days, bananas from 7 to 10 days, grapes from 7 to 14 days, and sapodilla from 4 to 7 days (Figs. S1-S3). Highly perishable animal products showed remarkable improvements, with meat storage life increasing from approximately 0.45 days to 1.25 days and fish from 0.45 days to 1.33 days. Furthermore, the bacteriocin treatment also enhanced refrigerated storage time. Fruits and vegetable shelf life increased significantly, such as grapes from 10 to 18 days, tomatoes from 12 to 19 days and cucumbers from 13 to 20 days. Animal products benefited similarly, with meat storage life rising from 9 to 14 days and fish from 10 to 15 days (Figs. S4-S6).

Table 4
Shelf-life enhancement property of bacteriocin produced by *P. pentosaceus* HPM1

Name of foods	Shelf life at room temperature (days)		Shelf life at freezing temperature (days)	
	Control	HPM1	Control	HPM1
Tomato	7 ± 0	15 ± 1	12 ± 1	19 ± 1
Cucumber	9 ± 1	16 ± 1	13 ± 0	20 ± 1
Bitter gourd	7 ± 1	10 ± 0	10 ± 1	15 ± 1
Pointed gourd	5 ± 0	11 ± 1	9 ± 0	14 ± 1
Apple	12 ± 1	20 ± 1	20 ± 0	28 ± 1
Banana	7 ± 1	10 ± 0	9 ± 0	14 ± 1
Grapes	7 ± 1	14 ± 2	10 ± 1	18 ± 2
Sapodilla	4 ± 1	7 ± 0	7 ± 1	11 ± 1
Meat	0.45 ± 0.01	1.25 ± 0.02	9 ± 0	14 ± 1
Fish	0.45 ± 0.01	1.33 ± 0.02	10 ± 0	15 ± 1

The comprehensive assessment of preservation treatments revealed significant impacts on sample weight and pH across the various food matrices evaluated. Untreated samples consistently exhibited significant weight loss compared to their fresh ones, indicating moisture depletion (Tables S8, S13 and S18). In contrast, all food samples treated with the *P. pentosaceus* HPM1 bacteriocin exhibited remarkable weight stability (Figs. 7a, 8a, and 9a) and their strength to retain moisture during storage. pH analysis also indicated further spoilage of untreated samples; tomato, grapes, meat and fish tended to become alkaline, whereas cucumber, bitter gourd, pointed gourd, apple and banana tended to become acidic (Tables S9, S14 and S19). Importantly, there was no significant change in the pH level of treated samples compared to their original fresh conditions (Figs. 7b, 8b and 9b), indicating that the bacteriocin was very effective in preserving this important biochemical parameter.

Furthermore, the nutritional value of the food products was assessed through the measurement of carbohydrate (Tables S10, S15 and S20), protein (Tables S11, S16 and S21), and fat concentrations (Tables S12, S17 and S22). Untreated commodities exposed to spoilage exhibited statistically significant reductions in these nutrient levels compared to their fresh counterparts. In stark contrast, vegetables, fruits, meat, and fish treated with the *P. pentosaceus* HPM1 supernatant maintained nutrient levels that were approximately equivalent to those of the fresh samples, with no significant differences observed. Overall, the HPM1 bacteriocin treatment consistently emerged as the superior approach for maintaining the physiochemical and nutritional quality of all food commodities tested. (Figs. 7c-e; Figs. 8c-e; Figs. 9c-e).

Cell Cytotoxicity of Bacteriocin by MTT Assay

The cytotoxic profile of the bacteriocin produced by *P. pentosaceus* HPM1 was evaluated via the MTT assay across a concentration range of 10 µg/mL to 100 µg/mL. Treatment with the bacteriocin at all concentrations resulted in cell viability percentages consistently above the untreated blank control, with values reaching as high as approximately 140% at 100 µg/mL (Table S23 and Fig. 10). These results indicate that the bacteriocin has no cytotoxic effect; it appears to support cellular metabolic activity. Conversely, the addition of 1 µM H_2O_2 (positive control) induced a significant ($p < 0.05$) decrease in cell viability into 20%. The contrast between the high viability observed with the bacteriocin treatment and the cell death induced by the H_2O_2 positive control confirms that the bacteriocin is entirely non-toxic and biocompatible within the tested concentration range.

Discussion

The preceding study established that the microbial ecosystem promotes the fermentation of beuli dal bari, which is composed of beneficial lactic acid bacteria [38]. In this experiment, specific LAB strains were successfully isolated from beuli dal bari. The uniformity in colony morphology across all isolates strongly indicates the presence of a certain LAB community under fermentation conditions. Therefore, the pure culture HPM1 was

designated for further detailed exploration. When the CFCS of isolate HPM1 was analysed against a broad panel of foodborne pathogens, encompassing both gram-positive (*E. faecalis, B. cereus, S. aureus, C. botulinum*) and gram-negative species (*P. aeruginosa, V. cholerae, K. pneumoniae, E. coli*), it yielded highly encouraging results [39, 40, 41]. The observed variation in HPM1's inhibitory activity underscores the evidence of strain-specific secondary metabolites (e.g., bacteriocins or organic acids) production. [42] Furthermore, its antifungal potential against typical spoilage and pathogenic moulds and yeasts (*Aspergillus spp.* and *C. albicans*) [43, 44] provides more practical advantages. This significant ability to denature both prokaryotic and eukaryotic contaminants [45] grants HPM1 as a very promising applicant to use as a bio-preservative producer. In line with this, the antibacterial efficacy of HPM1 was statistically comparable to *L. plantarum* (the positive control) [46] against the majority of indicator strains. This observation indicates that HPM1 produces antimicrobial compounds functionally similar to those produced by the reference strain, strongly pointing towards bacteriocin biosynthesis. Particularly, HPM1 shows statistically superior activity against *C. botulinum*, suggesting unique potency against anaerobic food spoilage organisms. To establish the proteinaceous nature of these agents [47], the CFCS from both HPM1 and the positive control was treated with proteinase K. The total loss of antimicrobial activity upon treatment in both samples provides evidence that the potent, broad-spectrum activity is mediated by proteinaceous compounds [48], that's bacteriocins. After antimicrobial activity analysis through Arbitrary Unit (mm^2/ml) calculation against specific pathogens, such as *L. monocytogenes* (1776.10 mm^2 /ml) and *P. aeruginosa* (3195.66 mm^2 /ml), confirms that HPM1 belongs to the group of highly effective antimicrobial peptide producers [6, 49, 50] and is suitable for practical application in food preservation.

Preliminary morphological characters and biochemical profile provide sufficient results [13] for identifying HPM1 as a bacteriocin-producing LAB. Positive results for versatile carbohydrate fermentation (glucose and sucrose) infer HPM1 as a *Pediococcus* sp [51]. Subsequent 16S rRNA gene sequencing confirmed this taxonomic assignment, with the NCBI accession number PP341216, which has been submitted to secure data integrity. Overall, this strong scientific characterisation of HPM1 as *Pediococcus pentosaceus* reconfirms it as a bacteriocin-producer strain. Next, focus on isolating and characterising the specific bioactive peptides that show antimicrobial power.

To facilitate this goal, revealed that bacteriocin production by isolate *P. pentosaceus* HPM1 is linked to specific growth conditions. Optimum growth is found in the range of 35–40°C and pH 6.5-7.0 (slightly acidic to neutral) [53]. After an initial lag phase during the first 24 hours, it achieves an optimum level with exponential growth kinetics between 36 and 48 hours [52]. The described growth curve, which indicates a slow start, then fast growth, is normal bacterial kinetics when it produces different secondary metabolites. Upon optimisation of the growth conditions, the bacteriocin was isolated and purified from the cell-free culture supernatant. The first step was the 90% ammonium sulfate precipitation, which successfully concentrated the presumed proteinaceous material. This concentrate was successfully fractionated using reversed-phase High-Performance Liquid Chromatography (HPLC). The observation that antibacterial activity against *L. monocytogenes* was selectively localised to fractions F5 through F9 confirms the success of the partial purification and indicates that the active bacteriocin is distinct from other inactive precipitated components [27]. The application of MALDI-TOF mass spectrometry provided the first critical insight into the bacteriocin's molecular identity, yielding a precise monoisotopic mass of 1231.492 Da. This low molecular weight strongly suggests that the bacteriocin produced by HPM1 belongs to the class of lanthionine-containing peptides (Class I bacteriocin), which are characteristically small, post-translationally modified peptides [54, 55]. This initial structural data is essential to locate HPM1 as a well-investigated bacteriocin producer.

Thermal stability is one of the important factors for industrial use of bacteriocins, where pasteurisation and sterilisation-type procedures are applied. *P. pentosaceus* HPM1 produces a bacteriocin showed a remarkable thermostability even after extreme thermal stress, such as 121°C. While a marginal reduction in activity was observed with increasing time and temperature exposure, likely attributed to partial protein denaturation, the metabolite's ability to retain potent activity levels after autoclaving is highly significant. This flexibility suggests that the HPM1 bacteriocin possesses a compact, stable molecular structure, potentially stabilised by disulfide bridges or a high proportion of hydrophobic amino acids [56, 57]. In addition to its thermal stability, the *P. pentosaceus* HPM1 bacteriocin has a pH stability that increases its application in a variety of food matrices. The results indicate that the bacteriocin exhibits maximum antimicrobial activity at pH 6.0, which is approximately similar to its physiological production environment (pH 6.5). While the peptide displays a gradual decline in inhibitory potential when exposed to acidic (pH 4.0–5.0) or alkaline (pH 8.0–9.0) conditions, it is interesting that the bacteriocin does not totally lose its potency. Which suggest that while extreme ionisation states may cause minor conformational changes, the molecule remains effective enough to bind and permeate target microbial cell membranes. Although efficiency is maximum between pH 6.0 and 7.0 [21], the fact that *P. pentosaceus* HPM1 retains activity over a broader pH range highlights its working potential. These stability profiles place the *P. pentosaceus* HPM1 bacteriocin as a promising natural preservative.

These stability profiles of *P. pentosaceus* HPM1 bacteriocin directly strengthen the findings in food-matrix applications. Preservation assessment finds it is highly effective at inhibiting spoilage and pathogenic proliferation across a wide array of food matrices under both ambient and frozen storage conditions. The extension of shelf life for highly perishable items like fresh meat and fish at room temperature suggests that the bacteriocin effectively suppresses spoilage organisms relevant to these matrices. Moreover, the increased shelf life under frozen storage indicates that the bacteriocin retains its structural integrity and activity even after prolonged exposure to refrigerated temperatures, making it adaptable to cold chain distribution systems. The effect of HPM1 bacteriocin on vegetables, fruits, and animal products puts it in a favorable position to use as an effective and natural biopreservative to extend the food safety and minimise post-harvest wastage.

These results show that the *P. pentosaceus* HPM1 bacteriocin not only increases the shelf life but also good in protect the structure and nutrient parameters of various food components. The observed weight stability in treated samples suggests that by suppressing the proliferation of spoilage microflora, the bacteriocin effectively reduces the rate of enzymatic and microbial breakdown that typically leads to moisture loss and tissue

degradation [58]. The stabilisation of pH levels in treated samples is particularly notable. The fluctuations observed in untreated groups, alkalinization in protein-rich substrates (meat/fish) and acidification in carbohydrate-rich samples are characteristic markers of metabolic spoilage and microbial contamination. By inhibiting these microbial metabolic pathways [59], the HPM1 bacteriocin prevents the rapid shifts in the chemical environment that lead to organoleptic decline. Moreover, the insignificant alteration in carbohydrate, protein concentrations and fat percentage before and after preservation highlights the blockage of nutrient utilisation by the spoilage microbe, present in foodstuffs. These findings of prolonged shelf life and retained nutrient parameters [60] of foodstuffs make it a suitable alternative to use as a preservative at small and commercial levels.

Finally, to ensure consumer safety, assessing the cytotoxicity of potential food-grade antimicrobial agents has become a crucial need. In this study, the MTT assay was performed to compare the effects of the *P. pentosaceus* HPM1 bacteriocin with a known cytotoxic agent, H_2O_2. The positive control (H_2O_2) effectively validated the assay by inducing a drastic reduction in cell viability, a result consistent with its known ability to generate oxidative stress and trigger apoptosis [61]. In contrast, the bacteriocin-treated groups showed no signs of toxicity; rather, they exhibited a dose-dependent increase in metabolic activity. This outcome suggests that the *P. pentosaceus* HPM1 bacteriocin shows no negative impact on mammalian cell viability even at 100 µg/mL concentration. Furthermore, the absence of cytotoxic effects of the bacteriocin, contrasted with the significant cellular degradation observed in the presence of H_2O_2, strongly emphasised the suitability of this antimicrobial metabolite for safe employment in food systems.

Conclusion

This work finds that *Pediococcus pentosaceus* HPM1, isolated from beuli dal bari, produces a bacteriocin that is a promising natural alternative to chemical preservatives. It gives significant promise to enhance food safety and maintain quality in food preservation. This finding establishes a productive partnership between traditional food microbiology and modern industrial preservation methods by isolating the effective nontoxic antimicrobial agent from *P. pentosaceus* HPM1. The strong and stable activity against food spoilage microbes supports its employment under various processing and storage conditions. Its effectiveness in extending the shelf life of different food products (fruits, vegetables, meat, and fish) highlights its viable economic and environmental sustainability. Additionally, it is also useful as a preservation approach, especially as a surface treatment, which is convenient to the consumer. A benefit of having an easy removal with just a simple washing procedure coincides with the consumer needs of having a low amount of additive residues, and thus it is a good substitute of the traditional preservatives. Future studies will aim to elucidate its mechanism of action and further analyse its chemical structure to better define the uniqueness of the isolated bacteriocin.

Declarations

Conflict of Interest

The authors confirm that they have no competing financial interests or personal relationships that might have influenced the research presented in this paper.

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Author Contribution

Conceptualisation: P.S., H.K.J, M.K.P., P.S., S.S.G.; Methodology: P.S., H.K.J, M.K.P.; Validation: H.K.J, M.K.P.; Formal analysis: P.S.; Investigation: P.S.; Data curation: P.S., P.S., S.S.G.; Writing—original draft preparation: P.S.; Writing—review and editing: All authors; Supervision: H.K.J, M.K.P.; Funding acquisition: P.S. All authors have read and agreed to the published version of the manuscript.

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Figures

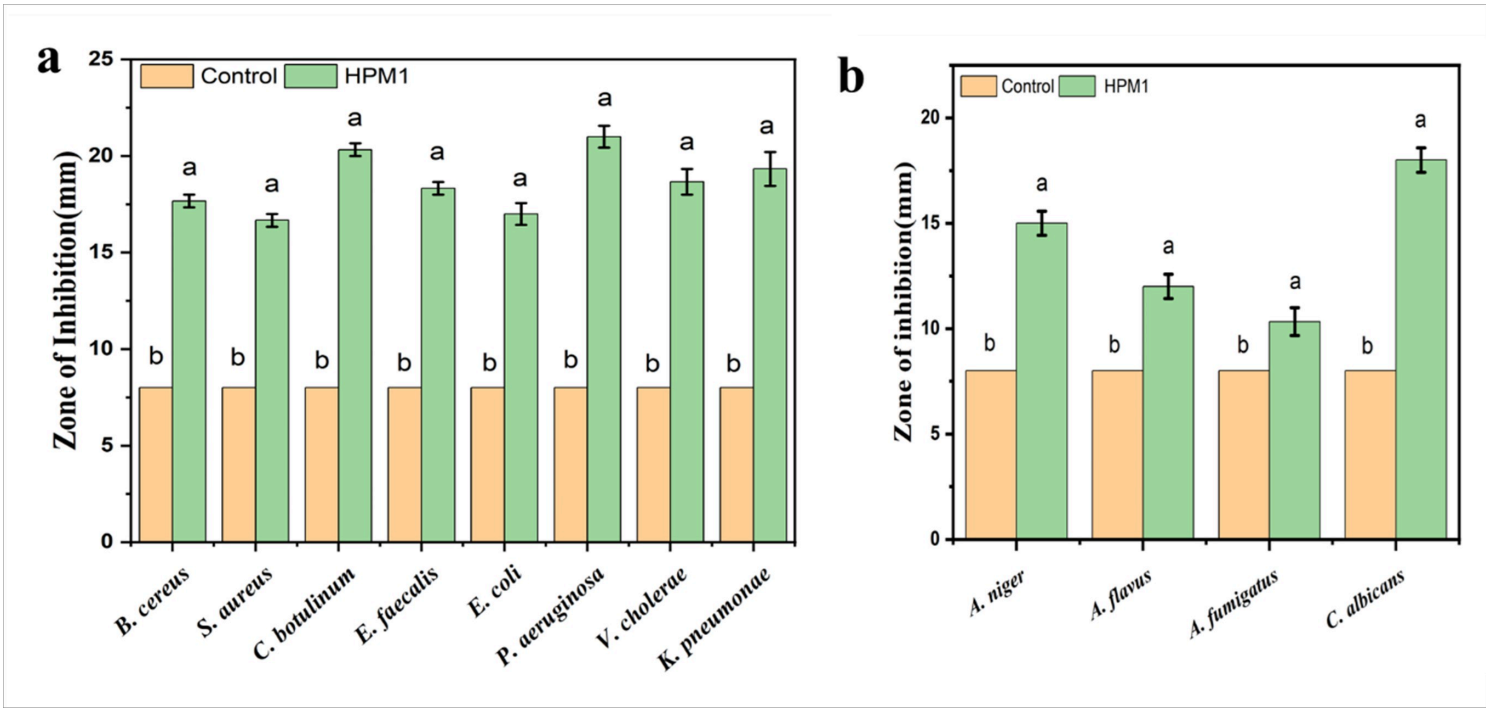


Figure 1

Antimicrobial efficacy of isolated HPM1 (a) antibacterial activity against selected food spoilage (indicator strains) bacteria (b) Antifungal activity against food spoilage fungus. Data are presented as the mean zone of inhibition (mm) ± standard error of the mean (SEM). Bars sharing different letters indicate statistically significant differences ($p < 0.05$).

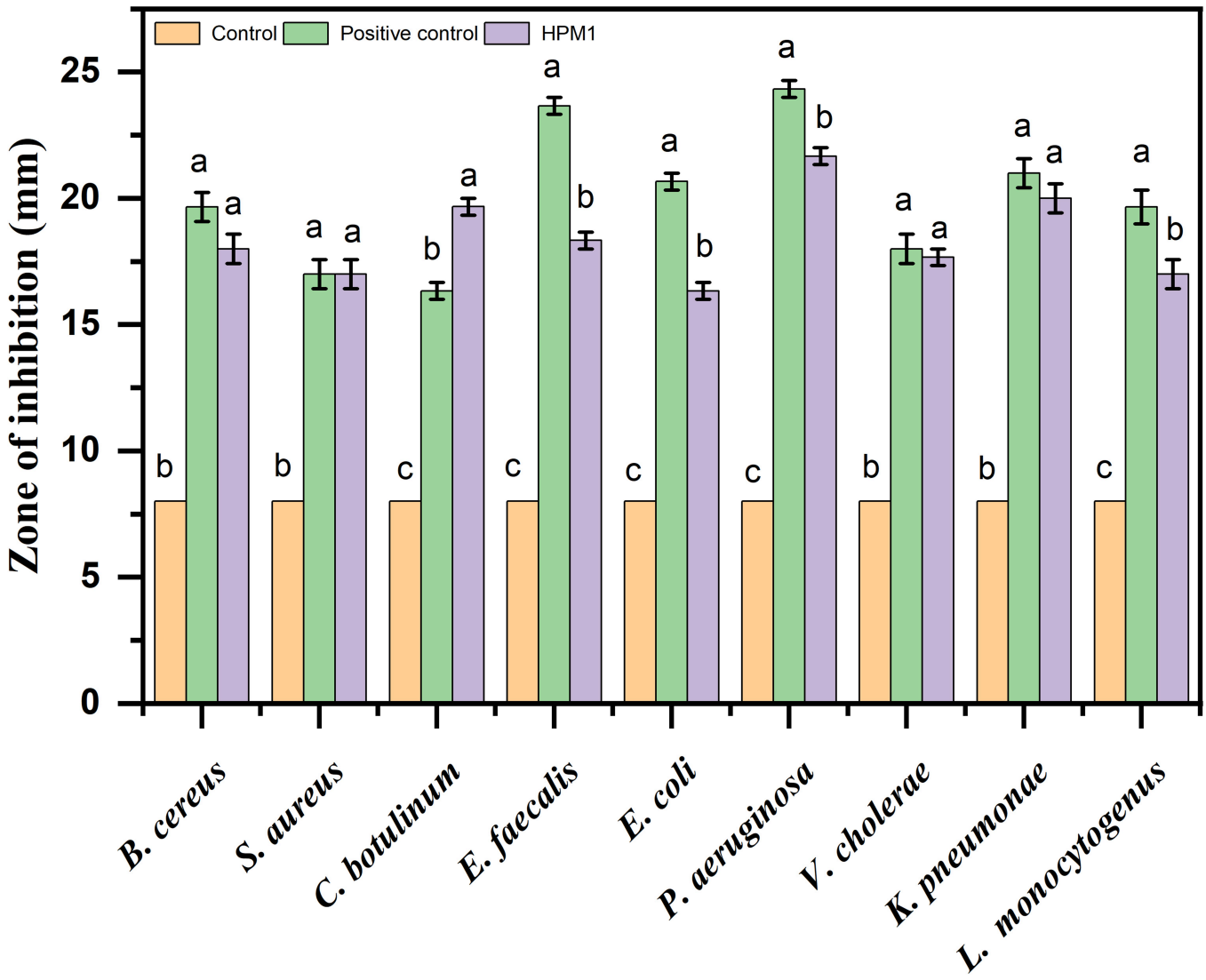


Figure 2

Comparative assessment of bacteriocin production by isolate HPM1 and positive control against indicator strains. Data are presented as the mean zone of inhibition (mm) ± standard error of the mean (SEM). Bars sharing different letters indicate statistically significant differences ($p < 0.05$) by Tukey's HSD post-hoc test following ANOVA

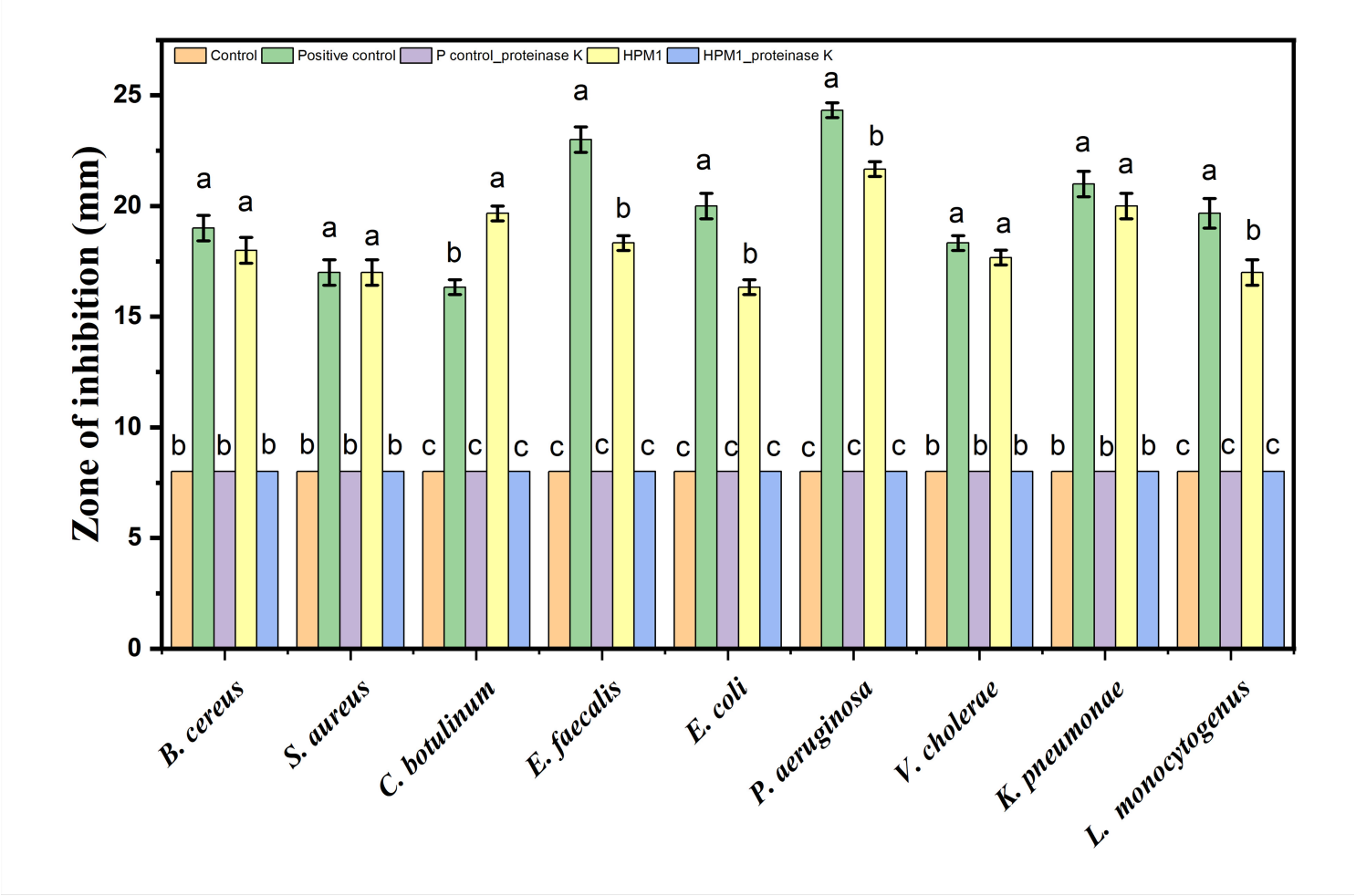


Figure 3

Confirmation of bacteriocin production by isolate HPM1 against indicator strains via proteinase K treatment. Data are presented as the mean zone of inhibition (mm) ± standard error of the mean (SEM). Bars sharing different letters indicate statistically significant differences ($p < 0.05$) by Tukey's HSD post-hoc test following ANOVA

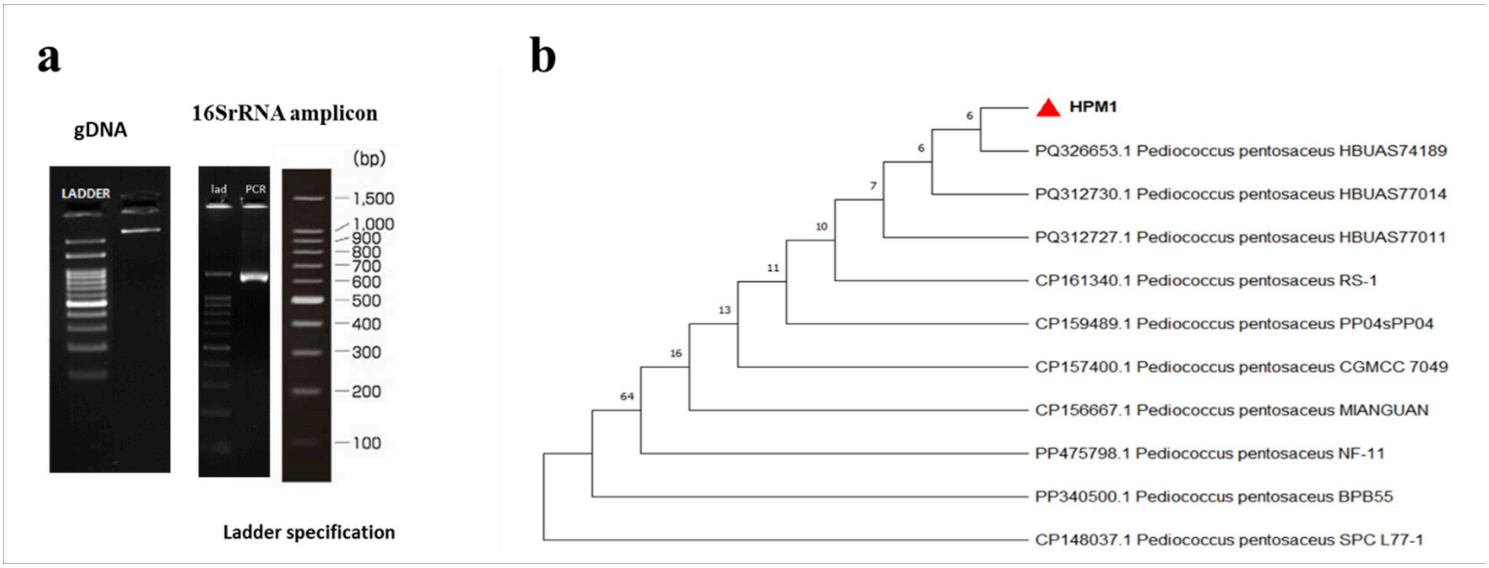


Figure 4

(a) 2% agarose gel visually displayed a single amplicon of 1500 base pairs, representing the 16s rRNA gene of the HPM1 isolates. (b) Phylogenetic trees constructed via the neighbour-joining method, where bootstrap support values (from 1000 replicates) are indicated on the branches

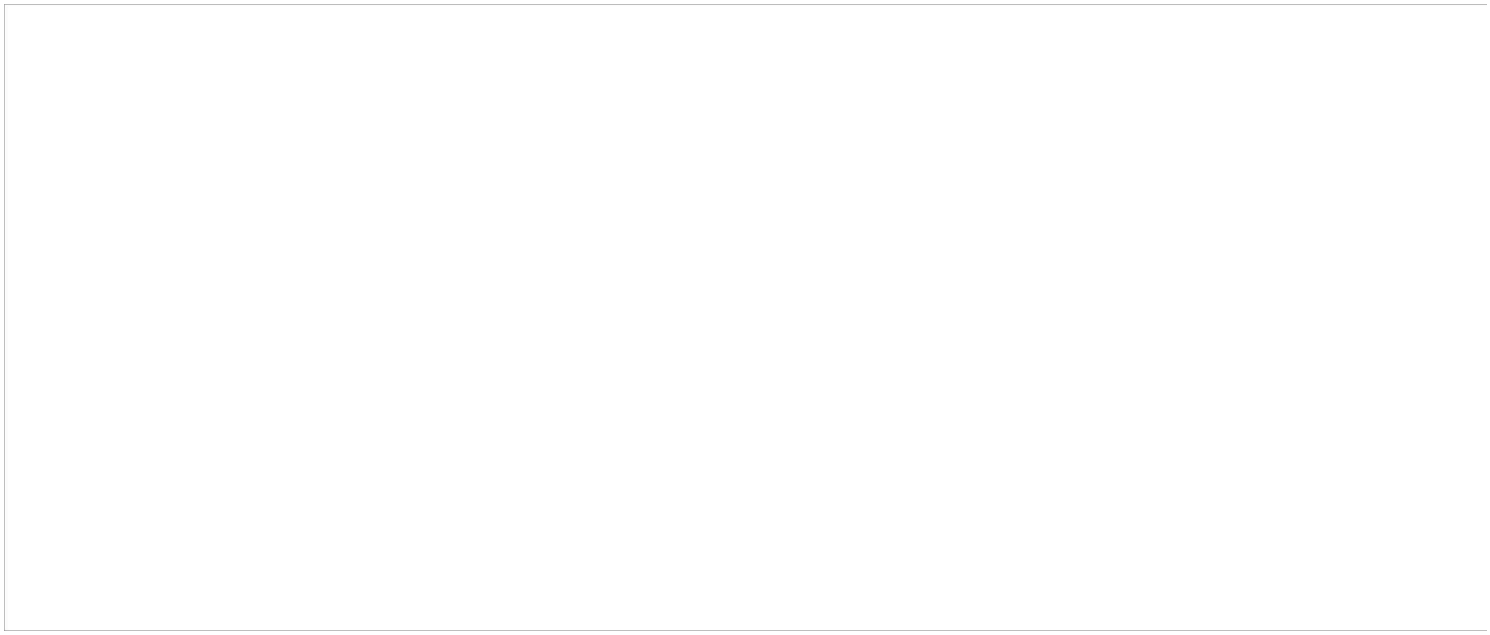


Figure 5

Growth kinetics of *P. pentosaceus* HPM1. (a) Growth profiles at different temperatures. (b) Growth profiles at different pH values. Optical density (OD) was measured at 560 nm over a 72-hour incubation period. Error bars indicate the standard deviation around the mean.

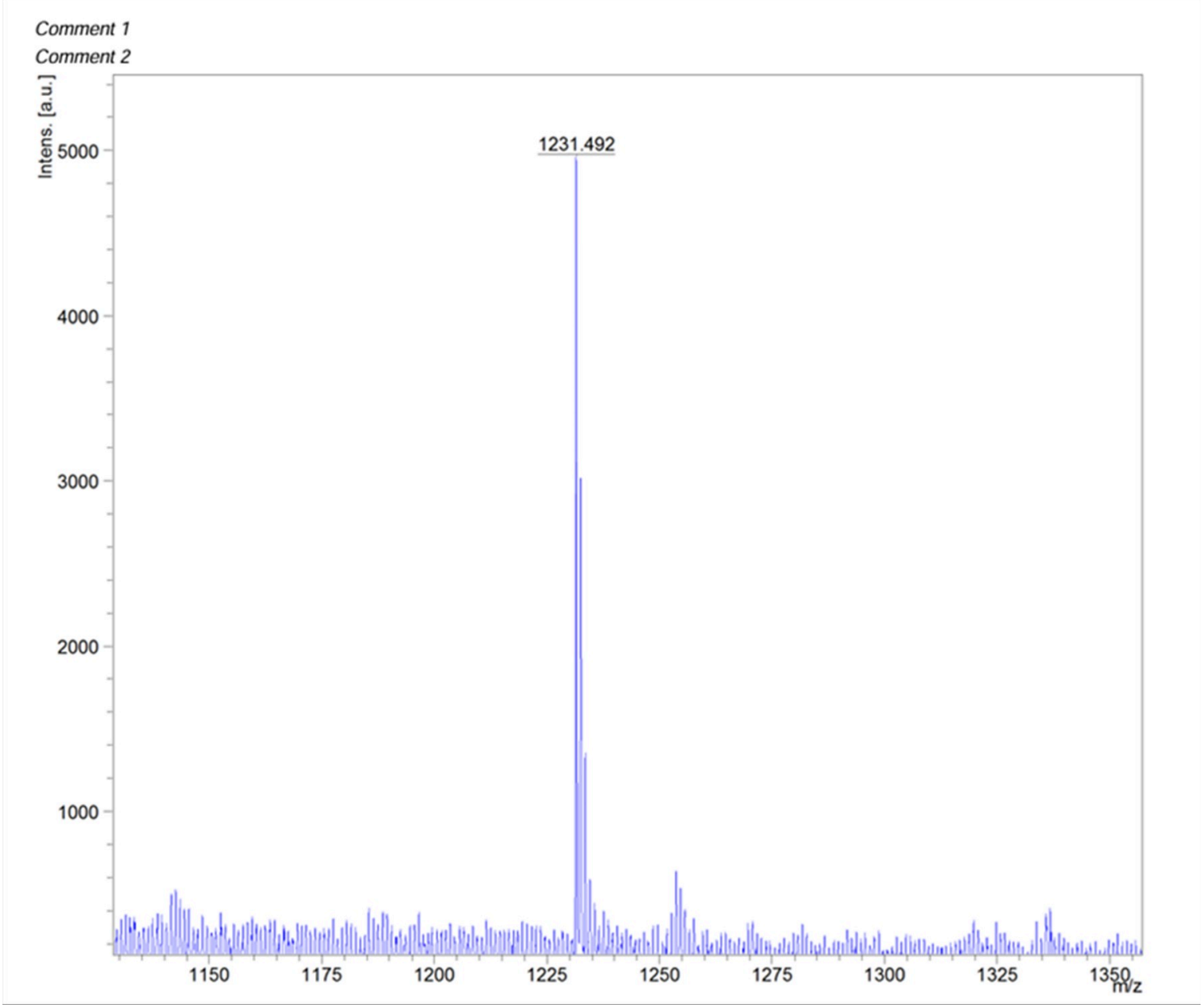


Figure 6

Mass spectrum of the bacteriocin produced by *P. pentosaceus* HPM1, confirming its molecular weight as 1231.492 Da.

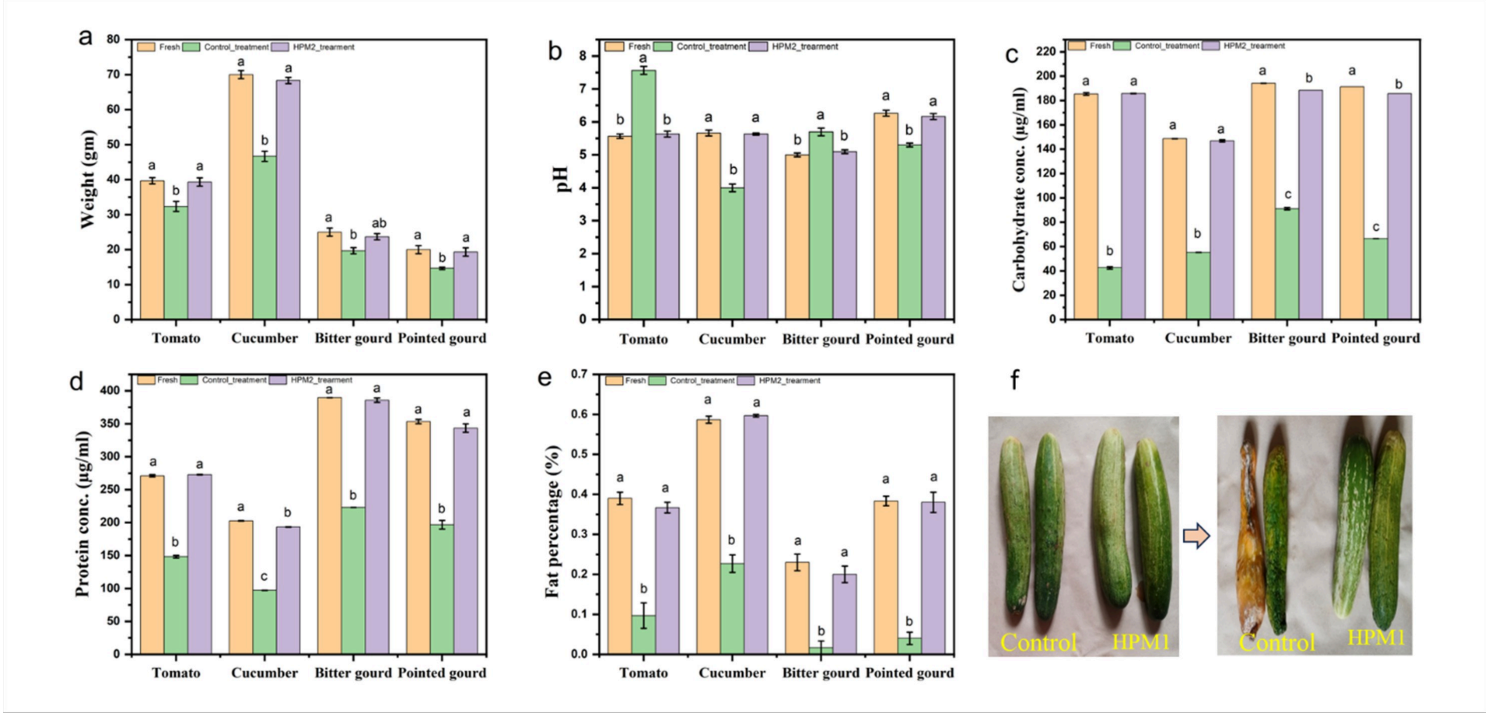


Figure 7

Impact of *P. pentosaceus* HPM1 bacteriocin preservation on the physical and nutritional parameters of selected vegetables (tomato, cucumber, bitter gourd and pointed gourd) before and after preservation. Bars sharing different letters indicate statistically significant differences ($p < 0.05$) by Tukey's HSD post-hoc test following ANOVA (a) Weight, (b) pH, (c) Carbohydrate concentration, (d) Protein concentration, and (e) Fat percentage. (f) Food preservation assessment of cucumber.

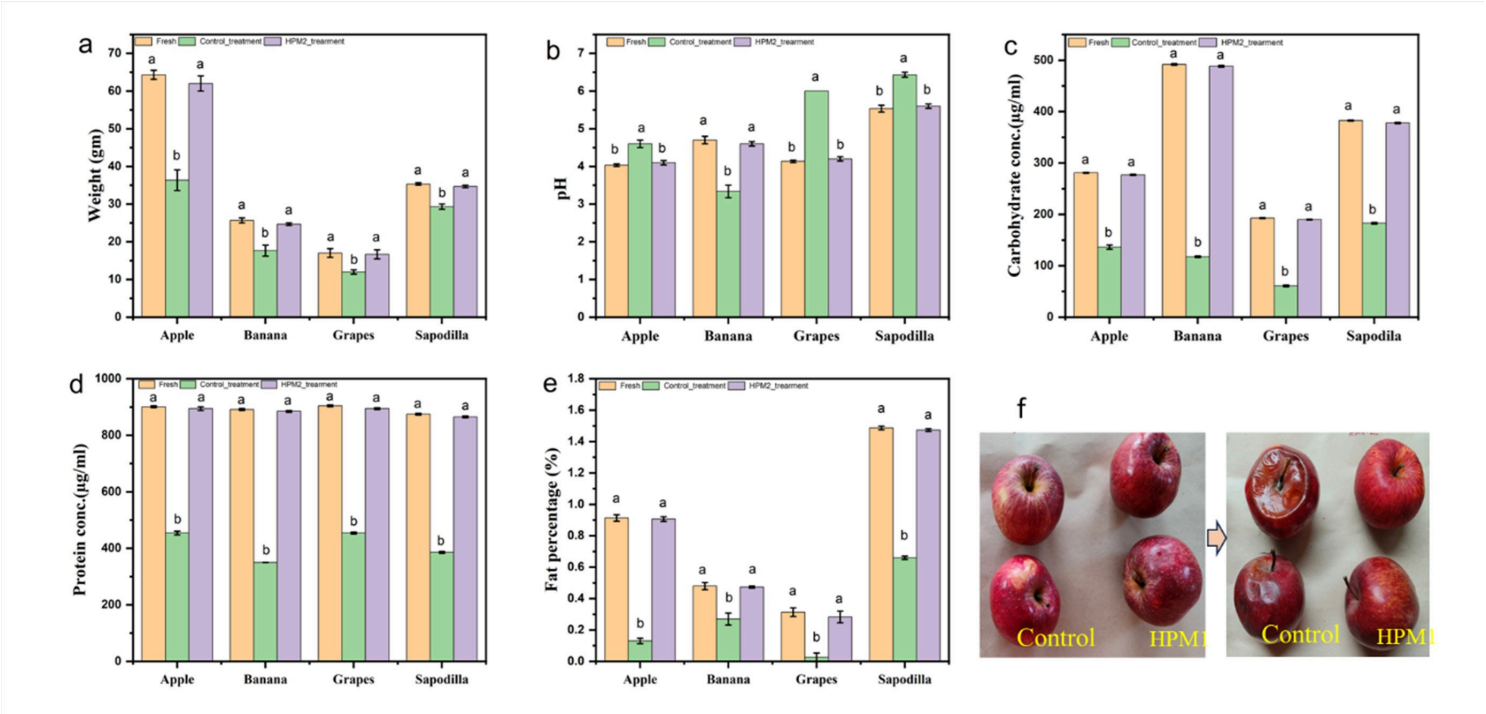


Figure 8

Impact of *P. pentosaceus* HPM1 bacteriocin preservation on the physical and nutritional parameters of selected fruits (apple, banana, grapes and sapodilla) before and after preservation. Bars sharing different letters indicate statistically significant differences ($p < 0.05$) by Tukey's HSD post-hoc test following ANOVA (a) Weight, (b) pH, (c) Carbohydrate concentration, (d) Protein concentration, and (e) Fat percentage. (f) Food preservation assessment of apple.

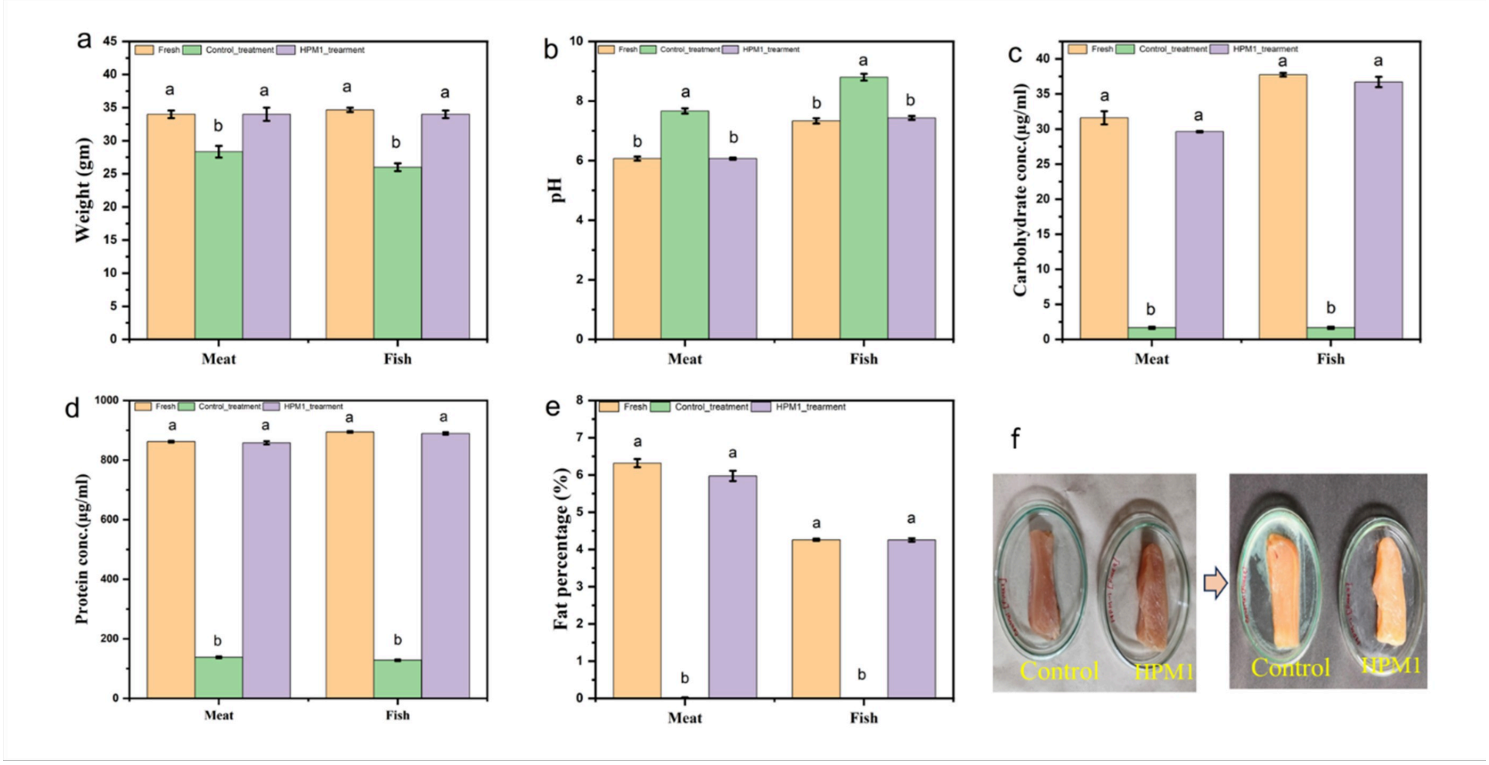


Figure 9

Impact of *P. pentosaceus* HPM1 bacteriocin preservation on the physical and nutritional parameters of selected animal products (meat, fish) before and after preservation. Bars sharing different letters indicate statistically significant differences ($p < 0.05$) by Tukey's HSD post-hoc test following ANOVA (a) Weight, (b) pH, (c) Carbohydrate concentration, (d) Protein concentration, and (e) Fat percentage. (f) Food preservation assessment of meat.

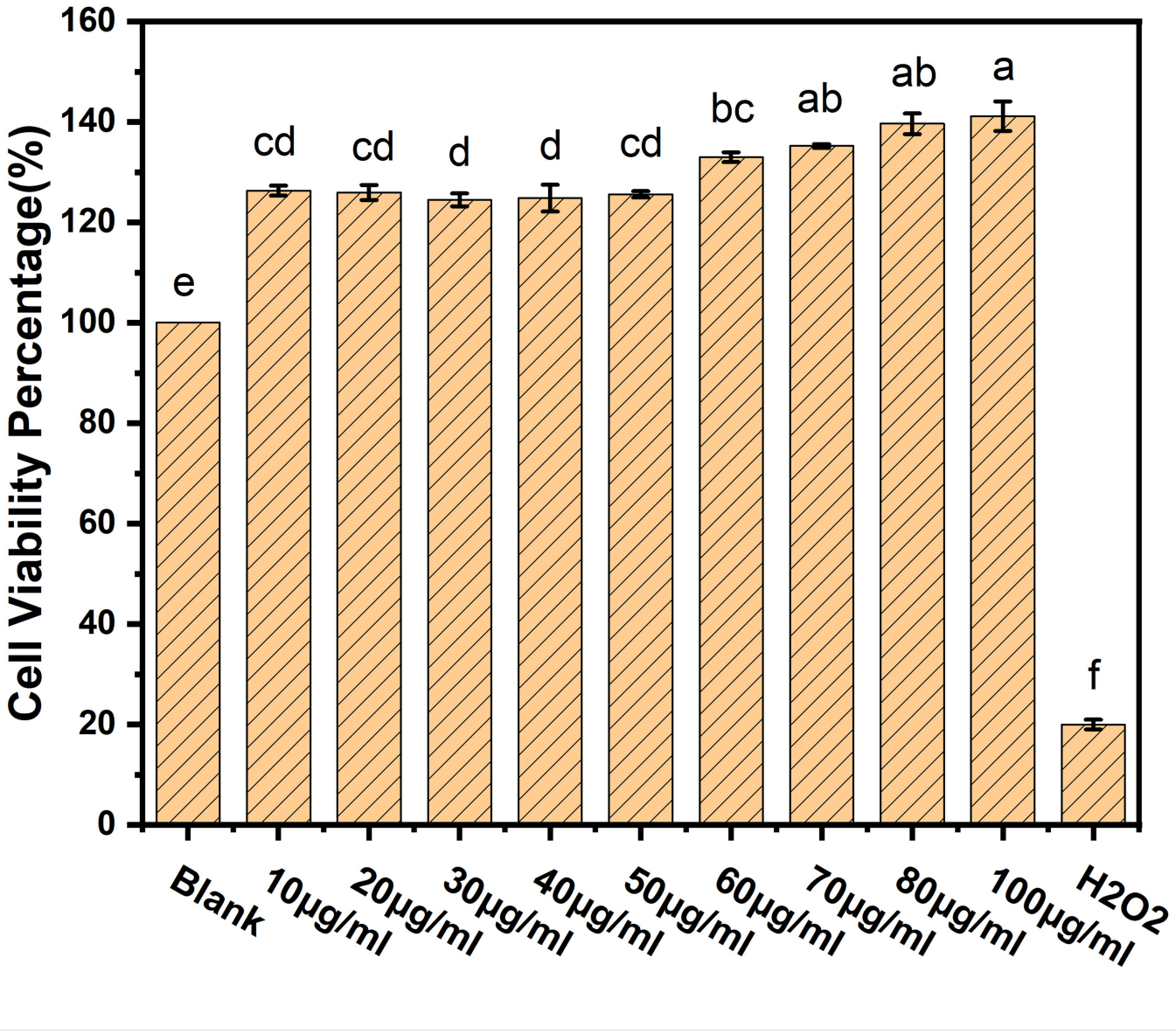


Figure 10

Cytotoxicity profile of *P. pentosaceus* HPM1-derived bacteriocin by MTT assay. Bars sharing different letters indicate statistically significant differences ($p < 0.05$) by Tukey's HSD post-hoc test following ANOVA

Supplementary Files

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- supplementary.docx
- image1.png