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Purification and characterization of plantaricin K-2 from *Lactiplantibacillus plantarum* K-2 isolated from locally fermented cabbage

Khonsuluv A. Sohibnazarova[1]*, Muzaffar Muminov[1], Iskandar Yakubov[2], Azimjon Abdunabiev[1], Shakhnoza Umarova[1], Jakhongirbek Gulomov[1], Yulduz Abduvokhidova[1], Urmonalieva Shakhlo[1], Ibragimova Shakhnoza[1], Akmal M. Asrorov[3], Fazliddin Kobilov[4], Bozorov Soyibjon[1], Dilbar Dalimova[1], Shakhlo Miralimova[4]

[1]Center for Advanced Technologies, Tashkent, Uzbekistan.
[2]Department of Botany and Genetics, Faculty of Biology and Ecology, National University of Uzbekistan, Tashkent, Uzbekistan.
[3]Department of Laboratory for Creating Technologies of Natural Drugs Based on Biopolymers, Institute of Bioorganic Chemistry of Uzbekistan Academy of Sciences, Tashkent, Uzbekistan.
[4]Department of Microbiology and Biotechnology of Probiotics, Institute of Microbiology of Uzbekistan Academy of Sciences, Tashkent, Uzbekistan.

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ABSTRACT

Bacteriocins are considered to be safe food preservatives, which have great potential to replace antibiotics due to their high efficacy against multidrug-resistant pathogens. In this work, the bacteriocin plantaricin K-2 was purified by three-stage purification – cation exchange chromatography, hydrophobic chromatography, and reversed-phase high-performance liquid chromatography with the purpose of establishing its practical value. The molecular weight of the peptide was 3937.05 Da by mass spectrometric analysis (Quadrupole Time-of-Flight mass spectrometry). The bacteriocin plantaricin K-2 was identified to belong to Class IIa and its physico-biochemical parameters were determined, and the 16S ribosomal DNA sequence analysis confirmed that the strain synthesizing it was *Lactiplantibacillus plantarum*. Plantaricin K-2 did not show antimicrobial activity when treated with proteases, but showed high thermal stability at high temperatures (121°C, 30 min). Production of plantaricin K-2 reached 6400 AU/mL during the stationary growth phase of *L. plantarum* K-2 cultivated in MRS at 37°C for 48 h. Although plantaricin K-2 is usually classified as a narrow-spectrum Class IIa bacteriocin, it showed relatively broad activity against food microbes and pathogenic microorganisms. The results of the study show that plantaricin K-2, belonging to a Class IIa bacteriocin, is a promising natural and safe biological object that could be used for preserving food as an antibiotic-resistant means in clinical applications.

1. INTRODUCTION

The use of bacteriocins from lactic acid bacteria (LAB) strains has several advantages over traditional antibiotics. In particular, the production of recombinant forms of the peptide, its potential for application in both the food and clinical industries, its high efficiency, and its relative lack of toxicity to eukaryotic cells have made these peptides very interesting and promising candidates for combating pathogenic strains [1-3]. Klaenhammer initially divided them into four classes: (I) lantibiotics – small molecules containing post-translationally modified peptides, lanthionine and β-methyllanthionine (e.g., nisin); (II) unmodified, small and thermostable peptides (e.g., pediocin); (III) large, thermolabile bacteriocins (helveticin and colicin); and (IV) complex bacteriocins linked to glycols or lipoproteins.

The Class IIa pediocin-like bacteriocins are considered among the most promising candidates for use as potential bioconservants in the food industry due to their strong anti-Listeria activity, which is associated with their common YGNGV motif at the N-terminus of the peptide [4,5]. In recent years, various researchers have reported a number of Class IIa bacteriocins from LAB strains, such as plantaricin LPL-1 [6], GA15 [7], SR18 [8], plantaricin 423 [9], plantaricin C19 [10], plantaricin IIA-1A5 [11], plantaricin LD1 [12], and BM1029; however, only one commercial product, nisin, is currently registered on the market [13]. In food preservation, more and more bacteriocins have been tested as natural antimicrobial agents due to their potent antibacterial properties [14]. Bacteriocins inhibit the growth of many foodborne pathogens and spoilage bacteria, such as *Staphylococcus aureus* [15], *Listeria monocytogenes, Escherichia coli, Pseudomonas spp*., *Bacillus spp.,* and *Clostridium* spp. They can also be used in various food products to increase shelf life [16].

**Corresponding Author:*
Khonsuluv A. Sohibnazarova,
Center for Advanced Technologies, Tashkent, Uzbekistan.
E-mail: xonsuluv91as @ gmail.com

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Another important aspect of this group of bacteriocins is their resistance to high temperatures and pressures. The low molecular weight of the proteins and the presence of disulfide (S-S) bonds are significant factors for their temperature resistance (heat stability). For example, the above-mentioned LD-1 plantaricin and LPL-1 [6], which have a molecular weight of up to 7 kDa, are both thermoresistant at 100°C and 121°C, while the IIA-1A5 peptide, which has a molecular weight of 9.5 kDa, is thermoresistant at 60–80°C [11]. Thus, the identification of potential LAB strains producing bacteriocins [17], as well as the identification and characterization of bacteriocins that meet the needs of the market, is still significant.

In addition, purified bacteriocin was partially characterized for use in the food industry as a food preservative. In recent years, special attention has been given to isolating and characterizing new bacteriocin-producing strains [18,19]. The purpose of this study was to screen and identify a powerful strain of LAB, as well as a bacteriocin. In the present work, an antimicrobial bacteriocin produced by *Lactiplantibacillus plantarum* K-2 was purified and its primary characterization was performed.

2. MATERIALS AND METHODS

2.1. Culture Conditions, Growth, and Isolation of Bacteria

Traditional homemade fermented cabbage was sliced and added to a saline solution, and 10 µL of the sample was inoculated into dishes with MRS agar medium (HiMedia, India) using a cup distribution technique. The Petri dishes were incubated at 37°C for 24 h, following the identification of bacteria. All indicator strains were cultured in Mueller–Hinton broth at 37°C and stored at −80°C in Mueller–Hinton medium (HiMedia, India) supplemented with 25% (vol./vol.) glycerol.

2.2. Screening of Bacteriocin-producing Strains of LAB

In this work, only those with high antimicrobial activity were selected from more than 110 previously studied Lactobacillus isolates. The highly active lactobacillus isolate was inoculated into 5 mL of MRS broth and cultured under anaerobic conditions at 37°C for 24 h. The cell-free supernatant was obtained by culture centrifugation at 7000 ×g for 20 min at 4°C and subjected to a test for antimicrobial activity by diffusion into agar wells after bringing the pH of the supernatant to 6.8 ± 0.2 using 1 M sodium hydroxide (NaOH). *L. monocytogenes* ATCC 1911 was used as an indicator strain, and trypsin or proteinase K was applied only on one side of the wells to ensure that the antimicrobial activity was due to the substances in the protein. The antibacterial activity of the isolates was evaluated by measuring the diameters of the inhibition zones. Bacteriocin-producing isolates with relatively high antibacterial activity were selected for subsequent studies.

2.3. Identification of the *L. plantarum* K-2 Strain

Identification of potential strains of LAB-producing bacteriocin was carried out in accordance with their cellular morphology, physiological characteristics, carbohydrate fermentation models, and Gram staining properties. The most active bacteriocin-producing strains, including *L. plantarum* K-2, were additionally identified by 16S ribosomal RNA (rRNA) sequencing using the following universal PCR primers: 16S rRNA–F: BAC27f–GAGTTTGATCMTGGCTCAG and 16 rRNA–R: BAC1492r–GGYTACCTTGTTACGACTT [20]. The results of 16S rRNA sequencing were aligned against NCBI database, and a phylogenetic tree was constructed using MEGA X software.

2.4. Growth Dynamics and Plantaricin Production

The *L. plantarum* K-2 strain was cultured in 10 mL of MRS broth to an OD_{600} = 2.0. Then inoculated in 1 L of MRS broth (pH 6.5) at 0.5% (vol./vol.) of the inoculate for 72 h at 37°C without stirring. Samples were taken every 6 h and cell density (OD_{600}), pH, and antimicrobial activity were assessed. Antimicrobial activity was calculated as activity units (AU/mL) using the dilution method. Furthermore, the activity of crude plantaricin K-2 against *L. monocytogenes* ATCC 1911 was tested using the spot-on-lawn agar method and the inhibition zones (mm) were measured.

2.5. The Process of Purification of Plantaricin K-2

L. plantarum K-2 was statically cultured in 1 L of MRS broth (HiMedia, India) for 48 h at 37°C by inoculation of 0.5% (vol./vol.) pure culture. After incubation, the culture was centrifuged at 7000 × g for 20 min at 4°C to pellet the bacterial cells. The resulting cell-free culture supernatant (CFC) was collected, and its pH was adjusted to 6.5, and the culture supernatant was incubated at +80°C for 15 min to inactivate proteases and avoid possible contamination.

2.5.1. Sequential purification of active fractions

For initial purification, the CFC was diluted 2 times using 20 mM phosphate buffer (pH 6.5) and applied to a pre-equilibrated SP-Sephadex C-25 cation-exchange column (20 mL; GE Healthcare, Sweden) equilibrated with the same buffer. The column was washed with two column volumes of 20 mM phosphate buffer (pH 6.5), and proteins were eluted stepwise using increasing concentrations of sodium chloride (NaCl) (0.1, 0.3, 0.6, 0.8, and 1.0 M) in 20 mM phosphate buffer, pH 6.5, at a flow rate of 1 mL/min. Eluted fractions were collected and assessed for antibacterial activity against the designated indicator strain. Active fractions were pooled and subjected to further purification.

Secondary purification was performed using a Sep-Pak C18 cartridge (0.5 × 2.5 cm; Waters, USA), pre-equilibrated with 1 M NaCl in 20 mM phosphate buffer (pH 6.5). Bound proteins were eluted using stepwise concentrations of acetonitrile (5%, 10%, 15%, 20%, 25%, 30%, 40%, 60%, and 80%) in 0.1% trifluoroacetic acid (TFA). Each fraction was dried, reconstituted in sterile distilled water, and again tested for antibacterial activity.

Finally, the active fraction from the Sep-Pak C18 purification was subjected to high-resolution purification using reverse-phase high-performance liquid chromatography (RP-HPLC). The sample was loaded onto a Zorbax 300SB-C18 column (5 µm, 4.6 × 250 mm; Agilent Technologies, CA, USA) and eluted using a linear gradient of acetonitrile (in 0.1% TFA) from 0% to 80% over 35 min at a flow rate of 0.5 mL/min. Elution was monitored at 280 nm, and all collected fractions were screened for antibacterial activity against the indicator strain. Antibacterial activity was quantified in arbitrary units per milliliter of culture medium (AU/mL), where one AU was defined as the reciprocal of the highest two-fold dilution (in 20 mM phosphate buffer, pH 6.5) that produced a clear inhibition zone against the indicator strain. The protein concentration in each purification step was determined using the Lowry method [21]. Based on these results, key parameters, including specific activity and recovery rate, were calculated for each purification stage.

2.6. Determination of the Molecular Weight and Analysis of Plantaricin K-2 by Agar

Molecular weight determination was carried out in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

electrophoresis. A 15% separating gel was prepared accordingly. The gel was stained with diamond blue Coomassie R-250 to determine the size of the molecule, and washed for 60 min with a triple change of distilled water. Then, 25 mL of a soft agar medium inoculated with an indicator strain was applied after removing the water and incubated for 18–24 h to match the molecular weight and antibacterial activity. The molecular weight of plantaricin K-2 was determined by time-of-flight mass spectrometry as described in our previous work [2].

2.7. Antimicrobial Spectrum of *L. plantarum* K-2 and Plantaricin K-2

The spectrum of antimicrobial action against dozens of clinical and reference Gram-positive and Gram-negative strains and some strains of fungi for both *L. plantarum* K-2 and plantaricin K-2 (samples of 90 micrograms/mL from hydrophobic interaction chromatography) was tested using agar spot analysis. 5 µL of *L. plantarum* K-2 cells grown overnight in 10 mL of MRS broth (HiMedia, India) were applied to MRS agar, and after incubation at 37°C for 48 h, the cells were killed using chloroform vapors for 30 min, and an aliquot of indicator strains (~10^{6-8} CFU/mL) incubated in Mueller–Hinton agar broth for 24 h at 37°C was placed on a spot with 5 mL of semi-solid agar. Similarly, 10 µL of partially purified plantaricin K-2 (90 µg/mL) was applied to MRS agar and subjected to antimicrobial activity against the same indicator strains by point analysis, as described above.

2.8. Effect of Proteases, Temperature, pH, and Ethanol on the Activity of Plantaricin K-2

10 µL of pepsin (3,200-4,500 units/mg protein, Sigma-Aldrich, USA), trypsin (10,000 BAEE units/mg protein, Sigma-Aldrich, USA), α-chymotrypsin (≥40 units/mg protein, Sigma-Aldrich), papain (≥10 units/mg, Sigma-Aldrich, USA), and proteinase K (≥30 units/mg protein, Sigma-Aldrich USA) at 1 mg/mL doses were separately added to 100 µL of the active samples obtained by RP-HPLC and incubated at 37°C for 4 h. Samples treated with the same amount of 20 mM phosphate buffer (pH 6.5) were used as a positive control. To determine the thermal stability of plantaricin K-2, partially purified samples from hydrophobic interaction chromatography (HIC) (90 µL/mL) were exposed to temperatures of 80°C, 90°C, and 100°C for 15 and 30 min in a thermostatic water bath and in an autoclave at 121°C for 15 min, and unincubated samples were used as a control. Solutions of partially purified bacteriocin with a pH ranging from 2 to 12 were separately prepared to test the pH stability using hydrogen chloride or NaOH and incubated at 37°C for 4 h. The pH of all the samples was adjusted to 6.5, and the samples were diluted until all the samples reached the same concentration (2.5 µL/mL) before analysis for antimicrobial activity. The samples were not subjected to pH changes but were at the concentration used as a positive control. To test the effect of ethanol, various concentrations of ethanol (5, 10, 15, 20, and 25%) were prepared in bacteriocins by adding 96% ethyl alcohol. Positive and negative samples were prepared by adding a proportional amount of sterile distilled water to aliquots of bacteriocin or ethanol to sterile distilled water, respectively. All samples were analyzed for their antimicrobial activity against the indicator strain by diffusion in agar wells, and the results are presented in mm transparent inhibition zones.

2.9. Statistical Analysis

All experiments were performed in triplicate (n = 3) and repeated at least 3 times independently to ensure reproducibility. The results are expressed as mean ± standard deviation. Statistical analyses and graphical representations were performed using GraphPad Prism (version 9.5.1).

3. RESULTS

3.1. Screening of Bacteriocin-forming Strains of LAB

In this work, we demonstrated that a strain of *L. plantarum* isolated from fermented cabbage exhibited relatively high antimicrobial activity against several indicator strains, including *L. monocytogenes* ATCC 1911 and *S. aureus* strains. However, *L. plantarum* K-2 strain isolated from home-fermented cabbage showed greater antimicrobial activity against *L. monocytogenes* ATCC 1911 than the other strains and was therefore selected as the target strain for further characterization [Table 1].

3.2. Identification of the *L. plantarum* K-2 Strain

The stained cells of LAB had the shape of a rod, without spores, when examined under a microscope, and were also determined to be Gram-positive. Other characteristics, including the nature of carbohydrate fermentation (data not shown here), suggested that the strain belongs to the genus *L. plantarum* species. *L. plantarum* K-2 16S rRNA gene amplified using universal primers and the nucleotides were sequenced. The resulting 16S rRNA nucleotide sequence was compared with that in the NCBI reference database using BLAST algorithms, and the 16S rRNA sequence of L. plantarum showed 99% similarity. Our isolate highlighted in red is MT753855.1 (*L. plantarum* strain K-2), which is located in the same group as other *L. plantarum* strains, in particular JCM 1149 (China and Great Britain).The upper cluster of the tree shows strains related to the *L. plantarum* species, while the lower branches contain representatives of *Lactobacillus fabifermentans, L. herbarium, L. xiangfangensis*, and other species. The results of this phylogenetic analysis indicate that our isolate has a close genetic relationship to the *L. plantarum* species. The phylogenetic tree was constructed using the algorithm of combining neighbors in the MegaX program [Figure 1]. The obtained results confirm the molecular identification of the isolate and scientifically substantiate its inclusion in the *L. plantarum* group.

3.3. Growth Dynamics and Production of Plantaricin K-2

The growth dynamics of *L. plantarum* K-2 showed that the strain entered the stationary phase after 12 h of incubation, with culture density remaining relatively stable from 12 to 72 h [Figure 2]. Similarly, the pH of the culture medium decreased sharply from 6.5 to 3.65 within the first 12 h, followed by only slight changes throughout the remainder of the incubation period, reaching a final value of approximately 3.5.

Maximum inhibitory activity was observed between 30 and 48 h, during which the largest inhibition zones and the highest activity (up to 6400 AU/mL) were recorded. After 54 h, a clear decline in activity was noted, likely due to bacteriocin degradation, instability, or reduced production in the later stages of growth. These findings suggest that optimal bacteriocin production by *L. plantarum* K-2 occurs between 30 and 48 h of incubation, corresponding to the early stationary phase of growth.

3.4. Purification of Plantaricin K-2

Plantaricin K-2 was purified by three-stage chromatography using cation exchange chromatography on SP-Sephadex C-25, hydrophobic chromatography on a Sep Pak C18 cartridge, and high-performance liquid chromatography. The results obtained at different stages of purification are shown in Table 2.

Table 1: Antimicrobial activity of *L. plantarum* strain K-2 and plantaricin K-2.

Indicator strain	*L. plantarum* K-2 strain[a]	Plantaricin K-2[b]	Antibiotic resistance[c]
Gram-positive bacteria			
Listeria monocytogenes ATCC 1911	20±0.05*	25	-
Staphylococcus aureus D-5	20±0*	22	OFX, C, CRO, LVX, DOC, AM
Staphylococcus aureus ATCC 29923	32±0.5	-	OX
Staphylococcus aureus D-2	20±0.28*	8	OFX, CIP, C, FOS
Staphylococcus aureus 0359446/wood	25±0.25*	21	PO, K
Staphylococcus epidermidis D-3	0	-	PO, CLR, TET, CO
Bacillus subtilis 5	16±0.47	-	-
Gram-negative bacteria			
Proteus mirabilis CAT-0103	35±0.25	-	PO, CLR, TET, OX
Proteus morganii 399	20±0.25*	16	PO, CLR, OX
Klebsiella pneumoniae B-1823	10±0	-	ST, CE, PIP, OX
E. coli 477	28±0.25	-	OX
E. coli CAT-0200	20±0.28*	10	-
P. aeruginosa 003841/114	32±0.5*	17	PO, CLR, OX
P. aeruginosa ATCC 27853	18±0.5	-	OX
P. aeruginosa D-1	28±0.5	-	TET, CO
P. aeruginosa D-2	26±0.5	-	OFX, PO, CLR, C, AN
Serratia marcescens 367	20±0.25	-	PO, OX
Enterococcus faecalis CAT-0203	25±0.25*	12	OX
Fungi			
Candida albicans CAT-0204	-	-	
Candida tropicalis CAT-02041	-	20	PO, K, ST, CE, CLR, CO, AN

Legend: [a]The values represent the diameter of the inhibition zone (mm) measured by the agar spot lawn diffusion assay. Data are presented as mean±standard deviation (SD) of three independent experiments. [b]The activity of plantaricin was tested by the agar spot lawn method, and the results were recorded. [c]AN: Amikacin; RI: Rifampicin; OFX: Ofloxacin; PO: Polymyxin; K: Kanamycin; ST: Streptomycin; CE: Cefotaxime; CLR: Clarithromycin; CIP: Ciprofloxacin; TET: Tetracycline; CO: Co-trimoxazole; C: Chloramphenicol; PIP: Piperacillin; OX: Oxacillin; CRO: Ceftriaxone; LVX: Levofloxacin; DOC: Doxycycline; AM: Ampicillin; FOS: Fosfomycin. *The active compound was identified as plantaricin based on its inactivation by the enzyme proteinase K. "-" Not detected. *L. plantarum*: *Lactiplantibacillus plantarum, S. aureus: Staphylococcus aureus, E. coli*: *Escherichia coli*, *P. aeruginosa: Pseudomonas aeruginosa*

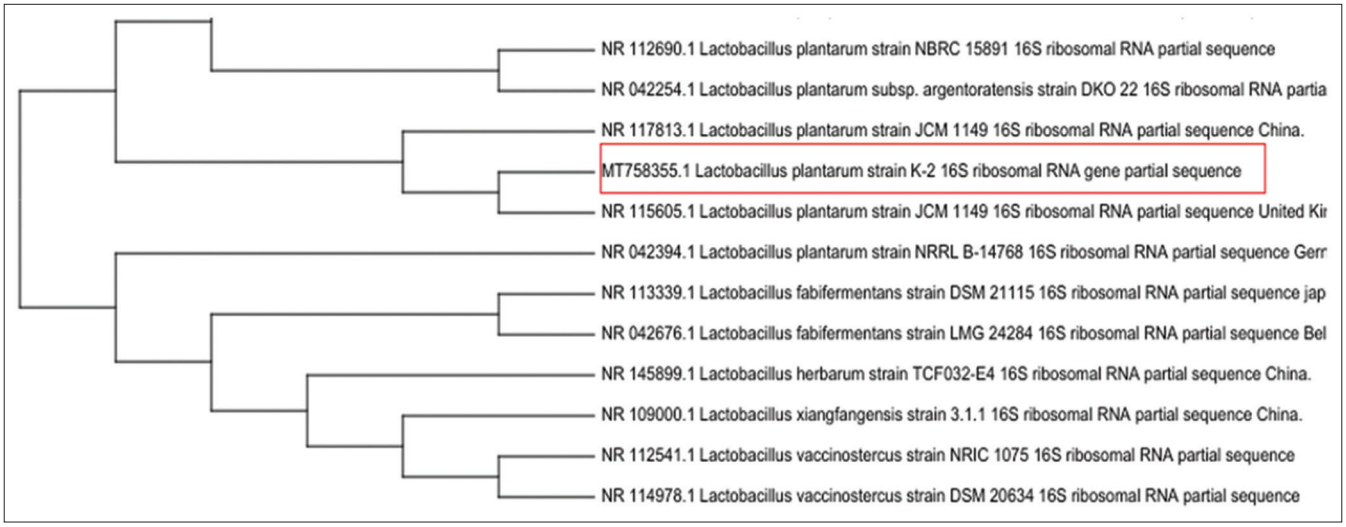

Figure 1: Phylogenetic tree of the 16S ribosomal RNA gene of lactic acid bacteria.

To purify crude plantaricin, the *L. plantarum* K-2 culture broth was diluted two-fold with 20 mM citrate buffer (pH 4.5) and applied to a SP-Sephadex C-25 column. The fractions eluted with 1 M NaCl solution were determined to have antibacterial activity in an agar spot assay. A 34.61-fold increase in purification and a 70.28% yield were achieved in this purification step. Next, the active fractions were subjected to hydrophobic interaction chromatography on a Sep-Pak C18 cartridge. Elution was performed using 30-50% acetonitrile in 0.1% trifluoroacetic acid, and the active fractions showed increased activity. At this step, the specific antibacterial activity of plantaricin

Table 2: Purification of plantaricin K-2 from *L. plantarum* K-2 strain.

Purification stages	Volume, mL	Activity (AU/mL)	Total activity, AU	Total protein, mg	Specific activity (AU/mL)	Purification, fold	Yield, %
Cultural supernatant	1000	514.29	514289.28	4432	116.04	1	100
SP Sephadex C-25	150	2409.68	361452.60	90	4016.14	34.61	70.28
Sep-Pak C18 (HIC)	5	7134.03	35670.14	5.5	6485.48	55.89	6.94
RP-HPLC	1	6692.00	6692.00	0.700	9560.54	82.39	1.30

RP-HPLC: Reverse-phase high-performance liquid chromatography, HIC: Hydrophobic interaction chromatography

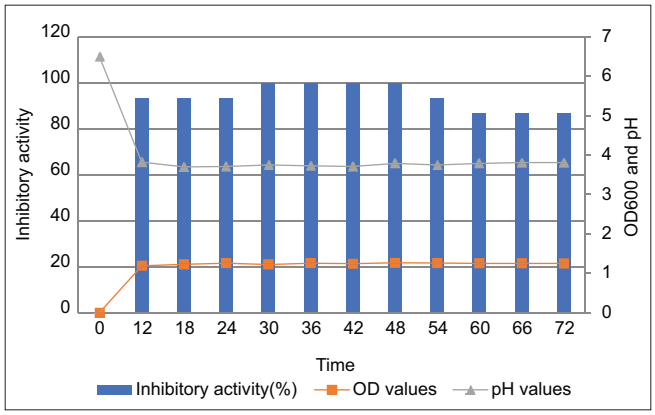

Figure 2: Dynamics of the *Lactiplantibacillus plantarum* bacterial growth and formation of plantaricin K-2. Blue columns stand for inhibitory activity; orange circles mean OD values and grey circles show the pH values.

K-2 increased by 55.89-fold, and 6.94% of the initial total activity was recovered.

Finally, the most active fractions were further purified by RP-HPLC, yielding a single peak showing antibacterial activity against the indicator strain, *L. monocytogenes*. This final purification step resulted in an 82.39-fold increase in specific activity, yielding 1.30% relative to the starting material.

3.5. Molecular Weight Analysis of Plantaricin K-2

Interestingly, two bands and two corresponding inhibition zones were observed in samples taken after hydrophobic interaction chromatography, one in the middle with weight markers of 100 and 60 kDa and the other below 8 kDa in SDS-PAGE and agar overlay analysis, respectively [Figure 3]. However, the molecular weight of fraction III from RP-HPLC was determined to be approximately 4 kDa using SDS-PAGE and overlay analysis, and the additional inhibitory activity between 100 and 60 kDa may be related to the aggregation of the peptide or binding to larger molecules. Mass spectrometric analysis of purified plantaricin K-2 revealed that its molecular mass was 3937.05 Da.

3.6. Antimicrobial Spectrum of *L. plantarum* K-2 and Plantaricin K-2

The antibacterial spectra of both *L. plantarum K-2* and plantaricin K-2 are shown in Table 1 together with the resistance of the indicator strains to antibiotics. *L. plantarum* K-2 strains have a very wide spectrum of antimicrobial effects, inhibiting both gram-positive and gram-negative bacterial strains, but no antimicrobial activity was detected against clinical isolates *Candida albicans, Candida tropicalis,* and *Staphylococcus epidermidis* D-3.

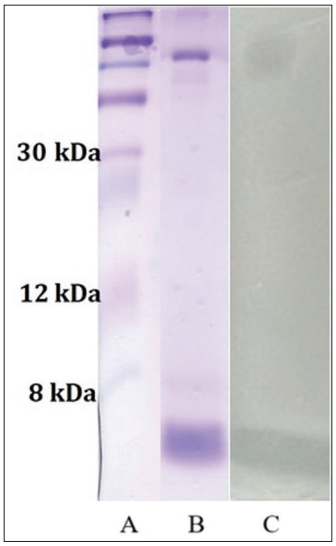

Figure 3: Electrophoretic analysis and antimicrobial activity of purified K2 peptide. A: Marker proteins, B: Purified K-2 peptide, and C: *In vitro* activity.

Plantaricin K-2 isolated from strain *L. plantarum* K-2 was active against several Gram-positive strains and particularly against strong activity against *L. monocytogenes*; for example, the inhibition zones for *L. monocytogenes* ATCC 1911 and *S. aureus* D-5 were 25 mm and 22 mm respectively. However, it should be noted that, consistent with its narrow-spectrum classification, plantaricin K-2 did not show activity against other Gram-positive bacteria (*S. aureus ATCC, S. epidermidis D-3, and Bacillus subtilis* 5), Gram-negative bacteria (*Proteus mirabilis CAT-0103, Klebsiella pneumoniae B-1823, and E. coli 477* and all tested *Pseudomonas aeruginosa* strains except *P. aeruginosa* 003841/114) as well as *Serratia marcescens 367 and C. albicans*. Interestingly, although no antimicrobial activity was observed against *C. tropicalis* in general, plantaricin K-2 showed an inhibition zone of 20 mm [Table 1].

3.7. Effect of Temperature, pH, Proteases, and Ethanol on the Activity of Plantaricin K-2

The effect of heat treatment, pH, and enzymes on bacteriocin activity is shown in Figure 4a and b and Table 3.

The antimicrobial activity of bacteriocin K-2 was maintained after heat treatment from 25°C to 100°C for 30 min; however, the antimicrobial activity was significantly reduced by 20% when incubated at 121°C for 30 min [Figure. 4a]. This indicated that bacteriocin K-2 was stable to high temperatures, but the activity was sensitive to high-temperature sterilization (121°C) [Figure 4a].

Notably, plantaricin K-2 could maintain most of its activity between pH 4.0 and 10.0, but completely lost its activity under harsh conditions

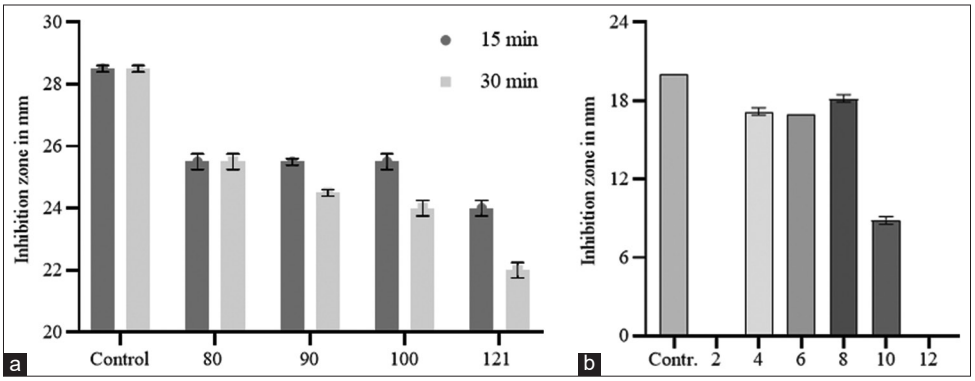

Figure 4: Effect of temperature (a) and pH (b) on the antimicrobial activity of plantaricin K-2. The error bars in the figure represent the (standard deviation) of the results of three independent experiments (n = 3) for each point.

Table 3: The effect of protein-degrading enzymes.

Peptide/ enzymes	Pepsin	Trypsin	Proteinase-K	Papain	α-chymotrypsin
K-2 bacteriocin	Cleaved	Cleaved	Cleaved	Cleaved	Cleaved

No significant effect was recorded in the preparation of various ethanol solutions in aliquots of plantaricin

at pH 2 and 12 [Figure 4b]. Accordingly, the optimal pH for the manifestation of inhibitory activity is usually between 4.0 and 8.0. All proteases showed complete loss of inhibitory activity after treatment with plantaricin [Table 3]. This indicates a high sensitivity of the peptide under study to pepsin, trypsin, α-chymotrypsin, papain, and proteinase-K. This is also due to the proteolytic degradation of the peptide under the influence of enzymes, which requires the development of additional measures to ensure its stability during *in vivo* use.

4. DISCUSSION

In this paper, we report the purification and characterization of plantaricin K-2, a bacteriocin produced by *L. plantarum* K-2 isolated from traditional Uzbek homemade fermented cabbage. The study systematically evaluated the peptide's production, physicochemical properties, and antimicrobial spectrum. These findings not only confirm the potent inhibitory activity of plantaricin K-2 against *L. monocytogenes* and other Gram-positive pathogens but also contribute to the global diversity of bacteriocins derived from LAB.

Several plantaricins, including LPL-1 [22], plantaricin P1053 [23], plantaricin W3-2 [24], and SH1a [25], have previously been identified from *L. plantarum*. The molecular weight of plantaricin K-2 (3937.05 Da) determined in this study is comparable to other plantaricins reported from *L. plantarum*, which likewise exhibit notable thermostability. Examples include bacteriocin B391 from *L. plantarum* B391 [26], JLA-9 from *L. plantarum* JLA-9 [27], and ZJ20208 [28]. Consistent with earlier findings, our results confirm that *L. plantarum* K-2 produces a functional plantaricin with strong antimicrobial activity that suggests its potential as a starter culture and highlights plantaricin K-2 as a promising candidate for application as a natural biopreservative in the food industry.

Plantaricins represent a structurally diverse family within Class II bacteriocins. Some belong to the pediocin-like Class IIa group, such as plantaricin 423 [29], plantaricin LPL-1 [22] which is small (<10 kDa), heat-stable, and carries the conserved N-terminal "YGNGV" motif strongly linked to anti-Listeria activity [30,31] that other plantaricins are Class IIb two-peptide systems (e.g., PlnEF, PlnJK, and PlnNC8) [31,32] that require complementary peptides for full activity [33], while additional members fall into Class IId [34,35] or other subclasses [36]. Although the full amino-acid sequence of plantaricin K-2 remains to be determined, its low molecular weight, high thermal stability, and strong anti-listerial activity closely parallel the functional profile of Class IIa bacteriocins. When considered as a whole, these comparisons place plantaricin K-2 within the broader structural and functional diversity of plantaricins, while also underlining its potential relevance as a representative of the Class IIa subgroup.

Purification of bacteriocins typically begins with ammonium sulfate precipitation to concentrate crude protein extracts, followed by chromatographic steps such as ion-exchange chromatography, hydrophobic interaction chromatography, and reverse-phase HPLC to achieve further purification. These strategies have been widely applied to *L. plantarum* bacteriocins, for example, plantaricin ZJ5 was purified using ammonium sulfate precipitation, cation-exchange, HIC, and RP-HPLC to obtain a high-purity peptide with effective activity against *S. aureus* and *E. coli* [37]. Reviews also confirm that combinations of ammonium precipitation, IEX, HIC, and RP HPLC are standard for isolating different bacteriocins [38]. In contrast, we adopted a modified approach starting with IEX, followed by HIC, and polishing with RP HPLC. This order offered several advantages. Beginning with IEX allows direct loading of (diluted) sample into the column without the need for salt precipitation that requires intermediate desalting or extensive buffer exchange, thus reducing sample handling time and loss. Furthermore, both HIC and RP HPLC utilize gradients of organic solvents which enhanced resolution while also facilitating downstream drying and solvent evaporation, yielding stable, storage-ready bacteriocin preparations. Overall, by employing the IEX → HIC → RP HPLC sequence, we achieved a high-purity plantaricin K-2 preparation with minimized buffer exchange steps, improved operational efficiency, and maintained antimicrobial activity, hereby demonstrating that a precipitation-free, fully chromatographic workflow can be sufficient for bacteriocin purification.

Plantaricin K-2 exhibited strong activity against *L. monocytogenes* and other Gram-positive bacteria such as *S. aureus*, comparable to activities reported for other *L. plantarum*-derived bacteriocins (e.g., plantaricin LD1, Q-7, LD4, and 149) [12,36,39]. Similar activity profiles have been reported for plantaricins such as YKX in other studies [40,41]. Given the limited intrinsic activity of bacteriocins

against Gram-negative bacteria, combining plantaricin K-2 with permeabilizing agents (e.g., EDTA and sodium tripolyphosphate) or physical treatments (temperature, pulsed electric field, and high hydrostatic pressure) is advisable to control Gram-negative spoilage organisms in foods [41,42]. The natural production of plantaricin K-2 by the K-2 strain also supports its use as a starter culture for fermented and acidic products, leveraging both bacteriocin production and organic acid generation for improved food safety.

Plantaricin K-2 demonstrated high thermal stability, consistent with several other plantaricins such as JLA-9 and J23 [27,43]. However, its antimicrobial activity was sensitive to proteolytic enzymes, a limitation shared by many Class II bacteriocins, indicating that protective strategies such as encapsulation or formulation will be necessary to preserve activity in protease-rich environments. Despite this, the peptide's broad pH tolerance, thermostability, and potent anti-listerial activity underscore its potential as a safe, natural, and environmentally friendly preservative. Importantly, this study provides the first characterization of a plantaricin from traditional Uzbek fermented cabbage, thereby expanding the known geographic and ecological diversity of *L. plantarum*-derived bacteriocins. Taken together, our results highlight plantaricin K-2 as a promising candidate for food biopreservation.

5. CONCLUSION

First study to isolation a thermally stable plantaricin K-2 produced by the *L. plantarum* K-2 strain isolated from local fermented cabbage has been established to be a potential antimicrobial peptide. Bacteriocin K-2, with a MW of 3937.05 Da, was found active against food-borne pathogenic bacteria and some fungi, pH-dependently stable, thermostable, and stable to surfactants. Moreover, it revealed high activity against various clinical strains of pathogens. On the other hand, the protein purified in this work was found to be cleaved by proteases. Our findings provide new insights into the industrial potential of this thermostable bacteriocin. Therefore, plantaricin K-2 may be considered a promising natural and safe biological preservative for application in the food industry. Further studies will focus on sequencing the whole genome of this strain to identify genes responsible for bacteriocin production.

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7. CONFLICTS OF INTEREST STATEMENT

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as potential conflicts of interest.

8. CONSENT FOR PUBLICATION

All authors agreed and given their consent for publication

9. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

The data presented in this study are deposited in NCBI (MT753855.1 https://www.ncbi.nlm.nih.gov/nuccore/1870026693). Further inquiries can be directed to the corresponding author. All the data are available with the authors and shall be provided on request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI) tools for writing and editing the manuscript, and no images were manipulated using AI.

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