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Research Article

Bioprospecting Bacteriocinogenic Lactic Acid Bacteria from Algerian Raw Goat Milk and Traditional Cheeses

Naima Boutaleb[1], Abdelbasset Mechai[2*], Rabah Arhab[1], Manel Debabza[2]

[1]Laboratory of Natural Substances, Biomolecules and Biotechnological Applications, Faculty of Exact Sciences and Life and Nature Sciences, University of "Larbi Ben M'hidi" Oum El Bouaghi, Oum El Bouaghi 04000, Algeria
[2]Laboratory of Bioactive Molecules and Applications, Faculty of Exact Sciences and Life and Nature Sciences, University of Echahid Cheikh Larbi Tebessi, Tebessa (E.C.L.T.U) 12000, Algeria

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ABSTRACT

Artisanal fermented dairy preparation represents one of the oldest and most prevalent methods of food preservation, having enabled rational management of valuable food resources for millennia across various cultures worldwide, particularly those of the southern Mediterranean countries. Bacteriocinogenic lactic acid bacteria (LAB) that are contained in traditional fermented milk are considered good sources of promising antimicrobial bioactive compounds. A total of 58 LAB were isolated and identified based on phenotypic characteristics and then tested for their antibacterial effectiveness against both Gram-positive and Gram-negative bacteria that cause food spoilage. Eight strains among selected LAB produce bacteriocin-like inhibitory substances (BLIS), which have a broad antibacterial spectrum against tested species. The strains were identified by 16S rRNA gene sequence analysis. All generated BLISs were fully inactivated by proteolytic enzymes while remaining unaffected by catalase, indicating their proteinaceous nature. The BLIS produced by *Lactiplantibacillus plantarum* NBC101174 was concentrated by the addition of ammonium sulfate to a final concentration of 80%, which allowed for an increase in the specific activity of the bacteriocin from 21.05 to 106.00 AU/mg, increasing the specific factor of 5.04 fold. The inhibitory substance produced by *Lpb. plantarum* NBC101174 exhibits a bactericidal effect, leading to cell lysis and a 99.9% lethality rate against the indicator strain. The findings of the current study could increase our understanding of bacteriocinogenic LAB diversity. It could also be concluded that LAB isolated from Algerian fermented milk provides a promising source for bacteriocins that can be used safely as bio-preservation of various foods produced under different storage conditions.

1. Introduction

The consumption of artisanal cheese and fermented milk products is one of the oldest traditions associated with livestock farming, as these dairy products are produced using traditional artisanal methods, prepared with milk from cows, goats, and occasionally sheep. For centuries, these artisanal goods have enhanced food availability in Algeria, and traditional dairy products hold a prominent status among other fermented milk preparations. Mostly made in rural areas for domestic use, these handmade products were greatly valued for their refreshing properties (Becila *et al.* 2022).

The most widely consumed Algerian fermented milk items produced using traditional methods are fermented milks (such as Raib and Lben), cheeses (Igounenes and Aghoughlou) in the North-Central region, and (Jben, Klila, Takammarit, Bouhezza, M'chouna, and Medghessa) in the North-Eastern region, as well as fatty dairy products (e.g., Zebda or butter, Smen or Dhan) in the South-Eastern region of Algeria (Leksir *et al.* 2019; Medjoudj *et al.* 2019).

* Corresponding Author
E-mail Address: abdelbasset.mechai@univ-tebessa.dz

One of the foremost pressing issues confronting scientists today is the contamination of food by pathogens, which are a frequent cause of foodborne diseases (Imade *et al.* 2021). Chemical additives have been employed extensively to preserve food during the past century, but because of their toxicity, they can seriously harm human health (Ng *et al.* 2020). Natural bio-preservative products are in great demand due to the growing demand for food free of chemicals and the limited ability of current food preservation techniques to prevent foodborne diseases. This new technique intends to improve food safety and quality while extending the shelf life of food items.

An alternative to control pathogenic bacteria is the use of selected lactic-acid bacteria (LAB) with antimicrobial activity as a protective culture (Todorov *et al.* 2022).

Indigenous LAB from fermented milk products are considered a potential source of antimicrobial compounds, as these products are frequently investigated to isolate and identify strains capable of producing various bacteriocins with varying antibacterial activity, which can inhibit foodborne pathogens and increase food safety (Martin *et al.* 2023).

The preservation effects of LAB are ascribed to many mechanisms, including competition for nutrients and space, as well as the formation of organic acids and other substances such as ethanol, H_2O_2, diacetyl, reuterin, and bacteriocins. (Woraprayote *et al.* 2023). It has been well documented that bacteriocin producers are bacteria belonging to the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus*, and *Weissella*. The previously mentioned genera have been found to be engaged in Algerian fermented milk, indicating that Algerian fermented milk could be a promising source of bacteriocin-producing species (Metrouh *et al.* 2022).

There is limited data on the diversity and antimicrobial potential of LAB from Algerian fermented dairy products.

In the present paper, we report the selection and identification of bacteriocinogenic lactic acid bacteria (LAB), as well as the characterization of an antimicrobial compound produced by *Lactiplantibacillus plantarum* NBC101174, which exhibits a broad inhibitory spectrum against various foodborne pathogens.

2. Materials and Methods

2.1. Sampling and Indicator Bacteria

A total of 16 samples of dairy products were used, including 10 samples of traditional cheese (Jben) produced in small-scale dairies and 6 goat milk samples collected from different regions in Tebessa, Algeria. Indicator bacteria: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 35984, *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 9372, *Micrococcus luteus* ATCC 4698, *Listeria monocytogenes* ATCC 7644, and *Escherichia coli* ATCC 25922, were used as test organisms, which are the most common food pathogenic and spoilage bacteria.

2.2. LAB Isolation and Biochemical Characterization

About 25 g of each sample cheese was combined with 225 mL of 0.1% w/v peptone water, and the mixture was homogenized by vortexing at 280 rpm for three minutes. For goat milk samples, 1 mL was added to 9 mL sterile diluent and homogenized by vortex mixing. Then, serial dilution from 10^{-2} to 10^{-5} was made in a 15 mL universal bottle. 0.1 mL of the homogenized suspension was aliquoted and inoculated onto selective media: (a) M17 agar (Merck, Darmstadt, Germany), incubated for 48–72 hours at 30°C; (b) De Man–Rogosa–Sharpe (MRS) agar (Quelab, Montréal, Canada), incubated at 30°C and 37°C for 48 hours under aerobic conditions and at the same temperatures in anaerobic jars for 72 hours. Three to four distinct single colonies were randomly chosen from each cultivated plate. The chosen colonies were examined for morphological traits, including Gram staining and colony morphology, gas production test, catalase test, and Arginine hydrolysis. Gram-positive and catalase-negative bacilli/ cocci were chosen and stored at -20°C in MRS or M17 broth containing 15% (v/v) glycerol (Merck).

2.3. Initial Screening Assay for Antibacterial Activities using the Spot-on-the-lawn Method

Fifty-eight LAB isolates were initially evaluated for antagonistic activity using the spot-on-the-lawn method as described by Schillinger and Lucke (1989), with specific modifications implemented. Overnight cultures of the isolates were inoculated onto MRS agar plates and incubated for 24 hours at 30°C to promote colony formation and metabolite synthesis. About 5×10^7 cfu/mL of the indicator strain *Staphylococcus aureus* ATCC 25923, was added into 100 mL of soft TSA (Trypticase soy agar) containing 0.7% agar and subsequently poured over the plate where the isolated LAB were cultured. Following a 24-hour incubation at 37°C, the diameter of the inhibitory zones was measured from the edge of the zone using a calliper and recorded in millimeters.

2.4. Presumptive Identification of LAB Strains

All isolates were phenotypically identified presumptively up to the Genus level. Gram-positive, catalase-negative bacilli and cocci were evaluated for growth at various temperatures (10°C, 15°C, and 45°C), pH degrees (4.4 and 9.6), NaCl concentrations (6.5% and 18%), and gas generation from glucose in MRS broth as described by Schillinger and Lücke (1987).

2.5. Screening Assay for Antibacterial Activities Using the Well-diffusion Method

2.5.2. Crude Supernatant Fluid Preparation

LAB strains were inoculated on the MRS broth medium for 24 h at 37°C. The bacterial cultures were centrifuged at 10,000 rpm/min for 10 min at 4°C. The recovered pellet underwent filtration and dialysis with a 0.01 mol/L potassium phosphate buffer (pH 6.6), followed by sterilization of the solution through a 0.45 µm pore size filter. The isolated fraction was identified as crude supernatant fluid (CSF). To ensure that the antimicrobial activity was not due to the acidity, the neutralized CSF (NCSF) was prepared by adjusting the pH of the CSF to 7 using 4 M NaOH and subsequently stored at -20°C for future applications.

2.5.2. Antibacterial Activity

The antibacterial activity of selected LAB was evaluated using the well-diffusion method (WDM) as outlined by Schillinger and Lücke (1989), with minor modifications. Each indicator strain underwent overnight cultivation in Tryptic Soy Broth (TSB). Subsequently, the bacterial cultures were streaked onto the surface of Mueller-Hinton agar, achieving a turbidity that corresponds to the 0.5 McFarland standard. Wells of 6 mm diameter were made in soft agar (0.8%) seeded with the indicator strains, and 75 µL of NCSF was poured into the wells. The plates were incubated overnight at 37°C, and zones of inhibition were recorded. Based on the inhibition growth zone diameters, antimicrobial activity was characterised and categorised as follows: slight ($6<x\leq8$ mm diameter), medium ($8< x\leq11$ mm), high ($11< x\leq13$ mm), and extremely high ($x>13$ mm).

2.6. Effect of Heat, pH, and Hydrolytic Enzymes on Antimicrobial Activity

The impact of heat treatment on antibacterial activity was evaluated by subjecting the antimicrobial substance generated by the chosen LAB to temperatures of 60 and 80°C for 30 minutes, 100°C for 20 minutes, and autoclaving at 121°C for 15 minutes. Subsequently, the antimicrobial activity was assessed using WDM as previously outlined. The effect of pH on antibacterial activity was tested by adjusting the pH of the antimicrobial substance to values ranging from 1 to 10 using 1 M NaOH or 1 M HCl and incubation for 2 hours at 25°C. The pH was then neutralized (6.5) and the residual activity was estimated (Schillinger & Lücke 1989). To test its sensitivity to enzymes, the NCSF was treated separately with proteinase K (2.6 U/mg) and catalase (2,600 U/mg), both of which were acquired from Sigma, in order to assess the effectiveness of hydrolytic enzymes on the antibacterial material. *Staphylococcus aureus* ATCC 25923 was used as an indicator strain, and WDM was used to measure activity at a final concentration of 0.5 mg/mL at pH 6.5.

2.7. Molecular Identification of Bacteriocin-Producing LAB

The entire genomic DNA of each LAB strain was extracted from 1.5 mL of overnight MRS broth culture utilizing the technique outlined by Van Hoorde *et al.* (2008). The amplification of the 16S rRNA gene was conducted utilizing 1,494 reverse primers (RPs) unique to bacteria and archaea. 5′-GGTTACCTTGTTACGACTT-3′ and 27 forward primer (FP) (specific to bacteria) 5′-AGAGTTTGATCCTGGCTCAG-3′. The PCR reaction comprises: 10 µL of 2× PCR master mix (Biomatik, Canada), 2.5 µL of 1 µM forward primer (FP), 2.5 µL of 1 µM reverse primer (RP), 6.5 µL of nuclease-free water, and 1.5 µL of template DNA. The PCR reaction conditions are as follows: initial denaturation at 94°C for 1 min; 30 cycles of 95°C for 30 s, 44°C for 30 s, and 72°C for 2 min, and a final extension step at 72°C for 4 min. An appropriate aliquot of each PCR amplicon was subjected to electrophoresis on a 1.0% agarose gel, followed by direct sequencing with a PRISM TaqDye Deoxy DNA sequencer of Applied Biosystems. A comparison for the similarity search of sequences was carried out using the NCBI (www.ncbi.nlm.nih.gov) (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic assessment of LAB isolates was conducted using the 16S ribosomal RNA gene sequence with MEGA software version 11, using the neighbor-joining method suggested by Saitou & Nei (1987).

2.8. Semi-Purification of the Antagonistic Compound Produced by *Lpb. plantarum* NBC101174

Among a number of LAB isolated from traditional fermented milk products in Algeria, *Lpb. plantarum*

NBC101174 was chosen because of its activity against the indicator strain. Ammonium sulphate precipitation (700 g/L) was used to concentrate the bacteriocin in the supernatant fraction obtained from this strain. After overnight agitation at 4°C, the precipitate was centrifuged at 10,000×g for 30 minutes. The precipitate was then diluted in 0.05M sodium acetate buffer at pH 5.0 and dialysed overnight at 4°C using a 1 kDa cut-off membrane against the same buffer. The inhibitory activity was assessed prior to and after purification, quantified in activity units per milliliter (AU/mL) with the formula:

$$AU/mL = (1,000 / V) \times D$$

D represents the dilution coefficient, while V denotes the volume of the spotted NCSF, which is 50 mL.

One AU was defined as the reciprocal of the highest serial two-fold dilution at which the indicator strain showed a distinct zone of growth inhibition (Cui *et al.* 2020). The protein concentration of the CSF was measured using the formula provided by Lowry *et al.* (1951).

2.9. Mode of Action of the Semi-Purified Antagonistic Compound

The mode of action of the compound produced by *Lpb. plantarum* NBC101174 against *L. monocytogenes* ATCC7644 was performed as described by Deraz *et al.* (2007). Briefly, the indicator strain was grown at 37°C in BHI broth. After 4 hours of incubation, cells were harvested and resuspended in sterile 20 mM sodium phosphate buffer to yield approximately 10^8 cfu/mL, and then separated into test and control samples. After that, 256 AU/mL of semi-purified preparation was introduced, and the two tubes were incubated at 37°C for 24 hours. The optical densities at 600 nm and cell counts (colony-forming units (cfu/mL)) were assessed at 2-hour intervals. Each test was conducted in triplicate, and the rate of killing was calculated following the method outlined by Deraz *et al.* (2007) as follows:
% lethality = [((initial viable cells) - (final viable cells)) / (initial viable cells)] × 100.

3. Results

3.1. Antimicrobial Screening and Phenotypic Characterization of LAB Isolates

A total of 84 isolates were obtained from 16 Algerian traditional fermented milk samples (Jben and goat milk) collected in different regions of the north-east of Algeria. Of these, 58 strains were identified as catalase-negative and classified as Gram-positive cocci arranged in pairs or long chains, as well as bacilli in pairs or chains, and cocobacilli. They were grouped and preliminarily identified to the genus level based on physiological and biochemical tests (Table 1). Using the spot-on-lawn method, 28 of the total isolates were reported to exhibit antimicrobial activity against the indicator bacteria, *Staphylococcus aureus* ATCC 25923. At this stage, the potential inhibitory action of hydrogen peroxide and organic acids was not ruled out. After neutralising and treating with catalase to remove lactic acid and hydrogen peroxide, only 17 isolates inhibited the development of indicator bacteria using WDM. From these 08 strains showing antimicrobial

Table 1. The preliminary classifications of all LAB isolated from Algerian dairy fermented milk products

Genus (number of isolates)	Source	Cell shape	Temperature tolerance			NaCl tolerance		pH tolerance		Catalase test	Voges-proskauer	Arginine hydrolysis	CO_2 from glucose
			10°C	15°C	45°C	6.5%	18%	4.4	9.6				
Group 1 *Entrococcus* (7)	Jben cheese/ goat milk	Cocci	-	+	+	+	-	+	+	-	+	+	-
Group 2 *Lactococcus* (14)	goat milk	Cocci	-	+	-	-	-	-	-	-	+	+	-
Group 3 *Pediococcus* (11)	goat milk	Cocci	-	+	+	+	-	-	-	-	±	±	-
Group 4 Homofermentative *Lactobacillus* (10)	Jben cheese/ goat milk	Rods	-	+	±	+	-	+	+	-	-	-	-
Group 5 Heterofermentative *Lactobacillus* (06)	Jben cheese/ goat milk	Rods	-	+	-	+	-	-	-	-	-	±	+
Group 6 *Leuconostoc* (10)	Jben cheese	Cocci/ovoid	-	+	-	+	-	±	-	-	-	-	-

(+) positive, (-) negative, (±) response varies between species

activity that disappear after treatment with proteinase, were selected for further study. Antimicrobial compounds from the 08 producing strains were tested against other strains typically linked to foodborne pathogens and bacteria that cause fermented food to deteriorate in order to assess their inhibitory efficacy better. All antagonistic compounds from the selected strains strongly inhibited Gram-positive bacteria (the diameters of the inhibition zones ranged from 6.36±0.22 mm to 12.87±0.21 mm) (Figure 1). The highest antimicrobial activity was shown by *Lpb. plantarum* NBC 101174 against *Listeria monocytogenes* ATCC 7644. *Enterococcus faecium* NBJ250769, *Enterococcus durans* MMJ140294, Lpb. plantarum NBC 101174, *Ln. mesenteroides* subsp. mesenteroides NBZ150464 and *Lactococcus lactis* subsp. lactis AMZ100864 showed antimicrobial activity against *Escherichia coli* ATCC 25922 with inhibition zone diameters that ranging from 08.0±0.4 mm to 12.03±0.62 mm. Cluster analysis using the heatmap method demonstrated that three LAB strains (*Lpb. plantarum* NBC 101174, *Lc. lactis* subsp. *lactis* AMZ100864, and *Enterococcus durans* MMJ140294) exhibited notable antimicrobial activity against all indicator strains.

3.2. Influence of Hydrolytic Enzymes, pH, and Temperature on Antibacterial Efficacy

The treatment of CFSs from the eight strains with catalase did not lead to any changes in activity, indicating that the recorded inhibition activity was not attributed to hydrogen peroxide. However, all antibacterial compounds produced by the selected strains were completely inactivated after treatment with proteinase K, confirming their proteinaceous nature. Therefore, they are considered bacteriocin-like inhibitory substances (BLIS). All BLISs were shown to be resistant to heating at 60 and 80°C for 20 min (Figure 2), and most of produced BLISs have antibacterial

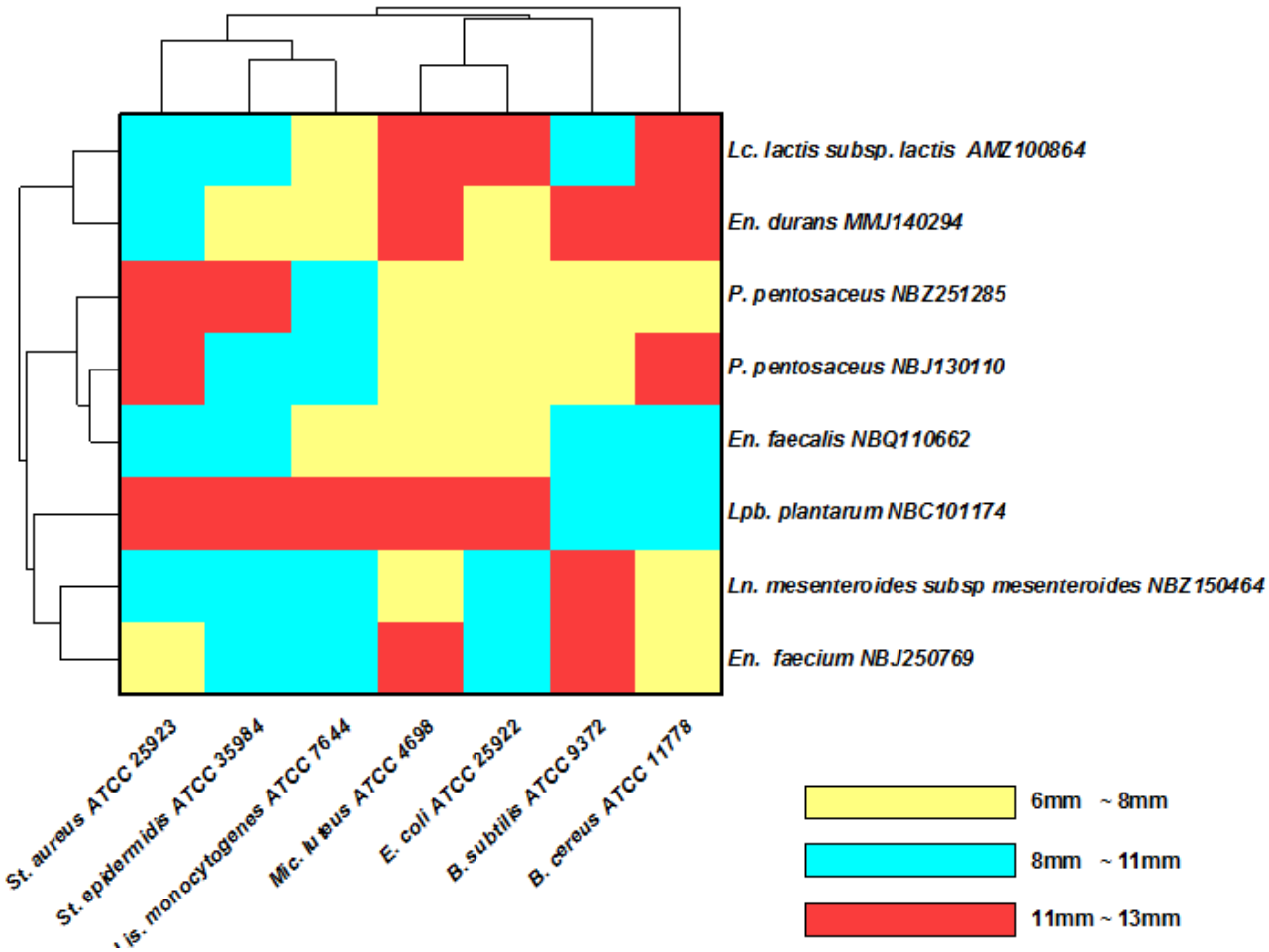

Figure 1. Heat map and hierarchical clustering for the antimicrobial activity (as measured by average zone diameter, mm) of selected lactic acid bacteria strains against bacterial indicator strains. Cluster analysis was conducted using originpro 2024b software

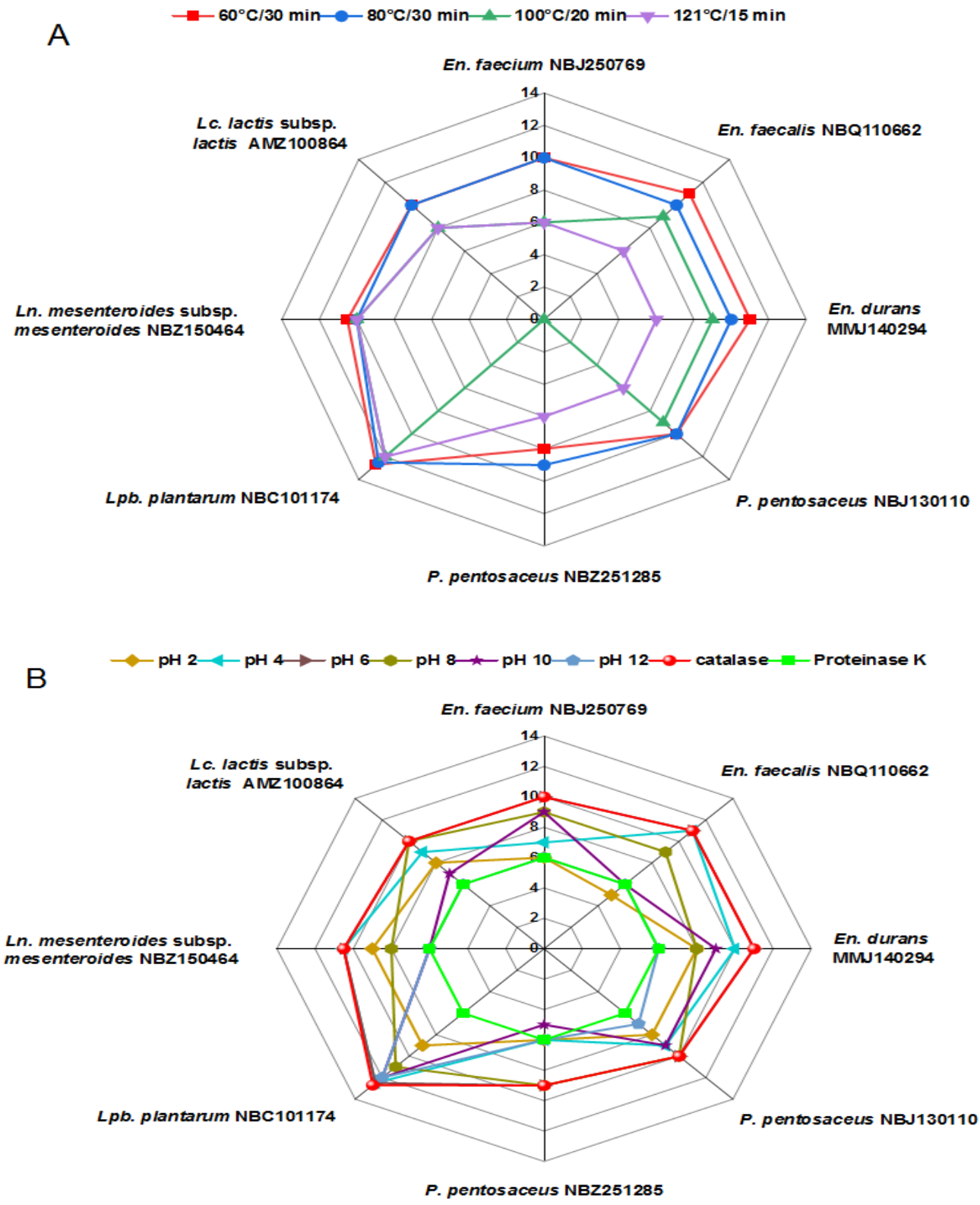

Figure 2. Effect of heat treatment (A), pH and hydrolytic enzymes (B) on the antimicrobial compounds produced in the supernatant by selected lactic acid bacteria the value (from 0 to 14) represents the diameter of the inhibition circle. All assays were conducted with *Staphylococcus aureus* ATCC25923 as indicator strain

activity at 100°C for 20 min. Our results have also shown that only BLISs from strains *Lpb. plantarum* NBC101174, *Ln. mesenteroides* subsp. mesenteroides NBZ150464 and *Lc.* subsp. *lactis* AMZ10086 maintains activity even after treatment at 121°C for 15 min. Nevertheless, BLISs were shown to be stable over a broad pH range (2 to10), and the highest bacteriocin activity was recorded under acidic conditions (pH 2-6), and activity decreased with increasing alkalinity (Figure 2).

3.3. Molecular Identification and Phylogenetic Analysis

A tree of phylogenetic relationships between strains was constructed using the 16S rRNA gene sequences (approximately 1,500 bp) and referred to the neighbor-joining method (Figure 3). Results showed that isolate NBC101174, as the representative of group 4 (Table 1), was placed in the cluster of the *Lactobacillus* genus. However, 16S rRNA sequence analysis of this strain showed 99% sequence similarity with *Lpb. plantarum*. The representative isolates of group 1 (NBJ250769, NBQ110662, and MMJ140294) were placed in the cluster of the *Enterococcus* genus and classified as *Enterococcus faecium, Enterococcus faecalis* and *Enterococcus durans*, respectively. The representative isolates of group 3 (NBJ130110 and NBZ251285) were placed in the *Pediococcus* cluster; they were identified as *Pediococcus pentosaceus* by configuration of a very well-defined cluster (100% bootstrap) with this species. Moreover, 16S rRNA sequence analysis of NBZ150464 indicated the highest identity with *Ln. mesenteroides* subsp. mesenteroides (group 6). Furthermore, 16S rRNA sequence analysis of AMZ100864 showed 99% sequence similarity with *Lc. lactis* subsp. *Lactis* (group 2).

3.4. Purification of the Inhibitory Substance Produced by *Lpb. plantarum* NBC101174

Bacteriocin purification is a typical approach for increasing its activity by specifically raising its level in the CFCS. The inhibitory substance in the CFCS was concentrated using ammonium sulfate (80%). The antibacterial activity and protein content have

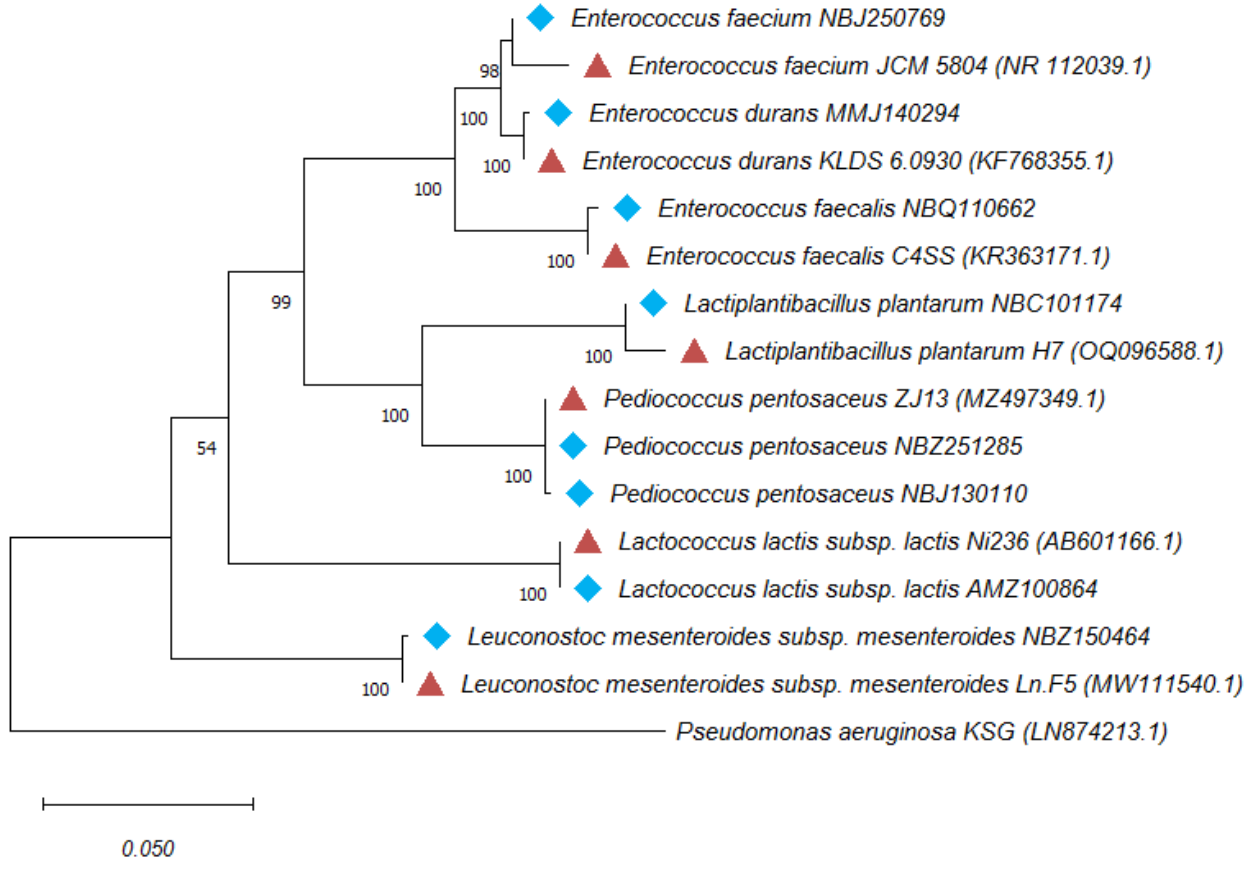

Figure 3. Neighbor-joining tree showing the phylogenetic relationships based on 16S rRNA gene sequences of LAB strains. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown. The sequence of *Pseudomonas aeruginosa* KSGT was used as an outgroup. Bar, 0.05 substitutions pernucleotide position

been measured, respectively, and the biochemical characteristics are given in Table 2. A total bacteriocin activity of 32 × 10^3 AU was obtained in CFCS and 25.6 × 10^3 AU was obtained after ammonium sulfate precipitation, which accounted for 80% recovery and 5.04 fold purification.

3.5. Properties of Plantaricin MTB23

The inhibitory substance produced by Lpb. plantarum NBC101174 was tentatively identified as a bacteriocin-like inhibitory substance (BLIS) and was designated as plantaricin MTB23. Plantaricin MTB23 activity spectrum precipitated by 80% ammonium sulphate was assayed by WDM against a wide range of microorganisms. This bacteriocin may inhibit several food spoilage bacteria and foodborne pathogens, including Gram-positive bacteria such as *L. monocytogenes, Staphylococcus aureus, Micrococcus luteus, B. cereus*, and *B. subtilis*, as well as Gram-negative bacteria like *Escherichia coli*. Complete inactivation activity was noted following the treatment of the partially purified plantaricin MTB23 with proteinase K, thereby confirming its proteinaceous nature. However, catalase treatment did not affect the antibacterial activity of partly purified bacteriocin, showing that the inhibition activity was not caused by hydrogen peroxide.

3.6. Mode of Action

The addition of an inhibitory substance produced by *Lpb. plantarum* NBC101174 to cell suspensions of *Listeria monocytogenes* ATCC7644, led to a marked decrease in optical density and cell viability compared to controls (Figure 4). A 3-log10 drop in cfu/mL was noted with bacteriocin within the initial 4 hours (99.9% lethality), while a 4-log10 decline was recorded after the eighth hour (99% lethality). However, within four hours, the optical density (OD_{600}) declined from 1.4 to

Table 2. Purification of plantaricin MTB23 produced by *Lpb. plantarum* NBC101174

purification stage	Volume (mL)	Arbitrary units (AU/mL)	Total activity (UA)	Protein concentration (mg/mL)	Total protein (mg)	Specific activity (AU/mg)	Recovery (%)	Purification factor
CFCS	1,000	32	32,000	1.52	1520	1520	100	1
Ammonium sulphate precipitation, 80% (dialysed and lyophilised)	50	512	25,600	4.83	241.5	241.5	80	5.04

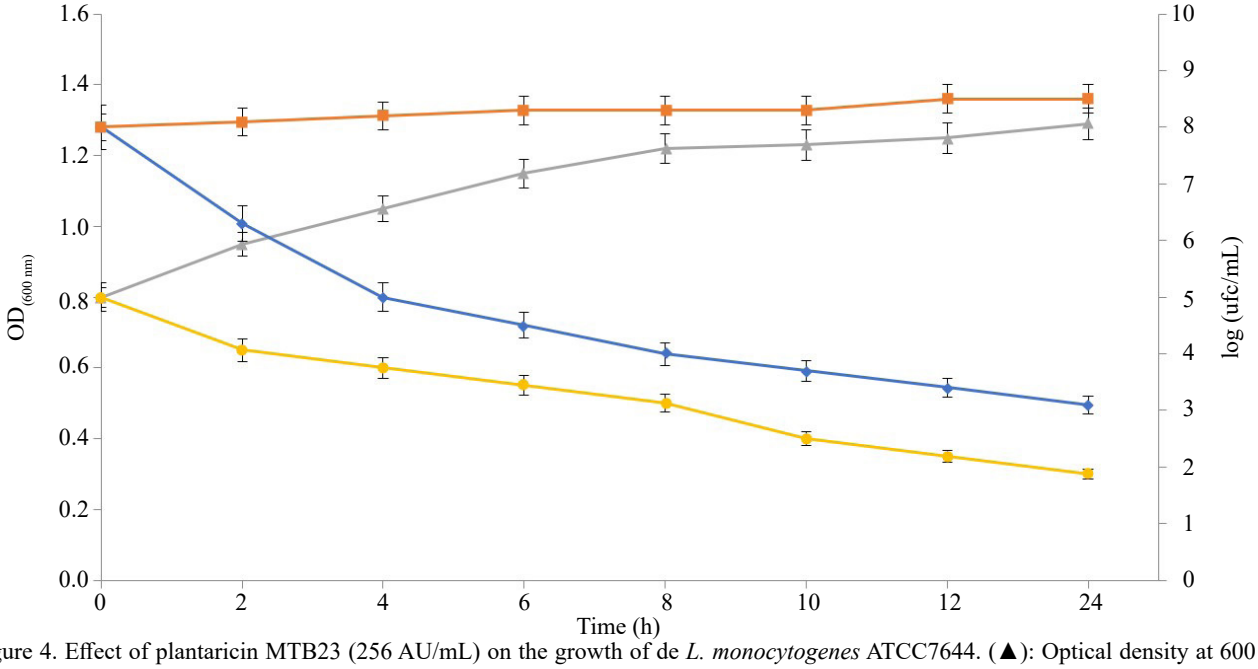

Figure 4. Effect of plantaricin MTB23 (256 AU/mL) on the growth of de *L. monocytogenes* ATCC7644. (▲): Optical density at 600 nm without bacteriocin; (●): Optical density at 600 nm with bacteriocin; (■): Viable cell count without bacteriocin; (♦): Viable cell count with bacteriocin

0.22. This indicates that Lpb produces the inhibitory substance. plantarum NBC101174 induced cell lysis via bactericidal activity.

4. Discussion

Bacteriocins produced by lactic acid bacteria have recently attracted much attention because of their safety and great inhibitory activity (Pei *et al.* 2018). Current outbreaks of food-borne infections have prompted the scientific community to focus on the antibacterial activity of bacteriocenogenic LAB, which can be isolated from a range of fermented dairy products under different environmental conditions.

In the current study, fifty-eight isolates were initially screened from a large collection of putative LAB to inhabit *S. aureus* ATCC25293. Among these, only 28 isolates showed significant inhibition and were characterized to genus levels according to morphological, physiological, and biochemical characteristics. They belonged to the genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus*, and *Enterococcus* spp. (Table 1). The presence of these genera is consistent with the findings of Girma and Aemiro (2021), who reported that *Lactobacillus, Enterococcus*, and *Lactococcus* spp. were the predominant species in Algerian dairy products. Eight of the twenty-eight LAB isolates were selected because of their bacteriocinogenic characteristics, and 16S rRNA gene sequence analysis revealed that they were: *Enterococcus faecium* NBJ250769, *Enterococcus faecalis* NBQ110662, *Enterococcus durans* MMJ140294, *Pediococcus pentosaceus* NBJ130110, *Pediococcus pentosaceus* NBZ251285, *Lpb. plantarum* NBC101174, *Ln. mesenteroides* subsp. mesenteroides NBZ150464, *Lactococcus lactis* subsp. lactis AMZ100864.

The chosen LAB strains exhibited a range of inhibitory activity, measuring from 6.36±0.22 to 12.87±0.28 mm against the bacteria tested (Figure 1). The majority of isolated CFS LAB demonstrated a more pronounced inhibitory effect on Gram-positive pathogens compared to Gram-negative ones, in line with the results from earlier research. (Haryani *et al.* 2023; Wang *et al.* 2023). This is due to structural and compositional differences in Gram-positive and Gram-negative bacterial cell walls. This observation clarifies the heightened sensitivity of Gram-positive indicator bacteria to nearly all LAB CFS.

In line with recent publications, our results also showed that the inhibition of the pathogenic bacteria varied by species and strain (Abushelaibi *et al.* 2017; Rahmeh *et al.* 2019). With the exception of the CFSs of *Pediococcus pentosaceus* NBZ251285 and *Ln. mesenteroides* subsp. mesenteroides NBZ150464, nearly all of the LAB CFS inhibited the development of Gram-positive pathogens (Figure 1). In previous studies, most LAB isolates had much stronger antibacterial action against *Staphylococcus aureus* ATCC 25923 than other indicator bacteria. BLIS from LAB is well-known for its narrow-spectrum antibacterial efficacy, particularly against similar Gram-positive bacteria (Rasid *et al.* 2022). Nonetheless, the influence of BLIS can be extended to Gram-negative bacteria by targeting the intracellular enzymatic system, compromising nucleic acids, and disrupting the bacterial cell membrane. Subsequent experiments have demonstrated that BLIS from LAB exhibits a broad-spectrum efficacy against both Gram-positive and Gram-negative bacteria (De Almeida Júnior *et al.* 2015; Bartkiene *et al.* 2019).

The results also revealed that BLIS's antibacterial activity against *Staphylococcus aureus* ATCC25923 was mainly stable at acidic pH (2-6). However, several BLIS lost their antibacterial activity under alkaline circumstances (pH ≥8). Furthermore, BLIS from *P. pentosaceus* NBJ130110 and *Lpb. plantarum* NBC101174 demonstrated the maximum antibacterial activity against *Staphylococcus aureus* ATCC25923 under a wide range of pH conditions (pH 2–12), mainly *Pediococcus pentosaceus* NBJ130110 and *Lpb. plantarum* NBC101174. According to Abanoz and Kunduhoglu (2018), the stability of bacteriocins across a broad pH range provides them with notable advantages as bio-preservatives in food products and fermented foods.

In this study, the BLIS stability of the eight promising isolates against *S. aureus* ATCC25923 was evaluated at different temperatures (60°C, 80°C for 30 min, 100°C for 20 min and 121°C for 15 min). The results (Figure 2) suggested that BLIS was produced by *Lpb. plantarum* NBC101174 showed the best antimicrobial stability against *S. aureus* ATCC 25923 at 121°C. These results corroborate previous studies in which most bacteriocins are produced by *Lpb. plantarum* NBC101174 are stable over a wide range of pH and after heat treatment, e.g., plantaricin ZJ5 (Song *et al.* 2014), plantaricin JLA-9 (Zhao *et al.* 2016), plantaricin YKX (Pei *et al.* 2021), bacteriocin W3-2 (Wang *et al.* 2023), and bacteriocin J23 (Zhang *et al.* 2018).

Compared with the control condition, which involved samples without enzyme treatment, the influence of proteinase K on the antimicrobial activities of BLIS demonstrated that treatment with this proteolytic enzyme entirely abolished the antimicrobial effect of the bacteriocin generated by all eight LAB strains. The loss of the antibacterial ability of the bacteriocin is linked to its proteinaceous component, hence confirming its proteinaceous origin (Heredia-Castro *et al.* 2015).

plantaricin MTB23 was purified by ammonium sulfate precipitation at a concentration of 80%. The specific activity of the concentrated antibacterial agent increased from 21.05 AU/mg to 106.00 AU/mg, with a yield of 80% and a purification factor of 5.04. Similarly, Chen *et al.* (2018) observed 1745.06 AU/mL activity for bacteriocin from *Lactobacillus plantarum* ZJ316 isolated from healthy infant feces after ammonium sulfate precipitation, and 3.48-fold purification was achieved. Wang *et al.* (2018) found 79.68% recovery and 4.2-fold increases in specific activity after ammonium sulfate precipitation for plantaricin LPL-1 purified from *Lactobacillus plantarum* LPL-1. The partially purified bacteriocins require additional processing to achieve a purified product suitable for food deterioration prevention and preservation. It is important to emphasize that over the last two decades, a wide range of novel plantaricins was identified from *Lb. plantarum*, such as *Lb. plantarum* B391 (bacteriocin B391) (Fernandes *et al.* 2017), *Lb. plantarum* LPL-1 (bacteriocin LPL-1) (Wang *et al.* 2018), and *Lb. plantarum* SF9C (plantaricin SF9C) (Butorac *et al.* 2020), *Lb. plantarum* KLDS1.0391 (plataricin MG) (Gong *et al.* 2010), *Lb. plantarum* W3-2 (plantaricin W3-2) (Wang *et al.* 2023).

Plantaricin MTB23, synthesized by *Lpb. plantarum* NBC101174 demonstrates potential as a broad-spectrum antimicrobial drug effective against both Gram-positive and Gram-negative bacteria, hence prolonging shelf life. However, their use is limited by several factors, including high costs of isolation and purification, low stability and solubility, and susceptibility to enzymatic degradation. Additionally, the specificity of some plantaricins may restrict their effectiveness against a broader range of pathogens.

The mode of action is one of the essential characteristics of a bacteriocin, allowing it to be applied effectively. The results indicate that plantaricin MTB23 exhibits a bactericidal effect on the sensitive strain, evidenced by a 96.8% reduction in the viable cell count (from 8.2×10^8 to 5.1×10^5 cfu/mL) of *Listeria monocytogenes* ATCC7644 in the presence of plantaricin MTB23. A comparable mechanism of action has been noted in other bacteriocins derived from LAB, e.g., plantaricin ZJ316 produced by *Lb. plantarum* ZJ316 (Chen *et al.* 2018), plantaricin LP 21–2 purified from *Lb. plantarum* SHY21–2 (Peng *et al.* 2021) and plantaricin SLG1 produced by *Lb. plantarum* SLG1 (Pei *et al.* 2018).

Lactobacillus plantarum NBC101174 is recognized for its potent bacteriocin production, which exhibits broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria, including pathogenic and spoilage strains. This strain's ability to produce multiple antimicrobial compounds enhances its effectiveness in controlling bacterial growth, making it a valuable resource for applications in the medical and food industries. The validation of its inhibitory compounds through gene sequencing further underscores its potential for antimicrobial product development.

Conflict of Interest

The authors have no conflict of interest to declare.

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