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CHARACTERIZATION AND ANTIMICROBIAL SPECTRUM OF BACTERIOCIN-PRODUCING PEDIOCOCCUS SPECIE MS3 ISOLATED FROM RAW MILK

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Abstract

Bacteriocin molecules are ribosomally synthesized peptides, which show antimicrobial activity against other bacteria belonging to the same species or across the genera. Pediococcus spp., are well-known for producing bacteriocin compounds which have important applications in the food and health sector. The purpose of this study was to evaluate the characteristics and understand the antibiotic spectrum of Pediococci species MS3 contributing to the advancements of novel antimicrobial agents. Thirty bacteriocin-producing Pediococcal strains were isolated using the spread plate method on MRS agar from raw milk, cheese, yoghurt, and green leafy vegetables. Among all 30 isolates evaluated for bacteriocinogenic potential, Pediococci MS3 isolated from raw milk was identified as the only pediocin producer. The Pediococcus specie MS3 showed inhibitory effects against gram-negative bacteria isolated from clinical/environmental/food samples. The Pedicoccus MS3 showed noticeable activity against gram-positive, gram-negative and fungal species i.e. C. albicans, Candida species and S. cerevisiae. The findings were crucial to support the applications of bacteriocin-producing Pedioccoccus MS3 in food industries for food preservation and prevention of spoilage associated with bacteria and fungi. Future research can expand on current research findings to enhance the understanding of bacteriocin-producing Pediococcus MS3 against bacterial and fungal spores.

Keywords: Antimicrobial, Characterization, Isolation, Pediococcus, Pediocin, Raw milk, Sensitive

INTRODUCTION

Bacteriocins are known for their potential antimicrobial activity against important pathogens and have wider applications in food and clinical industries (1). These bacteriocin molecules are ribosomally synthesized peptides, which show antimicrobial activity against other bacteria belonging to the same species or across the genera. This showed that bacteriocin has a wide-ranging spectrum of activity, which varies from a narrow spectrum to a broad spectrum of antimicrobial activity (2). Over the years, lactic acid bacteria have shown their potential to produce important antimicrobials like hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl acetaldehydes and bacteriocins. Lactic acid bacteria are also widely accepted due to their Generally Recognized as Safe (GRAS) status and safety in being used as a preservative in food and drug industries (3). *Lactococcus lactis* (lactic acid bacteria) is well-known for its ability to produce Nisin, which is a widely used bacteriocin in food industries. Nisin is an effective bacteriocin against important spoilage-causing bacteria like *Lactococcus monocytogenes* (4). The bacteriocin compounds isolated from lactic acid bacteria were found effective against pathogenic bacteria including *Staphylococcus aureus, Escherichia coli, and Salmonella spp.* (2, 3).

One such important lactic acid bacteria was *Pediococcus spp*., which produced bacteriocin compounds that have important applications in the food and health sector. Considering the morphology, *Pediococci* is gram-positive, facultatively anaerobic, non-motile, non-sporulating and non-catalase-producing lactic acid bacteria (5). Previous studies evaluated the role of *Pediococci* as a probiotic having the potential to improve growth in farm animals (7, 8), reduce the incidence of respiratory tract infections (5, 9) and improve lactose intolerance (10). Another study showed that *Pediococcus acidilactic iPFC69*, produced antimicrobial i.e.

pediocin which was effective against *S. aureus* causing their inhibition in fermentation processes. The bacteria were stable under high temperatures and low pH conditions, making them valuable in preservation processes (6).

However, there exists serious literature limitation on the properties and use of *Pediococcus spp.* isolated from raw milk despite the fact that *Pediococcus spp*. and their bacteriocins are known to possess antimicrobial properties. There is limited research done on their capacity to prevent both the foodborne as well as clinically associated bacteria, creating potential for the new strain identification and use in enhancing food safety and human health. Therefore, the current study focused on the characterization and antimicrobial spectrum of the *Pediococcus spp. (MS3)* which was isolated from raw milk samples. The purpose of this study was to evaluate the antimicrobial characteristics of the given *Pediococci spp.* and explore its potential for commercial and clinical antimicrobial use. The findings contributed to a deeper understanding of the antimicrobial spectrum of the *Pediococcus spp. MS3*, improving food safety and health status among the population.

MATERIALS AND METHODS

ISOLATION OF *PEDIOCOCCUS SPECIES*

A total of seventy-five samples containing cheese, green leafy vegetables, raw milk and yoghurt were used for the isolation of *Pediococcus spp.* Raw milk and solid food samples (1 mL and 1 g respectively) were prepared by performing serial dilutions. Each dilution in a volume of 1 mL was spread on de Man Rogosa Sharpe (MRS) medium (Merck, Darmstadt, Germany) agar plates and incubated at 30°C for 24 to 72 h in anaerobic conditions. Slimy colonies were isolated as the suspected *Pediococcus spp.*, further to identify and differentiate from *Lactobacilli* and *Leuconostoc* based on cell morphology, cultural biochemical characteristics and antimicrobial susceptibility testing are also performed. These are standard tests and their requirements are readily available in all microbiological labs.

IDENTIFICATION AND DIFFERENTIATION OF PEDIOCOCCUS SPECIES

BIOCHEMICAL AND CULTURAL CHARACTERIZATION

Catalase activity of *Pediococcus spp.* was determined by adding two drops of 3% (v/v) H_2O_2 on a clean glass slide. Aseptic transfer of culture onto the slide was performed using a sterile toothpick. Upon mixing immediate effervescence indicated a positive reaction. Gas production by bacteriocin-producing bacteria was assessed by propagating bacterial culture in MRS broth tubes, with Durham tubes in an inverted position at 37°C for 24 h. Cultural characteristics of isolated cultures were observed after incubating at 37°C for 24 h by streaking onto MRS, nutrient, and BHI agar plates.

GRAM STAINING

Pediococcus spp. after 24 h of growth in MRS medium was subjected to morphological characterization and through gram staining procedure and then observed through light microscopy under oil immersion.

VANCOMYCIN SUSCEPTIBILITY TESTING

The resistance of the isolates to vancomycin was assessed using the disc diffusion method. Organisms were grown in single colonies on Mueller Hinton Agar (MHA) and vancomycin (30 μg) discs were applied on these plates and incubated for 24 h at 37°C with 5-10% CO_2. The inhibition zone diameters of the tested discs obtained in this study were compared to the CLSI recommendations which categorize the zone diameter of ≥17 mm as susceptible to vancomycin, 15–16 mm as intermediately susceptible while pathogens with ≤14 mm were considered as resistant to the drug. This trait of vancomycin resistance can be significant to confirm the presence of *Pediococcus spp*.

SCREENING OF *PEDIOCOCCAL* ISOLATES FOR BACTERIOCIN DETECTION

Primary screening was performed using indicator strains, *Citrobacter species*, *Candida species, Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, Salmonella typhi,* and *Staphyloccus aureus*. The cross-streak method and agar spot test were used for the primary isolation of bacteriocin-producing *Pediococcus*. To confirm the *Pediococcal* activity, specified tests were performed by using specific volume of suspected strains. In Patch Test, BHI agar plates were overlayed with 3 mL of soft agar having 0.1 mL of the indicator strain and incubated at 37°C for 2 h for drying. A small loop of fresh colonies of *Pediococcus* spp. were taken using sterilized toothpicks and streaked over the indicator lawn. About twenty-four hours later, the plates were examined for the presence of small halos around the colonies indicating antimicrobial properties of the *Pediococcus strains*. For the Stab and Overlay Assay, *Pediococcus spp*. were stab directly on BHI agar plates and then incubated at 37°C for 24 h. Afterwards, the stabbed colonies were scraped off the plates and plates were exposed to chloroform vapours for 20-30 min. Plates were then overlaid with 3 mL soft agar seeded with 0.1 mL of indicator strain, further incubated at 37 °C overnight and observed for clear zone around producer strain.

In the Cross Streak Method, *Pediococcus spp*. cultures were aseptically streaked in a straight line from edge to edge of the BHI agar plates then the plates were inverted and incubated at 37°C for 24 h. After incubation, each plate was exposed to chloroform vapours in order to eliminate the bacterial growth. The indicator cultures were then streaked perpendicular to the *Pediococcal* streaks and the plates were incubated for a further 24 h at 37°C. Inhibition of growth was checked along the sides of the *Pediococcus spp*. streaks on the plates to determine the amount of antimicrobial activity.

In addition, Agar Spot Test was also performed to observe the bacteriocin activity of chosen *Pediococcus spp*. were determined by agar spot assay. *Pediococcus spp* were grown in BHI broth incubated for overnight at 37 °C. Cell free neutralized supernatants were prepared by centrifuging overnight cultures at at 10,000× g for 30 min. The cell sediments were discarded and bacteriocin preparation was spotted onto fresh indicator lawn, prepared by overlaying 5 mL soft agar containing 0.1 mL of indicator strains. Plates were observed after 24 h of incubation at 37°C and the diameter of the inhibition zone around the colonies was measured.

BACTERIOCIN CHARACTERIZATION

EFFECT OF TEMPERATURE

The effect of temperature on bacteriocin was carried out by exposing bacteriocin preparations (CFNS) to different temperatures 60°C, 80°C, 100°C, and 121°C for 60, 40, 20, and 15 min respectively placed into wells then incubated at 37°C for overnight. Each sample with a different thermostability was examined against *E. coli* by the agar diffusion method. Positive and negative controls were also run. Untreated bacteriocin was used as a positive control to check whether activity was retained while negative control consisted of sterile water to avoid interference. The exposure times were selected to facilitate direct relevance to food processing and sterilization conditions that exist in industrial sector.

EFFECT OF PH

Bacteriocin preparations CFNS were exposed to different pH values between 2-12 using 10 mM NaOH and 10 mM HCl maintained for 2 h. All samples were adjusted at pH 7 with sterile phosphate buffer and assayed for activity. The bacteriocin preparations with different pH values placed in wells incubated at 37°C overnight proceeded for examination against *E.coli* for the zone of inhibition.

EFFECT OF ENZYMES

The sensitivity of bacteriocin to enzyme treatments and fresh human saliva amylase was carried out using an agar well diffusion assay. Bacteriocin was treated with 1 mg/mL of enzymes amylase, lysozyme and trypsin. Lysozyme was dissolved in tris HCl buffer, pH 8.0. Bacteriocin preparations with each enzyme were placed in wells and incubated at 37°C overnight against *E. coli* as an indicator strain.

EFFECT OF CHLOROFORM

The 24 h stabbed cultures were exposed to chloroform vapors for 10, 20, and 30 min to observe the effect of chloroform on bacteriocin activity. Then, overlaid with sensitive cells and examined for the zone of inhibition against *E. coli*.

EFFECT OF ORGANIC SOLVENTS

The effect of organic solvent on bacteriocin was determined by mixing bacteriocin preparations with organic solvents (ethanol and methanol) equally at a concentration of 1.0 %. Each sample was incubated at 37°C for 30 min for the antimicrobial activity assay by agar well diffusion method. The bacteriocin preparations with either solvent were placed in wells, and incubated at 37°C for overnight to be examined against *E.coli* for zone of inhibition.

EFFECT OF UV RADIATION

A total of 1.3 mL of bacteriocin exponential cultures were placed in Petri plates and exposed to a UV germicidal lamp of 254 nm at a distance of 30 cm. The duration of exposure to UV radiation ranged from 0, 10, 20, 25, and 30 s. From each irradiated plate 1.0 mL was centrifuged at 10,000× g for 30 min and re-suspended in 6.0 mL of fresh medium. All cultures were incubated in the dark at 37°C for 24 h. After overnight incubation, cultures were centrifuged for antimicrobial activity assay by agar well diffusion method. The CFNS preparations with either of the doses were placed in wells, incubated at 37°C overnight and examined against *E. coli* for the presence of a zone of inhibition.

DETECTION OF LYTIC BACTERIOPHAGES

Two methods were used to examine if the CFNS of producer strains contained infectious or mutant phage particles that contributed to the observed inhibitory activity. Common water quality indicator species *E. coli* and *E. faecalis* were employed. Preliminary screening was performed on 24 h stabbed cultures of the producer strains; a disc of agar was crushed in sterile broth for the determination of activity by the agar well diffusion method. Further, stab and overlay assay was carried out as described with some modifications, where the indicator strain was spread on the opposite side of the plate so that it does not mix with the producer strain.

BACTERIOCIN TITER BY DIFFUSION ZONE ASSAY

The BHI-containing agar plates were overlaid with 3.0 mL soft agar with exponential phase indicator organisms. Then wells with a diameter of 6 mm were bored into each plate. A total of 100 µL of each two-fold dilution of CFNS preparation of producer *Pediococcal* stains were directly pipetted into each well. After 18-24 h of incubation at 37°C diameter of zones of inhibition were measured in mm. Bacteriocin activity or bacteriocin titre was expressed in arbitrary units (AU) or activity units /mL. One arbitrary unit is defined as the reciprocal of the last serial dilution exhibiting a clear zone of inhibition.

PLASMID CURING

A dilution from an overnight broth culture of *Pediococcal* strain in fresh BHI broth was prepared and incubated at 37°C for 1.5 h. A total of 0.04 mL of sample was added in each of the two tubes with 5.0 mL of BHI broth pre-warmed at 37°C and 43°C respectively. The tubes were then anaerobically incubated at the appropriate temperature for 5.5 h. The grown cultures were diluted and 0.1 mL was spread over BHI agar plates. After overnight incubation at 37°C colonies were scored and tested for the loss of antibiotic resistance (the counter selection marker) and bacteriocin production. The agar well diffusion assay was used to determine bacteriocin activity against *E. coli* indicator culture.

RESULTS

IDENTIFICATION AND CHARACTERIZATION OF ISOLATED PEDIOCOCCI

Thirty bacteriocin-producing *Pediococcal* strains were isolated using the spread plate method on MRS agar from raw milk, cheese, yoghurt, and green leafy vegetables. Fig. 1 (a) shows the frequency distribution of *Lactobacilli, Leuconostoc* and *Pediococci* among seventy-one catalase negative, 30 ug gram vancomycin resistant slime former on MRS agar.

All strains were observed to be Gram-positive cocci, implicated in pairs, tetrads or clusters and are of facultative anaerobic nature. They were catalase-positive, which is a remarkable characteristic in differentiating between pediocin-producing *Pediococcus spp.* and other lactic acid bacteria, which were catalase-negative. Also, all strains had a natural resistance to 30 μg of vancomycin, which is important to make these strains useful in food processing environment where vancomycin resistant pathogens may appear. In that sense, no gas production was seen in MRS broth, a fact that is a characteristic of *Pediococcus spp.*

Table I. Colony morphology, antimicrobial susceptibility, and biochemical, and cultural feature of the isolated strains from milk

Sample	Culture code	Growth on MRS agar (colony characteristics)	Catalase reaction	Vancomycin 30 μg susceptibility (resistant / sensitive)	Gas production in MRS broth	Gram staining from Thioglycollate Broth culture			Identified organism (*Pediococci**)
						Shape	Arrangement	Gram reaction	
Milk	MS 1	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 3	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 8	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 12	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 14	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 17	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 18	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 21	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 23	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 24	Slime, circular,	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*

		white, opaque							
Milk	MS 26	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 28	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 31	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 32	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 33	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 34	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 37	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 39	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 42	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 43	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 44	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 47	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 53	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 54	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 56	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 57	Slime,	-	R	-	Cocci	Tetrads,	+	*Pediococci*

		circular, white, opaque					clusters		
Milk	MS 59	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 60	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 61	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*
Milk	MS 68	Slime, circular, white, opaque	-	R	-	Cocci	Tetrads, clusters	+	*Pediococci*

*Most of the time prolonged incubation was required i.e. 48 h or 72 h; *Primary isolation was slime former, catalase-negative and 30 ug vancomycin resistant: *R, *R, Resistant to 30 ug vancomycin; *Number of isolated *Pediococcus*: 30

SCREENING FOR POTENT PEDIOCIN-PRODUCING ISOLATE

Among all 30 isolates evaluated for bacteriocinogenic potential *Pediococci MS3* isolated from raw milk was identified as the only pediocin producer as shown in Table II. Figs. 1 (b-d) illustrated that the patch test, stab inoculation, overlay test, agar spot test, and agar well diffusion assay assessed pediocin activity against single indicator cultures, whereas the cross-streak test evaluated activity against multiple sensitive cultures (Table II). After ruling out the antibacterial activity of Pediocin MS3, by treating it with strongly catalase-positive *S.aureus* it was identified as the bacteriocin producer. The nature and number of the indicator sensitive organisms are given in Table III.

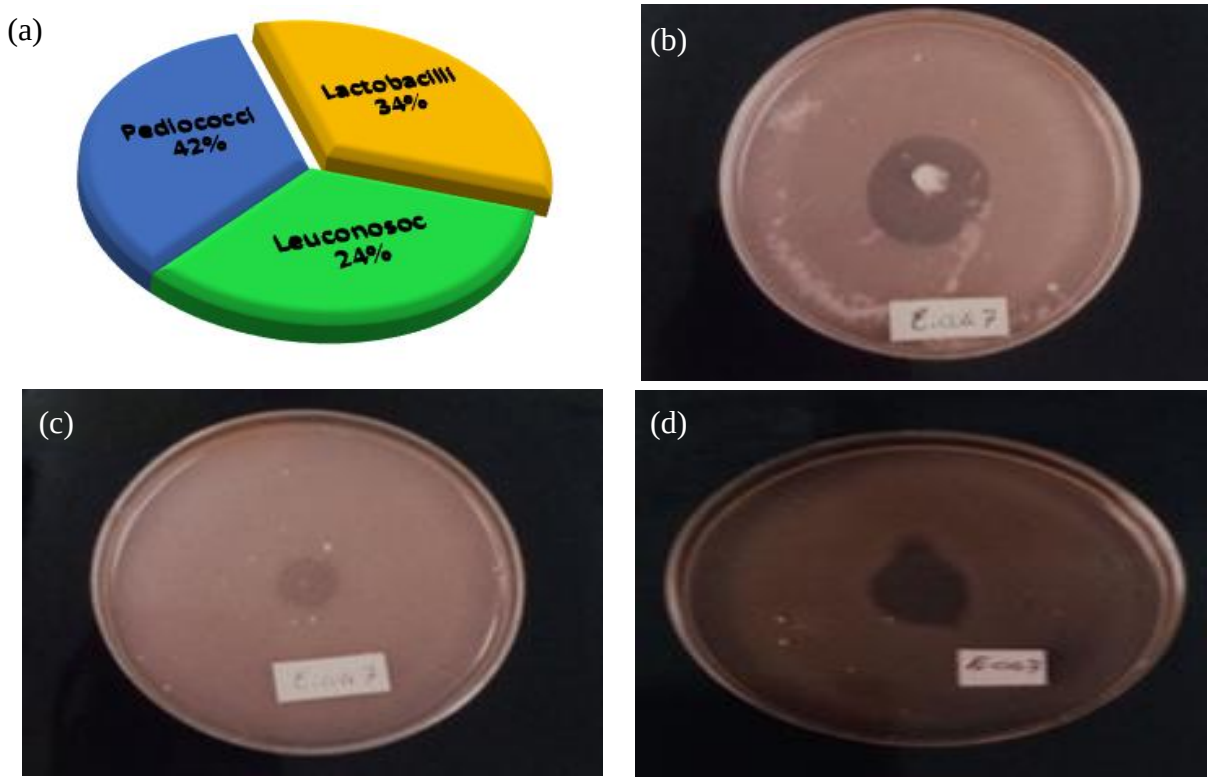

Fig. 1 (a). Frequency distribution of *Lactobacilli, Leuconostoc* and *Pediococci* among seventy-one catalase negative, 30 ug gram vancomycin resistant slime former on MRS agar; **(b).** Patch test demonstrating pediocin MS3 activity against *E. coli;* **(c).** Stab and overlay method demonstrating pediocin MS3 activity against *E. coli;* **(d).** Agar spot test demonstrating pediocin MS3 activity against *E. coli.*

Table II. Screening of *Pediococcal* cultures for bacteriocin

No. of tested *Pediococcus*	Culture code	*Primary screening		**Confirmation by other screening methods		
		Agar spot test	Cross streak method	Agar well diffusion	Patch test	Stab and overlay method
01	MS 1	-	-	NP	NP	NP
02	MS 3	+	+	+	+	+
03	MS 8	-	-	NP	NP	NP
04	MS 12	-	-	NP	NP	NP
05	MS 14	-	-	NP	NP	NP
06	MS 17	-	-	NP	NP	NP
07	MS 18	-	-	NP	NP	NP
08	MS 21	-	-	NP	NP	NP
09	MS 23	-	-	NP	NP	NP
10	MS 24	-	-	NP	NP	NP
11	MS 26	-	-	NP	NP	NP
12	MS 28	-	-	NP	NP	NP
13	MS 31	-	-	NP	NP	NP
14	MS 32	-	-	NP	NP	NP
15	MS 33	-	-	NP	NP	NP
16	MS 34	-	-	NP	NP	NP
17	MS 37	-	-	NP	NP	NP
18	MS 39	-	-	NP	NP	NP
19	MS 42	-	-	NP	NP	NP
20	MS 43	-	-	NP	NP	NP
21	MS 44	-	-	NP	NP	NP
22	MS 47	-	-	NP	NP	NP
23	MS 53	-	-	NP	NP	NP
24	MS 54	-	-	NP	NP	NP
25	MS 56	-	-	NP	NP	NP
26	MS 57	-	-	NP	NP	NP
27	MS 59	-	-	NP	NP	NP
28	MS 60	-	-	NP	NP	NP
29	MS 61	-	-	NP	NP	NP
30	MS 68	-	-	NP	NP	NP

*indicator / sensitive organisms used in primary screening methods: *Citrobacer spp., Candida spp., E. coli, Enterococcus faecalis, P. aeruginosa, S. typhi* and *S. aureus;***indicator / sensitive organisms used for the confirmation of pediocin production by *Pediococcus spp. MS 3: E. coli, E. faecalis;* Antagonistic activity of pediocin produced by *Pediococcus MS3* was further checked against other organisms and mentioned in Table VIII; *Brain heart infusion agar was used to check the bacteriocin activity

Table III. Nature and number of the indicator / sensitive organisms

Microorganisms	Blood	High Vaginal Swab	Throat swab	Pus	Urine	Other	Total
Gram-positive bacteria							
Bacillus species	-	-	-	-	-	80 (E)	08
Clostridium perfringens	03	-	-	-	-		03
Corynebacterium specie	-	-	01	-	10	-	01
Enterococcus faecalis	-	03	-	-	-	10 (M)	13
Lactobacillus species	-	-	-	-	-	06 (M)	10
Listeria species	-	-	-	-	-	01 (M)	06
Micrococcus specie	-	-	-	-	-	-	01
Staphylococcus aureus	12	-	-	-	-	-	12
Streptococcus pneumoniae	-	-	04	-	-	-	04
Streptococcus pyogenes	-	-	03	-	-	-	03
Gram-negative bacteria							
Acinetobacter specie	03	-	-	-	01	-	01
Citrobacter species		-	-	03	-	-	06
Enterobacter species		-	-	-	03	-	03
Esch		-	-	-	22	-	22
Klebsiella pneumoniae		-	-	-	08	-	08
Klebsiella species		-	-	-	09	-	09
Proteus mirabilis		-	-	-	03	-	03
Proteus vulgaris		-	-	-	02	-	02
Pseudomonas aeruginosa	03	-	-	03	-	-	11

Salmonella typhi	06	-	-	-	-	-	06
Salmonella paratyphi A	01	-	-	-	-	-	01
Fungi							
Candida albicans	-	06	-	-	-	-	06
Candida species	-	02	-	-	01	-	03
Saccharomyces cerevisiae	-	-	-	-	-	01 (FF)	01
		Grand Total					143

Key: E= environment; FF= fermented food; M= milk

ANTIMICROBIAL SPECTRUM OF PEDIOCOCCUS SPP. MS3

ACTIVITY AGAINST GRAM NEGATIVE BACTERIA

For Gram-negative bacterial pathogens, the *Pediococcus spp. MS3* strain showed the highest inhibition zone against the organisms. These are clearly demonstrated by the inhibition zones of *E. coli*: 30 mm, *Proteus mirabilis*: 18 mm, and *S. typhi*: 12 mm respectively. The high degree of *E. coli* inhibition is significant, as this bacterium causes food spoilage leading to foodborne diseases, indicating that pediocin MS3 offers potential in food safety management (Fig. 2 a, b, c).

ACTIVITY AGAINST GRAM-POSITIVE BACTERIA

It also showed fair activity against Gram-positive bacteria with zone of inhibition of 24 mm against *E. faecalis*: 20 mm against *Lactobacillus spp.* and 18 mm against *S. aureus* respectively. The activity against *S. aureus*, which is responsible for food-borne illness and hospital-acquired infection, adding to the strength of pediocin MS3 as an antimicrobial peptide.

ANTIFUNGAL ACTIVITY

It was observed that *Pediococcus spp. MS3* exhibited antifungal characteristics as well. The capacity to suppress fungal growth is of considerable importance to the food industry where fungi contribute towards deterioration of food products, with low shelf life and poor quality. Based on its ability to immediately inactivate both bacteria and fungi, pediocin MS can be marked as a perspective bio preservative with a rather wide spectrum of activity in food safety.

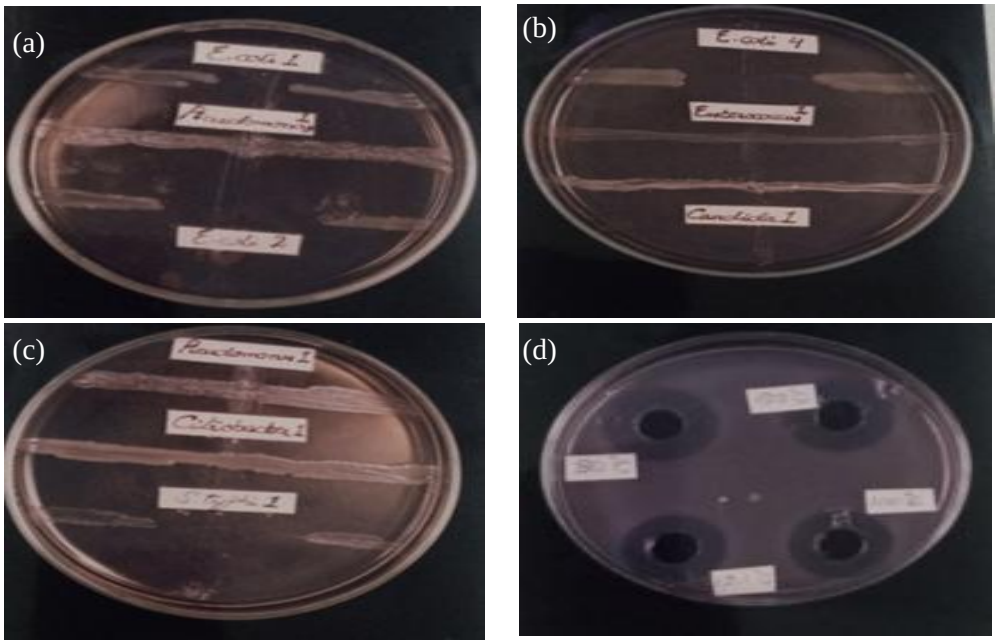

Fig. 2 (a). Cross streak method demonstrating pediocin MS3 activity against *E. coli* 1, *P. aeruginosa* 1 and *E. coli* 2. **(b).** Cross streak method demonstrating pediocin MS3 activity against *E. coli* 4, *E. Faecalis* 1 and *C. albicans* 1. **(c).** Cross streak method demonstrating pediocin MS3 activity against *P. aeruginosa* 1, *Citrobacter spp.* 1, and *S. typhi* 1. **(d).** Agar well diffusion method demonstrating pediocin activity against *E. coli* 7 after different temperature treatments.

Table IV. Spectrum of inhibition shown by bacteriocinogenic strains of *Pedococci spp. (MS3)* against Gram-positive & Gram-negative bacteria and yeast cells (by agar well diffusion method)

Microorganisms	Sensitive Strains / Tested strains	Average zone of inhibition (in mm)	Sensitivity in percentage % (sensitive strains x 100) / tested strains
Gram-positive bacteria			
Bacillus species	0/8	0	0.00
Clostridium perfringens	0/3	0	0.00
Corynebacterium specie	0/1	0	0.00
Enterococcus faecalis	7/13	24	53.84
Lactobacillus species	4/10	20	40.00
Listeria species	0/6	0	0.00
Micrococcus specie	0/1	0	0.00
Staphylococcus aureus	3/12	18	25.00
Streptococcus pneumoniae	0/4	0	0.00
Streptococcus pyogenes	0/3	0	0.00
Gram-negative bacteria			
Acinetobacter specie	0/1	0	0.00
Citrobacter species	0/6	0	0.00
Enterobacter species	0/3	0	0.00
Escherichia coli	14/22	30	63.63
Klebsiella pneumoniae	0/8	0	0.00
Klebsiella species	0/9	0	0.00
Proteus mirabilis	1/3	18	33.33
Proteus vulgaris	0/2	0	0.00
Pseudomonas aeruginosa	0/11	0	0.00
Salmonella typhi	1/6	12	16.66
Salmonella paratyphi A	0/1	0	0.00
Fungi			
Candida albicans	5/6	18	83.33
Candida species	1/3	20	33.33
Saccharomyces cerevisiae	1/1	22	100.00

*Source and characteristics of bacteriocinogenic strain of *Pediococcus specie (MS3)* are mentioned in the table no: 8.1; *Nature of indicator / sensitive strains are mentioned in the table no: 8.3; *Brain heart infusion agar was used to check the bacteriocin activity

PEDIOCOCCUS SPECIES MS3 TOLERABILITY TO PHYSICOCHEMICAL CONDITIONS

EFFECT OF TEMPERATURE

The thermostability of pediocin MS3 was evaluated at 4°C, 60°C, 80°C, 100°C, and 121°C respectively against indicator strain *E. coli* using the agar well diffusion assay (Fig. 2d). Pediocin MS3 showed maximum stability at a high temperature of 121°C by autoclaving for 15 mins. Notably, pediocin MS3 activity was unchanged by heat treatments at 60°C for 60 mins, 80°C for 40 min, and even after boiling at 100°C for 20 min with no reduction in the zone size. However, there was a slight decrease in the inhibition zone at 121°C (Table V).

Table V. Effect of different temperature on Pediocin activity by agar well diffusion method

Indicator organism	Temperature (C)	Time of exposure(minutes)	Pediocin activity (+/-)
Escherichia coli	60	60	+
	80	40	+
	100 (boiling)	20	+
	121 (autoclaving)	15	+

Key: +, activity retained (zone of inhibition); -, activity lost (no zone of inhibition)

EFFECT OF PH

Pediocin MS3 proved pH stability, maintaining its activity across a broad range of acidic to neutral pH levels from 2.0 to 10.0. However, its activity was particularly enhanced at alkaline conditions pH 11.0 to 12.0 (Table VI). This stability and activation were assessed using the agar well diffusion assay, against the *E. coli* strain.

Table VI. Determination of the effect of different pH on Pediocin activity by agar well diffusion

Indicator organism	pH	Pediocin activity (+/-)
Escherichia coli	2	+
	4	+
	6	+
	8	+
	9	+
	10	+
	11	-
	12	-
	Range	2-10

*Samples were adjusted to different pH with 10 mM NaOH and 10 mM HCl; *Samples were maintained at different pH for 2 h at 37℃; Key: +, activity retained (zone of inhibition); -, activity lost (no zone of inhibition)

EFFECT OF ENZYMES

Pediocin MS3 was treated with various hydrolytic enzymes, including trypsin, lysozyme, and β-amylase in human saliva. Its activity was assessed using the agar well diffusion assay with *E. coli* as the indicator strain. The antibacterial activity of pediocin MS3 remained unaffected by treatment with trypsin, lysozyme, and β-amylase (Table VII).

Table VII. Determination of the effect of different enzymes on Pediocin activity by agar well diffusion method

Indicator organism	Enzymes	Pediocin activity
Escherichia coli	Positive control	+
	Amylase (human saliva)	+
	lysozyme	+
	Trypsin	+
	Negative control	-

Samples were treated with enzymes for 1 h at 37 ℃; *Enzyme concentration, 1mg / mL; *Positive control, only pediocin; *Negative control, only enzyme; Key: +, activity retained (zone of inhibition); -, activity lost (no zone of inhibition)

EFFECT OF CHLOROFORM

The chloroform vapors, applied for 15 to 30 mins, do not affect pediocin MS3 activity. There was no change in the size of the zone of inhibition observed, indicating chloroform resistance (Table VIII).

Table VIII Determination of the effect of chloroform on prediction activity by stab and overlay method

Indicator organism	Treatment with chloroform for different time periods (min)	Pediocin activity
Escherichia coli	Positive control	+
	15 min	+
	20 min	+
	25 min	+
	30 min	+
	Negative control	-

*Samples were treated with chloroform at 37 °C; *Positive control, only pediocin; *Negative control, only saline; Key: +, activity retained (zone of inhibition); -, activity lost (no zone of inhibition)

EFFECT OF ORGANIC SOLVENTS

Pediocin MS3 was mixed with organic solvents including ethanol and methanol with a final concentration of 1.0% showing not to inactivate its activity. Control tests showed that the organic solvents alone had a negligible effect on the indicator strain (Table IX). The activity was assessed using the agar well diffusion assay with *E. coli* as the indicator strain.

Table IX. Determination of the effect of organic solvents on prediction activity by agar well diffusion method

Indicator organism	Treatment with chloroform for different time periods (min)	Pediocin activity
Escherichia coli	Positive control	+
	Ethanol	+
	Methanol	+
	Negative control	-

*Final concentration of organic solvents, 1.0%; *Samples were treated with solvents for 30 min at 37 °C; *Positive control, only pediocin; *Negative control, only saline; Key: +, activity retained (zone of inhibition); -, activity lost (no zone of inhibition)

INDUCTION BY UV LIGHT

Exposure to UV irradiation at 254 nm from 10 to 30 s does not enhance or elicit pediocin production indicating that pediocin synthesis is not influenced by short-term UV exposure within the tested parameters.

MEASUREMENT OF PEDIOCIN MS3 TITRE

The arbitrary units or activity units represent the activity of bacteriocin as the reciprocal of the highest serial dilutions showing inhibitory activity. Pediocin MS3 titration was performed against *E. faecalis* and *E. coli* by agar well diffusion assay. The titers were found to be 1,0240 and 5,120 arbitrary units, respectively (Table X).

Table X. Determination of the effect of pediocin titre in term of activity units or arbitrary units

Two-fold serial dilutions of pediocin	*Escherichia coli*	*Enterococcus faecalis*
Undiluted	+	+
1:2	+	+
1:4	+	+
1:8	+	+
1:16	+	+
1:32	+	+
1:64	+	+
1:128	+	+
1:256	+	+
1:512	+	+
1:1024	+	-
1:2048	-	-
AU / mL*	10240	5120

*Dilutions were made in Brain Heart Infusion broth; Key: +, zone of inhibition; -, no zone of inhibition

$$\text{AU / mL} = \frac{\text{Reciprocal of the highest dilution} \times 1000}{\text{volume of bacteriocin added}} \quad (1)$$

DETERMINATION OF PEDIOCIN MS3 DETERMINANTS

Pediococcus spp. MS3 was exposed to elevated temperature (43°C) for plasmid curing; verifying whether or not the genes responsible for pediocin biosynthesis existed on the plasmids or the bacterial chromosome. After curing, decrease in the inhibitory activity against *E. coli* was found through the agar well diffusion method, means that the genes of pediocin MS3 were located on plasmids. The plasmids are capable of transferring their characteristics horizontally across bacteria through conjugation, transformation or transduction. This form of horizontal gene transfer offers a means by which positive characters; such as antimicrobial peptides –spread among or between distinct strains or species of *Pediococcus.* Such transferability can be used to overproduce or redesign strains for specific uses, predominantly in apparatuses of food preservation or therapeutics.

Furthermore, plasmid localization enables expression of pediocin under certain environmental conditions because plasmid born genes have their own regulatory circuits. However, the location of these determinants on the extra-chromosomal state may also present some stability issues during the large-scale fermentation, which may require efficient control of the culture conditions in order to preserve the plasmid integrity and therefore yield consistent pediocin biosynthesis.

DISCUSSION

The current research contributed to the identification of thirty different bacteriocin-producing strains from raw milk using MRS agar. Among the isolates, *Pediococci spp. MS3* isolated from raw milk identified as the only pediocin producer. The researcher employed two different approaches i.e. assessment of pediocin activity against single indicator cultures and multiple sensitive cultures. The bacteriocin activity of pediocin against strong catalase-positive *S. aureus* resulted in the identification of *Pediococci spp. MS3* as a bacteriocin producer. A previous study by Xue et al. was also based on the characterization and isolation of bacteriocin produced by *Lacticaseibacillus casei KLS1*, which showed high efficacy against *S. aureus* and its biofilm formation (11). Ghazaei performed a similar study and found pediocin production by *Pediococcus pentosaceus* extracted from cheese. The *P. pentosaceus* strain proved to be effective against *S. aureus, Streptococcus pyogenes* and *Helicobacter pylori* (12).

The evaluation of the antimicrobial spectrum of *Pediococcus spp. MS3* identified a broad spectrum of its activity against gram-negative bacterial strains i.e. *E. coli, P. mirabilis* and *S.typhi*. In addition, gram-positive strains like *E. faecalis* (24 mm), *lactobacillus spp.* (20 mm) and *S. aureus* (18 mm) were also sensitive to pediocin MS3. The results also showed that ~~no~~ fungal strains including *C. albicans, Candida spp.* and *S. cerevisiae* were notably sensitive to the isolated pediocin MS3. In this regard, Amenu and Bacha indicated that bacteriocin produced by different lactic acid bacteria was effective against *E. coli, S. aureus*, and *L. monocytogenes.* Along with that, pediocin produced by *P. pentosaceus* showed a 34.13 ± 0.57 mm inhibitory zone against *P. aeruginosa* (13). Another study showed that the MIC value of pediocin was lowest against *S. aureus, P. aeruginosa,* and *K. pneumonia* (12).

The findings further highlighted the tolerance of *Pediococcus species MS3* against different temperatures, pH, enzymes, chloroform, organic solvents and UV light exposure. A significant finding was the high resistance of *Pediococcus spp. MS3* activity against heat which showed a decrease in the diameter of the inhibition zone at high temperature of 121°C. Furthermore, *Pediococcus spp. MS3* maintained its activity across a range of acidic to neutral pH. However, enhanced activity of pediocin was observed at alkaline pH mainly pH 11.0 to 12.0. Similarly, the bacteriocin produced by *Lacticaseibacillus paracasei* F9-02 retained their stability under different conditions and exposures, which included wide range of pH (2–10), temperature (40–121°C), enzymes (amylase, lipase and lysozyme), surfactants, metal ions ($CaCl_2$, $FeSO_4$, $ZnSO_4$, $MgSO_4$, $MnSO_4$, $CuCl_2$) and NaCl (2%–8%) (14).

The treatment of pediocin MS3 with trypsin, lysozyme, β-amylase, chloroform and organic solvents methanol and ethanol), also did not show any notable impact on the bactericidal activity. Todorov et al. assessed the impact of α-amylase, SDS, Tween 20, Tween 80, urea, and EDTA on the activity of pediocin and also not found any remarkable changes (5). The exposure to UV irradiation at 254 nm from 10 to 30 s did not enhance or diminish pediocin production indicating that pediocin synthesis was not influenced by short-term UV exposure within the tested parameters.

The researcher also assessed the activity of pediocin against indicator or sensitive organisms isolated from clinical samples of blood, pus, throat swab, high vaginal swab and urine. Among these, certain strains of *E. feaclis, Lactobacillus spp., Listeria species*, and *S. cerevisiae* were obtained from different environmental and food samples. The comprehensive approach helped in the assessment of pediocin's activity against bacteria having diverse natures and sensitivity levels. The pediocin MS3 isolated in the current research showed inhibitory activity against important gram-positive bacteria i.e. *Bacillus spp., C. perfringens, Corynebacterium species, E. faecalis, Lactobacillus species, Listeria species, Micrococcus species, S. aureus, S. pneumonia* and *S. Pyogenes*. In line with these findings, previous research by Sung et al. assessed the activities of pediocin, nisin and phenyl lactic acid produced by lactic acid bacteria. They found that pediocin was effective against gram-positive bacteria (*Bacillus cereus*) either in combination or individually, elucidating lethal effects (15).

The *Pediococcus specie MS3* showed inhibitory effects against gram-negative bacteria isolated from clinical/environmental/food samples. The inhibited gram-negative bacteria included *Acinetobacter species, Citrobacter species, Enterobacter species, Escherichia coli, Klebsiella pneumonia, Klebsiella species, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi* and *Salmonella para typhi A.* Thus study showed its

efficacy against a broad range of bacteria (15). Apart from bacterial species, the *Pedicoccus spp. MS3* characterized in the current study showed noticeable activity against certain fungal species i.e. *Candida albicans, Candida species* and *S. cerevisiae*. This finding was significant; since previous studies showed a limited understanding of the role of bacteriocin-producing *Pediococcus spp.* against fungal species. Future research can utilize this findings against different fungal species.

The limitation of our study includes small number of tested strains and further characterization of pediocin MS3 at molecular or protein level.

CONCLUSION

Pediocin-producer showed various important features such as thermostability and retaining activity at a wide range of pH, antibacterial activity against pathogenic bacteria and fungal agents that causes food spoilage. Thus making them outstand in the class of biopreservatives. This study has revealed that pediocin-producer found naturally in raw milk. *Pediococcus spp. MS3* and could potentially be utilized in real-time for food products. Pediocin, a potent antimicrobial can easily bind to food compounds and its direct application to food in different formulations such as powdered or liquid can be utilized for long-term preservation of food products in combination to other spoilage prevention methods. In the era with global threat of antimicrobial resistance, bacteriocin can be used as potent therapeutic.

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Conflict of Interest:

The authors declare no conflict of interest.

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