Molecular Characteristics of Iraqi *Lactobacillus plantarum* Isolates and Evaluate its Natural Bacteriocin(plantaricins) Antimicrbial Activity against Pathogenic Bacteria

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Abstract

In this study, local strains of *Lactobacillus plantarum* bacteria were isolated from traditional Iraqi raw milk and milk products. The present study was aimed to study the molecular aspects of Iraqi *Lactobacillus plantarum* isolates and to evaluate its natural plantaricins antimicrobial activity against pathogenic bacteria . For complete detection for *Lactobacillus* spp. the partial sequencing of 16S rDNA gene result was analyzed and compared with those in GenBank to find out the different in the sequence using the BLAST program (http:// www. ncbi. nlm.nih). The result was 17 samples diagnosed as *Lactobacillus plantarum*. Moreover 14 isolates of local *Lactobacillus plantarum* bacteria were show 100% similarity with those previously recorded in GenBank, while 3 isolates were found to display more than 99% similarity with *L. plantarum* strains were previously listed in GenBank. Moreover the study also reveals that all *L.plantarum* strains was showed antibacterial activity against three studied indicators bacteria and the *plnEF* gene (530bp) was detected in all 17 isolates of *L.plantarum*.

***Key words**: Lactobacillus plantarum, molecular,bacteriocin, antimicrobial, pathogenic bacteria*

Introduction

Bacteriain their life produce a different types of substances some of these substance have anti-bacterial activity. Plantaricins is a bacteriocin which is a uniquegroup of substances, which contains of aminorpoly -peptides with a bactericidal and moderately narrow anti-bacterial spectrum [1,2]. Currently,the newly purified bacteriocinshave a wide range ofa anti-microbial uses particularly in food preservation besides the fields of medication[3,4]. Moreover the lactobacilli are consider a common microorganisms which have been applied in manufacturing and human health, including food preservation and probiotics[5,6]. Additionally the Lactic Acid Bacteria (LAB), are broadlyused in the food production, especially in the fermented foods manufacture.Lactic acid bacteria are Gram positive bacteria , non-sporulating, anaerobic fermentative bacteria [7]. In addition to the previous application of antimicrobial bacteriocin and bacteriocin-like inhibitory substances (BLIS), that produced by lactic acid bacteria, they also show a good health promoting, for enhancing the nutritionrate, regulatorthe infection of intestinal, and reduce of pathogenic microorganisms[8].among the bestsignificantLAB which areapplied for making of fermented food products for example (meat, grass, and vegetable) is*Lactobacillus plantarum*. Several types of bacteriocins substances which produce by *L. plantarum,* was already described, as plantaricin (A), plantaricin (B), plantaricin (C), plantaricin (F), plantaricin (S) and plantaricin T[9].

Lactobacillus plantarum strains are produced plantaricin belong to class (IIb). These includetwo-peptide of bacteriocins , where in corresponding peptidesfor instance, PlnE/F and PlnJ/K which have a

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synergistic influencein contrast tothe target bacteria [10-12]. The majority of antibiotics have specific targets site ,in case of bacteriocins it eradicate the cells of microorganisms by destructive the cytoplasmic membrane or inhibition the biosynthesis of bacterial cell wall, or by both actions. Thebacteriocins have acomplicate mode of action, development of resistance against bacteriocins is mostly not reportable. One of the a significant limits within the widespread usage of most of the bacteriocins from Gram positive microorganisms is their incapability to eradicate the Gram-negative pathogens [13-15]. On the other handmany previous studies were attempt to isolate the *L. plantarum*from worldwide a variety of traditional cheese products, such as several Iranian ,Italian cheese varieties….etc [16-24].on the basis ofantimicrobial substances production and harmless characteristics, *L. plantarum* could be the suitable applicants for natural antimicrobial agent [25].Therefore, the present study was aimed to study the molecular aspects of Iraqi *Lactobacillus plantarum* isolates and to evaluate its natural plantaricins antimicrobial activity against pathogenic bacteria.

Materials andMethods

Samples collection and isolation Lactic Acid Bacteria (LAB)

One hundred twenty three samples of raw milk and traditional milk products (chess and yogurt) were used in this study. The samples of milks from caw and goat's milk were collected from farms located at the local north of Basrah province, while the cheese and yogurt samples were collected from local markets .The samples have been collected under aspect condition in sterile bottles and kept in an ice-box and instantlywastransported to thelaboratory ofMicrobiology.About 1ml of milk sample wasemixed thoroughly with 9 ml of sterilizedringer's solution (1:4)for 60s. A serial dilutions have been made, then 100 µlfromeach diluteness arespreadingon duplicate plated of MRS agar (HiMedia, India) .The MRSinoculatedculture were incubatedunder anaerobic conditions at 37°C for 48h.Then ten colonies from inoculated plates which analogous to the maximumdilutingare picked and subculturing forfurther tests [26].

On the other hand cheese and yogurt sampleswere collected from different local markets in which 1 gm. of sample was inoculated into 9 ml of MRS broth (Hi-Media, India) and incubated at 37º C for 48hrs.under anaerobic conditions. Then 1 loopful of broth culture was spread on MRS agar plates and incubated at 37°C for 48 hrs. under anaerobic conditions at 37°C[27].

For further characterization of LAB both Gram staining and catalase test were applied on all suspected isolates from raw milk, cheese, yoghurt samples [28,29],the single colonies were picked from subculturing on MRS agar medium.Only Gram-positive and catalase-negative isolates were taken as presumptive LAB and stored at 4 °C in MRS agar plates.

DNA extraction and PCR analysis

All suspect LAB isolates were initially cultured in MRS broth at 37 °C for 48hrs. Three ml of overnight culture were centrifuged at 14000 × g for 2min.,the supernatant was discard and the pellet cells were collected . The bacterial DNA wereextracted by using the Geneaidmicrobial DNA isolation Kit (Geneaid Biotech Ltd ,China), following the instructions of the manufacturer.

For confirmation and identification of *L. plantarum* , the PCR was used on previously extracted DNA samples .The PCR reaction was completed in 20 µl reaction mixtures with 1µl (10pmol/µl) for each primer, 5µl ready to use master mix (Bioneer /Korea) and the 5µl of DNA template. The final volume was adjustment by adding 8µl of nucleus free water to each reaction tube. The PCR conditions were initial denaturation of 95°C. for 5 min. followed by 34 cycle of denaturation of 95°C .for 30 s, annealing of 45°C.for 45s. and final extension at 72°C for 10 min. The PCR amplicons werethen run on 1.5% agarose gel staining with ethidium bromide to check the successful of amplification .Agarose gel was usedand visualized using a gel documentation system (E - graph – ATTO -Japan). Fragment size of approximately

319bp. positive gene was verified as positive for *L. plantarum*bacteria.

16S rRNA gene sequencing and phylogenetic analysis

The 5′ end variable region of the 16S rDNA was PCR amplified using 27F and 1525R primers (table 1). The PCR amplification reaction wasperformed in a total volume of 50µl, contains of 10µl master mix supplied from Bioneer /Korea and 10µl of genomic DNA , then 1µl of each primer was added to the reaction and the final volume was adjustment by adding the nucleus free water. Amplifications steps were carried out in a Thermal Cycler (Thermal Cycler, MJR), using the following program:

ThePCRconditionwasachieved with first denaturing phase of 95 °C for (5min) then 30 PCR cycles of 94°C (15s); 52°C(30s) and 72°C (2min). The final elongation cycle was conduct at 72 °C for (5min) . The PCR amplification products then electrophoresis using1.5% agarose gel already staining with ethidium bromide and subsequently visualize usinggel documentation system. The DNA ladder was used as a molecular mass marker. The PCR product then sequenced by (Macrogen Inc./ Seoul,South Korea Korea).The blast analysis areused then to distinguishacomparable sequences in the https://www.ncbi.nlm.nih.gov (NCBI) database. The phylogenetic tree were constructed by the neighbor -joining method using the software MEGA(Version 10).

Identification of genes encoding bacteriocin production

The oligonucleotides primers used in the current study are mentioned in table 1. The PCR was achieved on17 DNA samples using a master mix from Bioneer /Koreaunder the following conditions:the initial denaturation at 94ºC.for 3 min ,then 35 cycles of another denaturation step at 94ºC (1 min.); annealing step at 58.5ºC.(1 min) and elongation step at 72 ºC.(90 s.) and final elongation at 72°C (6 min.) .The amplicons of PCR were electrophoresed on 1.5% agarose gel.

Table 1: The sequences of oligonucleotide primer used in the current study

Primer name	Sequence (5'-3')	Size (bp)	Reference
16rRNA: L. plantarum	-TCGGGATTACCAAACATCAC- -CCGTTTATGCGGAACACCTA-	319 bp	30
16rRNA: 27F and1525R	27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ 1525R: 5′-AAGGAGGT GW T CCARCCGCA-3′	1525bp	31
planEF forward	-AGAGCACTATTAGGTAGTAAATAGCTGTGA- -AAATAACATCATACAA GGGGGATTATTT-	530bp	32

Evaluation the ability of identified strains to produced active bacteriocin

All 17 isolated were showed a significant bacteriocin producing , we select only one strain for further studies related to antibacterial activity and bacteriocin producing. Production of crude bacteriocin from isolated strain was tested according to method described previously [33].

The antibacterial effect of bacteriocin were screened *in vitro* for their antibacterial activity against: *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* using the paper disc-agar diffusion technique on Muller Hinton agar as a culture medium for antibacterial activity . The filter paper disks (6 mm in diameter) impregnated with different volume of crude bacteriocin (20, 40, 60 µl) and placed on the Petri plates. The plates were incubated for 24 hrs. at 37 °C. The inhibition zone diameters were measured in millimeters of inhibition zone around the disc . The pathogenic bacteria was supplied from Department of Microbiology, College of Veterinary Medicine, University of Basrah, Iraq.The percent bacteriocin activity was calculated using the following formula:

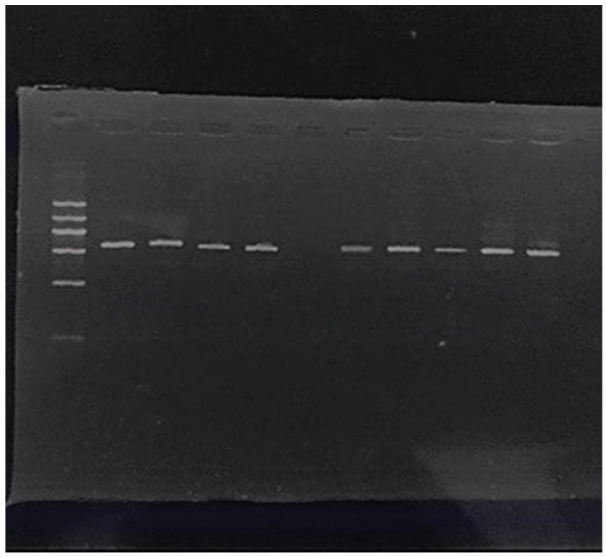
$$\text{Bacteriocin activity (\%)} = \frac{B-A}{A} \times 100$$

A = Disc diameter

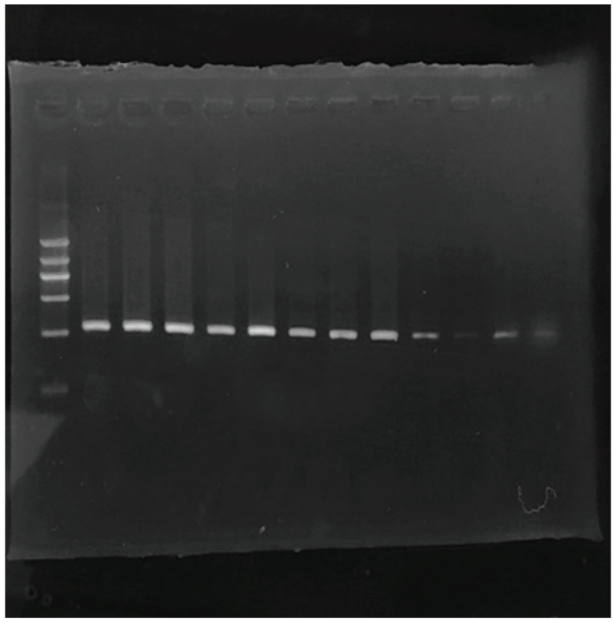
B = Inhibition zone diameter

Results

A total of 28 Lactic Acid Bacteria (LAB) were isolated from the123 collected samples of locally produced raw milk and traditional milk products (chess and yogurt). These samples originated from different animal sources, including cows and goats. All of the isolates were Gram positive and catalase negative (table 2). On the other hand the PCR technique was done for these samples The PCR results of 16rRNAspecific primers revealed that out of 28 only 23 isolates were diagnosed as*Lactobacillus plantarum* (figure 1)which reveals the bands of 319bp.s was clear observed on the 1.5% agarose gel analysis. Moreover the DNA of the 23 isolates weresent to Macrogen (Seoul, South Korea) with the primer (27F-1525R), Averagely1525bp was obtained per sequence, which was then compared with those in GenBank using the BLAST program (http://www.ncbi.nlm.nih). The result was 17 sample diagnosed as *lactobacillus plantarum* (table 2).In this table 14 isolates of local *Lactobacillus plantarum* bacteria were show 100% similarity with those previously recorded in GenBank, while 3 isolates were found to display more than 99% similarity with *L. plantarum* strains were previously listed in GenBank (table 3).The16S rDNA gene sequences results of 17 isolates were used to construct the phylogenetic tree using MEGA10 software. The Neighbor -Joining method was used for tree constructed and for confirmed the results of the homology analysis with the higher query cover (above 99%) of national samples as shown in figure (2) . For recognize between Iraqi samples and national sample from NCBI information a recording code was put before national sample with the country were it isolate.Finally the PCR results of plnEF gene specific primers revealed that all of the 17 isolates of *Lactobacillus plantarum* were have the plantaricin gene (figure 3). According to this figure the bands of 530bp was observed on the gel and represented the plnEF gene.



Figure(3) :Conventional PCR amplification of plnEF gene for Lactobacillus plantarum . Lane M: molecular marker (100bp), lane 7and 8 positive result for plnEF gene (530bp), lane F for control positive .



Figure(1): PCR amplification of 1.5% agarose gel electrophoresis. Lane1(M): molecular marker (100bp), lanes 2 to13 positive results for plnEF gene for *L.plantarum* gene (319bp).

Table 2: The number and percentage of *lactobacillus plantarum*recovered from milk &milk products samples.

Type of sample	Sample no.	No. of MRS culture	No. of PCR Positive	No. of positive sequences
Raw Milk &milk products	123	28	23	17
%	-	%22.76	82.14%	73.91 %

Table 3: The identification results of 17-strains of *Lactobacillus plantarum* by 16S rRNA Sequences

Sample No.	Source	Identities	GenBank ID	Country
1	Lactobacillus plantarum	99.39%	MT020400.1	China
2	Lactobacillus plantarum	100.00%	CP034997.1	Slovakia
3	Lactobacillus plantarum	100.00%	MG754629.1	China
4	Lactobacillus plantarum	100.00%	MF369880.1	China
5	Lactobacillus plantarum	100.00%	MT109312.1	Egypt
6	Lactobacillus plantarum	100.00%	MT109312.1	Egypt
7	Lactobacillus plantarum	100.00%	MT109312.1	Egypt
8	Lactobacillus plantarum	100.00%	MT109312.1	Egypt

Cont... **Table 3: The identification results of 17-strains of *Lactobacillus plantarum* by 16S rRNA Sequences**

9	Lactobacillus plantarum	99.89%	MN905459.1	China
10	Lactobacillus plantarum	100.00%	MT109312.1	Egypt
11	Lactobacillus plantarum	100.00%	MT109312.1	Egypt
12	Lactobacillus plantarum	100.00%	MG754552.1	China
13	Lactobacillus plantarum	100.00%	MT109312.1	Egypt
14	Lactobacillus plantarum	100.00%	MT109312.1	Egypt
15	Lactobacillus plantarum	99.71%	MT109312.1	Egypt
16	Lactobacillus plantarum	100.00%	MT109312.1	Egypt
17	Lactobacillus plantarum	100.00%	MH778541.1	China

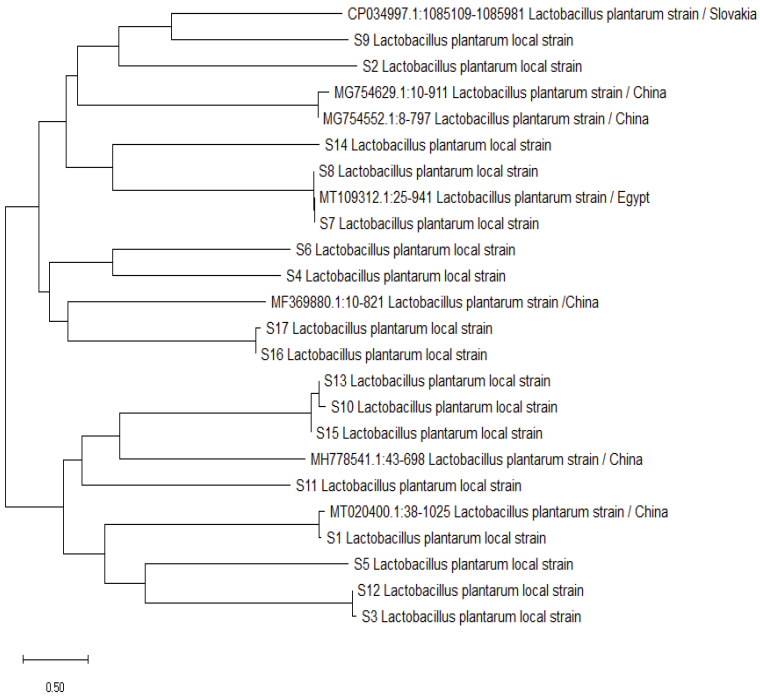


Figure2: Phylogenetic neighbor-joining(NJ) tree based on the16Sr DNA full-length sequences(~1.525kb) of the 17 selected *Lactobacillus plantarum* strains of raw milk and milk products samples based on the results of sequence alignment.

The S meaning sample and the numbers refer to the sample number.

The plantaricins antimicrobial activity was tested in three volumes against each pathogenic bacteria. Plantaricins revealed highest antimicrobial inhibitory activity in 40 and 60µl concentration against pathogenic tested bacteria : *Staphylococcus aureus*(136% and276.66% respectively) followed by *Pseudomonas aeruginosa*(1991.66%,252.66%) while the inhibitory activity against *E.coli* was 166.66% and 219.33% respectivelly(table 4,5 and 6).

Table 4: Antimicrobial activity of crude plantaricins at (20 µl,40 µl, 60µl) agains*S.aureus*.

Bacterial strain	crude plantaricins (µl)		
S.aureus	20µl	40 µl	60 µl
Mean Diameter of inhibition zone (in mm)	13.5	14.16	22.6
Mean diameter(mm) of inhibition zone (Ampicillin 25µg/ml)	12	13	20
Plantaricins activity%	125%	136%	276.66%

The disk diameter 6.0 mm.

Table 5: Antimicrobial activity of crude plantaricins at (20 µl,40 µl, 60µl) against *E.coli*.

Bacterial strain	crude plantaricins (µl)		
E.coli	20µl	40 µl	60 µl
Mean Diameter of inhibition zone (in mm)	12.5	16	19.16
Mean diameter(mm) of inhibition zone (Ampicillin 25µg/ml)	10	13	15
Plantaricins activity%	108.33%	166.66%	219.33%

The disk diameter 6.0 mm.

Table 6: Antimicrobial activity of crude plantaricins at (20 µl,40 µl, 60µl) against *P.aeruginosa*.

Bacterial strain	crude plantaricins (µl)		
P.aeruginosa	20µl	40 µl	60 µl
Mean Diameter of inhibition zone (in mm)	12.83	17.5	21.16
Mean diameter(mm) of inhibition zone (Ampicillin 25µg/ml)	13	15	18
Plantaricins activity%	113.83%	191.66%	252.66%

The disk diameter 6.0 mm.

Discussion

The raw cows and goats milk and their derivative products are considered a good source for isolation of lactic acid bacteria (LAB) which having the probiotic properties and have the ability to produce a novel bacteriocinogenic materials like plantaricins . In this context, there is a several previous studies were focused on isolation and molecular characterization of lactic

acid bacteria and study their activity against other pathogenic bacteria [34-36]. In the present study we try to isolate the local strain of *Lactobacillus plantarum*. and study their potential antibacterial activity. The results of primary isolation indicated that 28 out of 123 were isolated based on culturingDe Man Rogosa Sharpe (MRS) medium and catalase test ,and 23 isolated on the bases of PCR. This results were in line with the results of[37 and 38]. They reported that MRSagar was used as routinely medium for isolation of LAB and suitable for lactobacilli bacteriocin assay.

On the other hand the final diagnosis were based on sequencing results in which 17 isolates were confirmed as*L. plantarum*. Since the genome sequencing plays an important role in the accurate identification of bacteriocinogenic LAB [39]. The phylogenic tree was constructed based on 16S rDNA gene of local bacterial isolated and the results of sequences analysis were closely linked to those previously registered on the NCBI (National Centre of Biotechnology Information) from Slovakia, China, India and Egypt (figure.3). The phylogenetic analysis categorized all isolates into two main roots and each roots have different sub-roots or branching . In the figure (3) the first branch have shown the distribution of the national samples among with the Iraqi samples S9 and S2 in which S9 show extreme similarity with sample from Slovakia, while S2 share the same ancestor but it has a special branch. On the other hand the *Lactobacillus plantarum* local strain (S7 and S8) show more nearby with sample from Egypt. While *Lactobacillus plantarum* local strain S14 also share the same ancestor but it has the special branch. While the *lactobacillus plantarum* local strain (S5,S12,S3) were lacks to the national samples lead to distributed in special branch. Moreover, figure (3) also reveals that most *lactobacillus plantarum* local strain (S17,S16,S 6,S4, S13 ,S10, S15,S11,S1) show similarity with sample from China and this result was in line with [40].

In all genetics and molecular studies the phylogenetic tree was consider a good tool to see the kinship between species based on similarities or differences in physical properties such as sequence or genetic sequence of DNA or amino acid and protein [41], So the differences in the distribution of *lactobacillus plantarum* local strain could be attributed to the mutation in 16S rDNA gene that led to some variation in the Iraqi isolates . This mean the Iraqi isolates of *lactobacillus plantarum* were extreme similar to the national strains and share the same sequence of (16S rDNA) gene because this gene is more constant and have a slight mutation rate and evolution and need very long time to change so it is used in the classification of the microorganisms in the world. Detection based on 16S rDNA is consider a reference means for bacterial identification and taxonomic studies. Additionally the using of 16S rRNA gene as housekeeping genetic marker for taxonomic and bacterial phylogeny analysis could be attributed to the exist of 16S rRNA gene in almost all bacteria, moreover the 16S rRNA genes (1500 bp) are huge enough for informatics and genetic analysis purposes[43]

In the present study the primary screening for the ability of local *L. plantarum* isolates to produce antimicrobial activity (crude plantaricins) was assessed against three studied indicators *S.aureus, E.coli* and *P.aeruginosa* (table 4,5,6). In these tables the highest inhibitory activity was observed at 40µl and 60µl concentrations against *S. aureus* (166.66%, 219.33%) followed by *P. aeruginosa* (191.66% and 252.66%) while inhibitory activity reported against *E. coli* was (166.66% , 219.33%) for 40µl and 60µl respectively. The antimicrobial activity of crude bacteriocins have been reported to be inhibitory against several other bacteria[44- 46 and 33]. The results described in the present study are in agreement with the observations of 20 and 21 who reported a highest inhibitory activity of bacteriocin producing *Lactobacillus* spp. against*S. aureus*, [33] and against*S.aureus, E.coli* [47]furthermore, [35] was observed that *L.plantarum* isolates from raw cow milk samples had a powerful antimicrobial activity against a set of indicator microorganisms.

Moreover, the plantaricins-encoding genes was detected in all*L.plantarum* isolates by polymerase chain reaction (PCR). The results were revealed that all

tested *Lactobacillus plantarum* strains (no = 17) hold plnEF genes. The results were agree with the results of [48] based on the observation of the PCR product by using specific primers for amplified the plnEF in *Lactobacillus plantarum* bacteria .

Conclusion

We concluded that *lactobacillus plantarum* local strains have share the same DNA sequences similarity with samples from Slovakia, China, India and Egypt. Moreover all *L.plantarum* isolates were have the genes responsible for production of bacteriocins which a provide a natural substitute for antibiotics. However, more further studies are necessary toimprove quality and safety of these bacteriocins.

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