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STUDIES ON *LACTOBACILLUS* BACTERIOCIN FOR PRODUCTION AND CHARACTERIZATION AGAINST SOME PATHOGENIC AND FOOD SPOILAGE BACTERIA

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ABSTRACT

Bacteriocins produced by lactic acid bacteria (LAB) are a heterogeneous group of antibacterial proteins that vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties inhibitory substances include bakery and dairy products, cereals, and bread and cheese. Nisin is a natural bacteriocin produced by Lactococcus lactis.It has a broad inhibitory effect against gram positive bacteria. It can be destroyed be proteolytic enzymes that exist in food systems. We found that, minimum inhibitory concentration of free nisin was higher than encapsulated nisin in both culture media and cheese for L.monocytogenes and S. aureus. E. coli resisted to any form of Nisin in culture media but not in cheese. Nisin A is being used at the concentrations of 100-200 ppm in the preservation of, dairy products such as cheeses and milk. In addition, encapsulation protected nisin against cheese fat and protease. Reuterin is a water – soluble non- proteinaceous product produced by Lactobacillus reuteri. It has been described to have antimicrobial effect against certain gram- negative and gram- positive bacteria, yeasts, fungi, and protozoa. It inhibits Salmonella, Shigella, clostridium, Staphylococcus, Listeria, and Trypanosoma. The aim of present work was to study the combination of Nisin and L. reuteri against eight bacterias (Staphylococcus aureus, Salmonella typhi, Escherchia coli, Klebsiella pneuoniae, Pseudomonas aeruginosa, Bacillus subtilus, Bacillus cereus, Streptococcus spp.)Reuterin was isolated from L. reuteri during the anaerobic fermentation of glycerol using Gas Pack EZ Anaerogas pack container system on MRS agar. Minimum inhibitory concentration of bacterioncins (Nisin, crude Lactobacillus reuteri, Nisin + crude Lactobacillus reuteri) was studied using broth micro dilution method. Bacteriocins reuterin showed best synergism for both gram positive and gram negative bacteria used in the study. MIC of Bacteriocin (Nisin) alone against tested strains was determined to be 0.017+_0.001 to 0.40+_0.002 mg/ml. crude Lactobacillus reuteri alone was determined to be 0.016+_0.001 to 0.033+_0.001mg/ml which is 3 or 2 fold higher for MIC of Bacteriocins(Nisin + crude Lactobacillus reuteri) in combination i.e. 0.010+_0.001to 0.029+_0.001mg/ml. The synergistic activity of Biopreservative i.e. bacteriocins (Nisin and crude lactobacillus reuteri) with chemical preservative (sorbic acid) for pathogenic bacteria was evaluated using well diffusion assay. The diameter of inhibition zones increased on combination. The highest zone increased was seen in case of Bacteriocins in combination i.e. 29+_0.06 AB-3 (E.coli)

KEYWORDS - *Antimicrobil activity, Bacteriocin, Food Preservation, Lactic Acid Bacteria.*

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INTRODUCTION

Lactic acid bacteria (LAB) are food- grade microorganisms used for the production of numerous fermented food products to improve their flavor, texture and shelf-life.LAB proudce antibacterial compounds that include organic acids, diacetyl, hydrogen peroxide and bacteriocins,which are known to reduce food spoilage and growth or proliferation of pathogenic bacteria. Use of these naturally produced compound as food bio-preservative agents has therefore gained increasing attention in the food industry and now represents a promising way to preserve food without chemical agents, especially in ready-to-use.Bacteriocins may also find use in the preparation of products that are not submitted to sufficient thermal sterilizaton during their production, since they represent a risk of contamination by pathogenic bacteria such as *Listeria monocytogenes*, known in the art to be responsible for numerous worldwide outbreaks.

Lactobacilli are important organisms recognized for their fermentative ability as well as their helth and nutritional benefits (Gilliland, 1990). They produce various compounds such as organic acids, diacetyl, hydrogen peroxide,and bacteriocin or bactericidal proteins during lactic fermentations (Lindgren and dobrogosz, 1990) the antimicrobial properties of *Lactobacilli* are of special interest in developing strongly competitive starter cultures for food fermentation. *Lactobacilli* exert strong antagonistic activity against many microorganisms, including food spoilage organisms and pathogens .Producation of the primary metabolite, lactic acid and the resulting pH decrease is the main preserving factor in food fermentation. In addition, some strains may contribute to the preservation of fermented foods by producing other inhibitory substances, such as bacteriocins.

Control of both pathogenic and spoilage microbe in a variety of foods is important to guarantee food quality and safety. Recently, biopreservation has become a topic of interest [1]. This technique is used as an alternative to chemical additives for increasing self-life storage and enhancing safety of food by using natural microflora and their antimicrobial products [2]. Lactic acid bacteria are believed to be safe because they have been long established as the normal flora in fermented food; thus they have great potential for use in biopreservation. The preserving effects of lactic acid bacteria are due to the production of antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocin or related substances [3,4].

MATERIAL AND METHODS

Isolation of Microbes from Food Sample

Lactobacillus selection Agar

Sr.No	Compound	Amount for			
		100ml	250 ml	500 ml	1000 ml
1	Pancreatic Digest of Casein	1.0g	2.5 g	5.0g	10.0g
2	Sodium Acetate Hydrate	2.5 g	6.25g	12.5g	25.0g
3	Yeast Extract	0.5g	1.25 g	2.5 g	5.0 g
4	Acetic Acid	0.13 ml	0.33 ml	0.65ml	1.3 ml
5	Potassium Dihydrogen phosphate	0.6 g	1.5 g	3.0g	6.0 g
6	Ammonium citrate	0.2 g	0.5 g	1.0 g	2.0 g
7	Glucose	2.0 g	5.0 g	10.0 g	20.0 g
8	Magnesium Sulfate	58.0 mg	0.14 g	0.28 g	0.575 g
9	Manganese Sulfate	12.0 mg	30.0 mg	60.0 mg	0.12 g
10	Ferrous Sulfate	3.4 mg	8.5 mg	17.0 mg	34.0 mg
11	Polysorbate 80	0.1 g	0.25 g	0.5 g	1.0 g
12	Agar	1.5 mg	3.75 g	7.5 g	15.0 g
pH 5.5 +/- 0.2					

Isolation of Pure Culture

- Three sets of Lactobacillus selection agar plate of desired medium for each of the samples to be tested were prepared and labeled according to their dilutions (10^{-2}, 10^{-3}, 10^{-4}).

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- 100 mg of each sample and 900 ul of sterile water was added to create the 10^{-1}, 10^{-2},10^{-3},10^{-4} dilutions at different concentration.
- Aseptically 100ul of different dilutions were spread in the respective plate.
- All plates were incubated in an inverted position for 24 to 48 hours at 37°C.
- Pure distinct culture colonies were picked and streaked into new agar plate and incubated further in an inverted position for 24 to 48 hours at 37°C.

Gram Staining

Microbial culture were heat- fixed on the slide and stained for 1 minute with crystal violet (2% Crystal violet, 0.8% Ammonium Oxalate in 50% ethanol) staining reagent. Slides were washed with water and flooded with the mordant (Gram's iodine, 3% Iodine/Iodide Mixture in water). After 1 minute. Slides were washed with water and treated with decolorizing agent (95% ethanol). After decolorization slides were counterstained with safranin (0.25% in ethanol) for 30 seconds to 1 minute. Slides were then washed with water until no color appears in the effluent and then blot dry with absorbent paper. Slides were then observed under oil immersion using a Brightfield microscope (100X) and images were captured using Nikon Optiphot microscope equipped with Amscope MU1000 Camera.

DNA Sequencing and Phylogenetic Analysis Service

DNA Isolation

1. Centrifuge 1 ml of the overnight grown culture at 8000 rpm for 5 min ,or until a compact pellet forms . Discard the supernatant .
2. Resuspend pellet in 567 ul TE buffer by repeated pipetting . Add 10 ul Lysozyme (10mg/ml) and incubate for 30 mins at 37°C.
3. Add 30 ul 10% SDS and 5 ul of RNAse (10 mg/ml). Mix thoroughly and incubate 1 hr at 37°C .
4. Now add 3ul proteinase K (10mg/ml) and mix thoroughly and incubate 1 hr at 37°C.
5. Add an equal volume of phenol/chloroform/isoamyl alcohol, extract thoroughly , and centrifuge at 8000rpm for 5 min.
6. Transfer the supernatant to a fresh tube . Repeat the step if necessary.
7. Add 70ul of 3M Sodium Acetate and add 1.2 ml of chilled ethanol to precipitate the nucleic acids . Shake the tube back and forth until a stringy white DNA precipitate becomes clearly visible .
8. Centrifuge at 8000rpm for 5 min to pellet down the precipitated DNA.
9. Wash the DNA with 70% ethanol and centrifuge at 8000rpm for 5 min at room temperature to repellet it. Repeat this step.
10. Carefully remove the supernatant and briefly air dry the pellet in laminar flow hood.
11. Redissolve the pellet in 50-200 ul nuclease free water.

PCR Conditions

Reaction Mixture (50ul)		Cycling Conditions		
Template DNA	100 mg	Initial Denaturation	2 minuts at 95°C	
Forward Primer	0.3 uM	Denaturation	30 seconds at 95°C	35 Cycles
Reverse Primer	0.3 uM	Annealing	30 seconds at 52°C	
Master Mix	25 ul	Extension	2 minutes at 72°C	
Nuclease Free Water	Valume makeup 50ul	Final Extension	15 minutes at 72°C	

Primer Details :

No.	Oligo Name	Sequence (5'-3')	Tm(°C)	CG –Content
1	27F	AGAGTTTGATCMTGGCTCAG	56.3	47.5%
2	1492R	CGGTTACCTTGTTACGACTT	55.3	45%

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Primer Details (For Sequencing):

No.	Oligo Name	Sequence (5'-3')	Tm(°C)	GC –Content
1	785F	GGATTAGATACCCTGGTA	56.3	47.5%
2	907R	CCGTCAATTCMTTTRAGTTT	55.3	45%

Sample ID	6

Sequence in FASTA Format

AGTTGTATTGGTCGTATCTGTTACTAGGGAACCGCTTGAATCTTGATTTAATTTTGAACGAGTGGCGGACGGGTGAGTA
ACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTGGAAACAGATGCTAATACCGCATAAATCCAAGAACCGCAT
GGCTCTTGGCTGAAAGATGGCGTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAACGGC
TCACCAAGGCAATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGAGACACGCCCAAACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCCGTGAGTGAAGAAGGCTTTCGGG
TCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAGAAAGCCAC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGGTTATCCGGA

Alignment Table

s.no	Description	Max score	Total score	Query cover	E value	Ident	Accession
1	*Lactobacillus rhamnosus* strain NBRC 3425	900	900	93%	0	99.40%	NR_113332.1
2	*Lactobacillus zeae* strain RIA 482	867	867	93%	0	98.19%	NR_037122.1
3	*Lactobacillus casei* strain NBRC 15883	854	854	89%	0	99.16%	NR_113333.1
4	*Lactobacillus paracasei* strain ATCC25302	854	854	89%	0	99.16%	NR_117987.1
5	*Lactobacillus casei* subsp.casei ATCC 393	854	854	89%	0	99.16%	NR_041893.1
6	*Lactobacillus paracasai* subsp. tolerans strain NBRC 15906	848	848	89%	0	98.95%	NR_041054.1
7	*Lactobacillus saniviri* JCM 17471 DSM 24301	743	743	91%	0	94.25%	NR_113289.1
8	*Lactobacillus camelliae* strain MCH3-1	732	732	89%	0	94.75%	NR_041457.1
9	*Lactobacillus brantae* DSM 23927 strain SL1108	721	721	90%	0	93.96%	NR_125575.1
10	*Lactobacillus sakei* subsp. carnosus strain CCUG 31331	610	610	90%	0	89.83%	NR_104208.1

Tree File

The evolutionary history was inferred using the Neighbor- Joining method [1]. The optimal tree with the sum of branch length= 0.20964036 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches[2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). there were a total of 1578 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [4].

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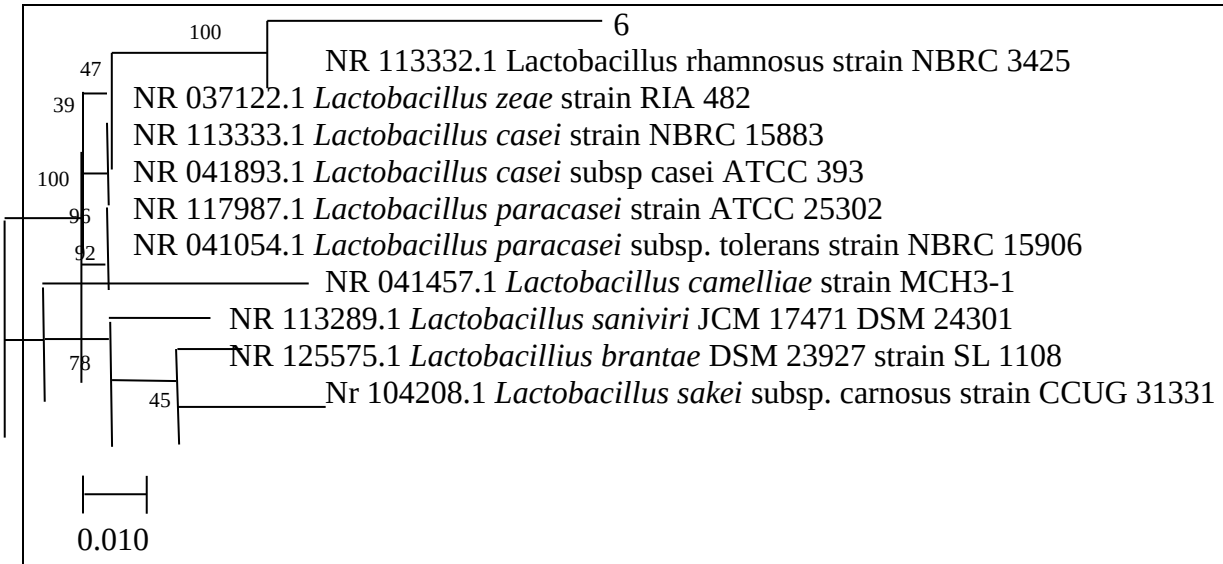

Distance Matrix

Distance Matrix

The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Maximum Composite likelihood model [3]. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequences pair (pairwise deletion option). There were a total of 1578 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [4].

		1	2	3	4	5	6	7	8	9	10	11
1	6		0.0093	0.0134	0.0150	0.0145	0.0154	0.0153	0.0235	0.0288	0.0282	0.0409
2	*Lactobacillus* rhamnosus strain NBRC 3425	0.0318		0.0028	0.0034	0.0035	0.0035	0.0035	0.0103	0.0126	0.0104	0.0156
3	*Lactobacillus* zeae strain RIA 482	0.0508	0.0088		0.0011	0.0024	0.0013	0.0026	0.0102	0.0132	0.0102	0.0159
4	*Lactobacillus casei* strain NBRC 15883	0.0575	0.0115	0.0020		0.0023	0.0000	0.0025	0.0104	0.0133	0.0104	0.0159
5	*Lactobacillus paracasei* strain ATCC 25302	0.0555	0.0113	0.0070	0.0070		0.0025	0.0006	0.0105	0.0144	0.0101	0.0158
6	*Lactobacillus casei subsp. casei ATCC 393*	0.0595	0.0122	0.0027	0.0000	0.0077		0.0026	0.0105	0.0133	0.0104	0.0160
7	*Lactobacillus paracasei* subsp. tolerans strain NBRC 15906	0.0597	0.0122	0.0081	0.0081	0.0007	0.0088		0.0104	0.0142	0.0100	0.0158
8	*Lactobacillus saniviri JCM 17471 DAM*	0.0978	0.0469	0.0467	0.0476	0.0480	0.0484	0.0477		0.0136	0.0059	0.0155

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	24301											
9	*Lactobacillus camelliae* strain MCH 3-1	0.1230	0.0582	0.0602	0.0614	0.0663	0.0617	0.0661	0.0653		0.0126	0.0191
10	*Lactobacillus brantae* DSM 23927 strain SL1108	0.1204	0.0470	0.0468	0.0477	0.0457	0.0480	0.0455	0.0254	0.0602		0.0125
11	*Lactobacillus sakei subsp.* carnosus strain CCUG 31331	0.1771	0.0736	0.0753	0.0763	0.0751	0.0767	0.0755	0.0747	0.0894	0.0581	

Inference: *Lactobacillus rhamnosus* sp.

RESULT AND DISCUSSION

In the last few decades, tremendous interest has swelled in the potential use of bacteriocins from lactic Acid Bacteria (LAB). The bacteriocins produced by this group of bacteria are considered potent bio-preservative agents and their application in food is currently the subject the subject of extensive research.

The present investigation highlights the isolation and characterization of bacteriocin producing *Lactobacillus* sp. isolated from Dosa (appam) batter, curd, sauces and cheese. Five Isolated bacteriocinogenic *Lactobaillus* sp. were characterized and identifed on the basis of their morphological, physiological and biochemical characteristics out of five, two were idenified as *L. plantarum* and coded as p1 and P2 due to their different potential in bacteriocin activity and other were identified as *L. casai, L. brevis, L. fermentum.*

Antimicrobial activity of the bacteriocins produced by the *Lactobacillus* sp. in this study was not due to hydrogen peroxide or acidity, as activity was not lost after treatment with catalase or peroxidase or adjustment of pH to 7.0.

Production of bacteriocin was studied in both aerobic and anaerobic conditions. Anaerobic condition was found suitable for maximum production of bacteriocin by all isolates whereas the aerobic condition did not support growth of *Lactobacillus* sp. (as they are anaerobic or microaerophilic bacteria) as well as the production of bacteriocin.

Bacteriocin production by the test isolates displayed secondary metabolic kinetics because all bacteriocin were produced during the pre-and early exponential growth phases, reached a maximum level at late stationary phase. Some reports indicate that bacteriocins are produced throughout the experimental growth phase and not solely during late logarithmic or early stationary phase (Joerger and Klaenahammer, 1986 Piard et al, 1990).

Optimization of bacteriocin production process was carried out by taking different parameters such as different carbon and nitrogn sourcer, pH, Temperature, Salt concentration and optimized parameter was determined by Arbitrary unit.

Supplementation and/or replacement of carbon and nitrogen sources demonstrated that larger quantities of bacteriocin could be produced by addition of glucose (2.0%) while addition of other carbon sources had on effect or adverse effect on production and in case of nitrogen source maximum production was achieved by addition of Tryptone , yeast extract and meat extract together in the medium..Maximal activity in composed medium was achieved at initial pH ranging from 6-8 while extreme alkaline and acidic pH did not support the bacteriocin production and optimized temperature was 30.c.

In the optimized conditions, the bacteriocin was produced at its maximum and the purifed bacteriocin could be directly used as bioprservative. Optimization of bacteriocin production will help to reduce their production cost and it could be available commercially (comparatively at low cost) to reduce or replace the addition of chemical preservatives.

All bacteriocin gave same results after treatment with enzymes. Complete inactivation of antimicrobial activity from all *Lactobacillus* sp. was observed after treatment of bacteriocin with proteinase k. trypsin and pepsin confirming its proteinaceous nature . Loss in antimicrobial activity by treatment with a-amylase suggesting that bacteriocin could be glycosylated. Lipase caused only a slight redution of bacteriocin activity, indicating that besides the proteinaceous subunit, some lipid components may also involve in antibacterial activity.

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SDS PAGE of bacteriocins showed that the molecular weight of bacteriocin from *L. plantarum* P1and P2 were approximately same and other have comparatively high molecular weight. Complete inactivation by proteinases and some loss of bacteriocin activity with lipase and amylase showed that these molecular weight of protein contribute major part in the total molecular weight of bacteriocins with some contribution of carbohydrate and lipid moieties.

During the purification procedures, each step resulted in considerable loss of peotein concentration while specific activity increased. At 60% saturation with ammonium sulphate highest increase in activity was observed for *L. plantarum* P1, *L. casai* , *L. fermentum* . While in the case of *L. brevis* and *L. plantarum* P2 it was achieved at 80% saturation . This agreed with the findings of lvanova et al. (2000). The increase in activity could be due to release of active monomers from bacteriocin complexes. During salt precipitation various amount of the protein was fractionated as a surface pellicle, this might be due to the association of bacteriocin molecules with the hydrophobic globular micelle like structure in the supernatant fluid. Similar observations have also been recorded for lactocin S and lactacin F (Muriana and klaenhammer,1991) The above fractions were subjected to ion exchange chromatography and production of active fraction of bacteriocin was achieved.

Thermal stability at 121.c for 20 min .was observed in case of bacteriocin produced by and it is important , if this bacteriocin is to be used as a food preservative, because many procedures of food preparation involve a heating step. The phenomenon of heat stability of LAB bacteriocins have been reported earlier for plantaricin A (Daeschel et al., 1990), Plantaricin C19 (Audisio, 1999), Plantaricin S (Jimenez-Diaz et al. 1990), Plantaricin 149 (kato et al., 1994), Plantaricin SA6 (Ralph et al., 1995), Plantaricin 423 (Van-Reenen,1998), pentocin TV35b (Okkers et al. ,1999), lactocin RN78 (Mojgani and Amirinia, 2007) and a bacteriocin produced by *L. brevis* 0G1 (Ogunbanwo et al. , 2003). The findings of this report are also in agreement with the above mentioned reports as we observed heat stability of *L. plantarum* P2 bacteriocin. The retention of activity by this bacteriocin after heating at 121^0 C for 60 min , place it within heat stable low molecular weight group of bacteriocins. This quality of the bacteriocin makes it superior in processed food stuffs where high heat is applied . While other bacteriocins were not heat stable. Andersson (1986) also reported loss of activity after heat treatment at 121^0 C for 15 min.

The activity of bacteriocin elaborated by the test isolates was also pH dependent. The bacteriocins produced by *L.brevis* and *L. plantarum* P2 were stable at acidic and alkaline pH as well as in high salt concentrations which make them an attractive applicant in food supplied i.e. they can be used in acidic foods like pickle, yogurt etc. while other showed stability only at acidic pH between 4-6. This was also shown by Reddy et. al., 1984; Abdel-Bar et. al., 1987 in two bacteriocins, namely bulgarican and lactobulgarican, isolated from *L. bulgaricus*, have, the highest activity and stability at pH 2.2 and 4.0 respectively, against a range of pathogenic and spoilage bacteria.

Increased antibacterial activity in the bacteriocin produced by *L. plantarum* P2 and L. brevis was observed in acidic pH specifically at pH 5. This may be due to the increase in net charge of bacteriocins at low pH might facilitate translocation of bacteriocin molecules through the cell wall. The solubility of bacteriocins may also increase at lower pH, facilitating diffusion of bacteriocin molecules.

CONCLUSION

The highly promising results of these studies underline the important role that functional, bacteriocinogenic *Lactobacillus* sp. may play in the food industry as starter cultures , co-cultures, or bioprotective cultures, to improve food quality and safety.

The characterization study of Bacteriocin from test isolates to exploit their potential make applicable them as suitable candidate for future application as a safe and efficacious biological preservative. The peculiar broad spectrum antibacterial characteristic, technological properties and especially heat and pH stability and salt tolerance capacity of *L. plantarum* , can positively has impact on their use as biopreservative, with a view to improving the hygiene and safety of the food products especially processed foods. However the pH stability and salt tolerance capacity of bacteriocin produced by *L. brevis* make it an attractive applicant in food supplies i.e. it can be used in acidic foods like pickle . The narrow-spectrum bacteriocins produced by *L. fermentum* could be used more specifically to selectively inhibit certain high-risk bacteria in foods without affecting harmless microbiota.

Bacteriocin producing lactobacilli with great potential could be directly used as starter culture or the concentrate from of these bacteriocins could also be

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used as biopreservative in the food industry and it can help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organolptic and nutrional properties . This can be an alternative to satisfy the increasing consumers demands for safe, fresh-tasting, ready-to-eat, minimally-processed foods and also to develop 'novel' food products (e.g. less acidic, or with a lower salt content).

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